ZÁRÓJELENTÉS,

11 KÖZLEMÉNY ÉS 3 KÖZLÉSRE ÖSSZEÁLLÍTOTT MUNKA ALAPJÁN
OTKA_K62317, 2006-2010

A von Willebrand faktor multimer - és konformáció függő aktivitása, vaszkuláris hematológia

Kérem a jelentésben foglaltak alapján született minősítést az OTKA kiegészítő eljárásban később módosítsa, figyelembe véve a később megjelent közleményeket.

TARTALOM

A VWF az érfal sérülésekor elérhetővé váló szubendothelium és a thrombocyták között teremt kapcsolatot nagy áramlási nyíróerők jelenlétében, így a thrombocyta adhézió egyik fontos résztvevője. A VWF és a thrombocyták egyidejűleg jelen vannak a keringésben ugyanakkor összekapcsolódásuk nem következik be, amíg a VWF kapcsolatba nem lép a szubendotheliummal és/vagy nagy áramlási nyíróerők nem lépnek fel. Ennek a hátterében VWF alegység egy-egy doménjén belüli kisebb és/vagy a domének illetve ismétlődő alegységek egymáshoz való viszonyának megváltozásából adódó nagyobb affinitás/aviditás változással járó konformációváltozások feltételezhetőek. Ezeket a változásokat az alábbi módon és módszerekkel tanulmányoztuk.

1. A lehető legkevesebb komponenst tartalmazó modell rendszert hoztunk létre, amelyben tisztított VFW és a thrombocytán lévő receptora, a GPIb közötti kölcsönhatást vizsgáltuk.

a. VWF tisztítására szolgáló eljárást dolgoztunk ki. A módszer három kromatográfiás lépésben (gélszűrés, anioncsere, affinitás kromatográfia) nagy tisztaságú VWF preparátum előállítására alkalmas. Röviden: az első gélszűrés (Sepharose 4FF) lépésben elválasztjuk a minta nagy molekulatömegű komponenseit. Ezt a frakciót anioncserélő oszlopon (Q-Sepharose HP) tovább tisztítjuk, majd affinitás kromatográfiás (HiTrap Heparin HP) lépéssel fejezünk be a folyamatot. A módszert FPLC rendszerre adaptáltuk, nem tartalmaz kicsapásos lépést, a tisztítás során végig Tris-HCl puffert alakalmazunk. Az alkalmazástól és a kiindulási anyagtól (pl.: thrombocyta lizátum, plazma) tisztaságától függően a lépések száma és sorrendje megváltoztatható.

b. A konformációváltozás létrejöttéhez a thrombocyták jelenléte is szükséges lehet, mivel feltételezhető, hogy maguk a thrombocyták is húzóerőt fejtenek ki a VWF-ra időleges kapcsolódásuk során. Ennek a hipotézisnek a vizsgálatához a thrombocytákat

funkcionálisan aktív GP Ib-vel fedett polisztirén gyöngyökkel helyettesítettük. Ehhez 3 μm átmérőjű karboxilát polisztirén gyöngyökhöz kovalensen 24B3 antitesteket kötöttünk. A 24B3 monoklonális antitest képes a glycocalicin (a GP Ib receptor extracelluláris protelítikus fragmense) aktív konformációban lévő megkötésére. Felhasználás előtt a 24B3 gyöngyöket glycocalicnnel inkubáltuk, majd mostuk; glycocalicin forrásként thrombocyta lizátum szűrletet használtunk. Flowcytometriás módszerrel ellenőriztük az antitestek jelenlétét a gyöngyökön, majd a gyöngyökön lévő 24B3 glycocalicin kötő képességét. Igazoltuk, hogy botrocetin (kígyóméreg, amely képes a GPIb és a VWF összekapcsolódásának indukálására) jelenlétében a gyöngyök felszínén VWF kötés mutatható ki. Ezzel igazoltuk, hogy a gyöngyök felszínén az immobilizált glycocalicin funkcionálisan intakt. Az általunk kifejlesztett módszer újdonsága a korábbi próbálkozásokhoz képest, hogy a gyöngyök felszínén a a glycocalicint a megfelelő orientációban és konformációban rögzítettük. A módszer hasznosságát jelzi, hogy - sajnáltos módon munkacsoprtunktól függetlenül - az általunk végzett kísérletekkel párhuzamosan nagyon hasonló eljárást alkalmazó publikáció jelent meg (Tadayuki Yago et al., J. Clin. Invest. 2008; 118:3195-3207), illetve kerekedelmi forgalomban ehhez hasonló gyöngyökön alapulóVWF:RCo teszt is megjelent.

Előadás: Udvardy ML, Kappelmayer J, Hársfalvi J**:** ***Glycocalicin coated beads for studying platelet GPIb function,*** Platelets, 2007; 18(1): 98, 2006

Jelentős eredmény**, a VWF nagynyomású folyadékkromatográfiával történő tisztási eljárásának és a thrombocytát modellező gyöngy GP-Ib fragmenssel való fedésének kidolgozása. Tervünk a két termék alkalmazása a jövőben kifejlesztendő diagnosztikai célú reagens kitben.**

2. A VWF molekulák változó számú dimer alegységekből állnak össze nagy VWF multimer molekulává. Méretüket azonban a keringésbe kerülve specifikus proteolízis csökkenti. A keringésben megtalálható VWF molekulák így változó méretűek és jellegzetes méretbeli (multimer) eloszlást mutatnak. A molekula méret – az aviditás változása miatt – alapvetően befolyásolja a VWF-thrombocyta kölcsönhatást. Csak a nagy oligomerek képesek hatékonyan thrombocyta adhéziót közvetíteni, ugyanakkor a szokásosnál nagyobb oligomerek thrombosis hajlamot fokozhatják. Új eljárást dolgoztunk ki a VWF multimer eloszlásának pontosabb leírására. Ehhez továbbfejlesztettük a VWF elektroforézis technikáját (borát pufferek és merkaptolízis együttes használata) és új értékelési módszert dolgoztunk ki, amelyben VWF gélelektroforézis során nyert denzitometrás görbéket elemeztük. A multimerek méretének jellemzéséhez első lépésben a görbe alatti terület felső 25 százalékához eső vándorlási távolságot határoztuk meg. Az ehhez a vándorlási távolsághoz tartozó molekulaméretet - amelyet a mintában található oligomer csúcsok és a hozzájuk tartozó molekulatömeg összefüggése alapján számítottunk ki - tekintettük a minta legnagyobb multimereit jellemző értéknek. A módszer segítségével elemeztük von Willebrand betegek mintáit, egészséges egyének plazma illetve thrombocyta lizátum mintáit vizsgáltuk. Összevetettük a multimer analízis eredményét a VWF funkcionális tesztekkel (VWF kollagén kötő kapcitás, VWF risztocetin kofaktor aktivitás) és igazoltuk a multimer méret és a funkcionális teszt eredmények közötti összefüggést

(r^2=0,42 ill. 0,43).

Közlemény 1: Udvardy, ML; Szekeres-Csiki, K; Harsfalvi, J ***Novel evaluation method for densitometric curves of von Willebrand Factor multimers and a new parameter (MMW) to describe the degree of the multimerzation,*** Thromb Haemost. 2009;102(2):412-7. If: 3.80

Szabadalom: Udvardy, ML és Harsfalvi, J**, Multimerizáció mértékét meghatározó szoftver.** bejelentés alatt**,** 2010.

Jelentős eredmény**, az, hogy a VWF multimerizáció fokának analizálására kidolgozott szoftver alkalmazható bármeilyen szakaszolt növekményt (multimerizáció vagy polimerizáció) leíró csúcsgörbe halmaz értékelésére.**

A VWF konformáció függő kötőhelyeinek megismerésére fág technológiát alkalmaztunk. Arteriás áramlási körülményeket modellezve VWF-t kötöttünk műanyag felszínhez és azt vizsgáltuk, hogy az immobilizáció hatására szabaddá váló molekularész(ek) 7-es és 12-es peptid inzertek lehetséges variációit tartalmazó fágkönyvtárból milyen szekvenciájú peptidet/ket kötnek meg. A szelekciós körben kötött fágokat ne specifikus elúció után sokszoroztuk. A specificitás növelése érdekében további két szelekciós kör uán ssDNS analízissel meghatároztuk az inzertek aminósav szekvenciáit, majd különböző molekulaszerkezet összehasonlító programok és szekvencia bank alkalmazásával kerestük a peptid szakaszt az élő szervezet ismert vagy jósolt molekuláiban. Kollaborációban szintetizáltattuk azt a peptidet, amelyik legnagyobb valószínűséggel mutatott hasonlóságot/azonosságot a kollagén molekula egyszakaszához szakaszához. A fágok, és a petid is gátolja a kollagén VWF kölcsönhatást és a thrombocyta adhéziót is csökkenti.

Közlemény 2: Szanto T, Vanhoorelbeke K, Toth G, Vandenbulcke A, Toth J, Noppe W, Deckmyn H, Harsfalvi J**.** ***Identification of a VWF peptide antagonist that blocks platelet adhesion under high shear conditions by selectively inhibiting the VWF-collagen interaction.*** J Thromb Haemost. 2009 Oct;7(10):1680-7. If: 5,94

Jelentős eredmény**, hogy a peptiddel csökkenthető a kollagén felszínen, artériás áramlási körülmények között képződő thrombus mérete. Ezért a peptid potenciális thrombocyta adhéziót és aggregációt gátló gyógyszer része kehet.**

A VWF molekula multimerizációja a VWF molekula multimerizációjának fokától függő aktivitása a kollagénhez való kötődésének mértékét is befolyásolja. A VWF kollagén kötő képességének (VWF:CB) a vizsgálatára a VWF kollagén kötő módszer (VWF:CBA) alkalmas. A módszerek (többnyire saját laboratóriumi, de kapható a kereskedelmi forgalomban is) különböző kollagéneket, különböző módon alkalmaznak. A módszervalidálási folyamat során irodalmi felmérést készítettünk.

Közlemény 3: Szekeres-Csiki Katalin, Udvardy Miklós László, Varga-Fekete Tímea, Hársfalvi Jolán**.** ***Von Willebrand-faktor és laboratóriumi diagnosztikai szerepe*** Orvostudományi Értesítő, 2008; 81 (1): 45-48 www.orvtudert.ro

Az irodalom áttekintése után saját módszert dolgoztunk ki a VWF:CB mérésére. Humán placentából származó III-as és I-es típusú kollagént alkalmaztunk.

Megállapítottuk, hogy a nemzetközi gyakorlatban nagyon különböző módon készült kollagén felszínek közül a leggyakrabban használt ecetsavas oldatból történő fedés igen bizonytalan, esetenként semmilyen kollagén réteget biztosít.

Közlésre összeállítva (Melléklet 1.): Shlomit Mendelboum Raviv1, Katalin Szekeres-Csiki, Attila Jenei, Janos Nagy, Boris Shenkman, Naphtali Savion and Jolan Harsfalvi. ***Effect of coating conditions on collagen matrix formation: Von Willebrand factor and platelet binding.*** Thrombosis and Haemostasis

Azt is megállapítottuk hogy az I-es III-as timupú kollagenek VWF kőtő képessége nem csökkent.

Közlésre összeállítva: (Melléklet 2.) Szekeres-Csiki Katalin, Udvardy Miklós László, Bézi Andrea, Hársfalvi Jolán. ***VWF bindig activity of human collagen Type I and III. (JTH ?)***

A **klinikai kutatásban** a VWF molekula mennyisége és multimerizáltságának mértékétől függő aktivitására kidolgozott módszereinket alkalmaztuk és hozzájárultuk a VW betegség diagnosztikájának előrelépéséhez, a multimerek méretének quantitatív meghatározásával. A klinikai esetek feldolgozását, a genetikai analízist kollaborációban végeztük.

Közlemény 4: Szanto, T., Schlammadinger, A., Salles, I., Pareyn, I., Vauterin, S., Harsfalvi, J., Vanden Bulcke, A.M., Deckmyn, H. And Vanhoorelbeke, K., ***Type 2b Von Willebrand Disease in Seven Individuals from Three Different Families: Phenotypic and Genotypic Characterization.*** Thromb Haemost, 2007; 98(1): 251-4. If: 3,501

Közlemény 5: Szanto, T., Schlammadinger, A., Staelens, S., De Meyer, S.F., Freson, K., Pareyn, I., Vauterin, S., Harsfalvi, J., Deckmyn, H. And Vanhoorelbeke, K., **The A/T1381** ***Polymorphism in the A1-Domain of Von Willebrand Factor Influences the AffinityoOf Von Willebrand Factor For Platelet Glycoprotein Ib alpha.*** Thromb Haemost, 2007; 98(1): 178-85. If: 3,501

Nagy tanulmányok igazolták, hogy az emelkedett VWF:Ag szint, a VWF növekedett aktivitása, fokozott thrombosis rizikót jelent, trombotikus mikroangiopátia megjelenéséhez vezethet. Különböző, mikroangiopátia veszélyét magával hordozó betegségekben vizsgáltuk a VWF multimer és szekréciója utáni hasító enzimének, a metaloproteinázok családjába tartozó ADAMTS-13-nak a szerepét.

Közlemény 6: Gombos T, Makó V, Cervenak L, Papassotiriou J, Kunde J, Hársfalvi J, Förhécz Z, Pozsonyi Z, Borgulya G, Jánoskuti L, Prohászka Z. ***Levels of von Willebrand factor antigen and von Willebrand factor cleaving protease (ADAMTS13) activity predict clinical events in chronic heart failure.*** Thromb Haemost. 2009 Sep;102(3):573-80, If: 3.803

Közlemény 7: Molvarec A, Rigó J Jr, Bőze T, Derzsy Z, Cervenak L, Makó V, Gombos T, Udvardy ML, Hársfalvi J, Prohászka Z. ***Increased plasma von Willebrand factor antigen levels but normal von Willebrand factor cleaving protease (ADAMTS13) activity in preeclampsia.*** Thromb Haemost. 2009;101:305-11, If: 3,501

Cirrhosisban vérzéses és trombotikus szövődmények egyaránt gyakoriak. Vizsgáltuk, hogy milyen szerepe van ezekben folyamatokban a VWF paramétereinek és az ADAMTS-13 enzimnek. Megállapítottuk, hogy az ADAMT-13 mennyisége és aktivitása a betegség legsúlyosabb fázisában csökkenést mutat, ezzel párhuzamosan a nagy multimerek többlete vagy hiánya nem volt jellemző. A VWF:Ag mennyiségének növekedése a fokozott endotél perturbáció következménye lehet. A VWF:CB is csökkent a betegség legsúlyosabb fázisában, ami utalhat a szintetizálódott VWF multimerizációjának hibájára.

Előadás: Tornai I, Papp M, Udvardy ML, Harsfalvi J***. VWF Functions, Adamts-13 Activity and Antigen Levels In Patients With Liver Cirrhosis.*** J Thromb Haemost 2007; 5 Supplement 2: P-T-207

A vénás és artériás körülményeket modellező, teljes vért alkalmazó áramlási kamráknak több típusa ismert. A párhuzamos lemezű és a viszkoziméterekből kidolgozott síkon forgó kúp rendszerű kamrák a leggyakrabban alkalmazottak. Az előbbinek a vér ígénye, attól függően, hogy milyen méretű a kamra nagyon különböző lehet. A vér mennyisége és az adhezív/thrombogén felszín méretének aránya is igen eltérő a kamrákban. Legkisebb vérigénye a síkon forgó rendszerű kamráknak van, azonban ezekben a kamrákban a felszín aránya a vér térfogatához képest igen nagy. A két rendszerű kamrát egyidejűleg alkalmazva, megvizsgáltuk, hogy az eredmények iterpretálhatóságát ez hogyan befolyásolja. Megállapítottuk, hogy a síkon forgó kúp klinikai kutató laboratóriumi körülmények között alkalmas a vaszkuláris hematológiai betegségek patomechanizmusának vizagálatára.

Közlemény 8: Szarvas, M., Oparaugo, P., Udvardy, M.L., Toth, J., Szanto, T., Daroczi, L., Vereb, G. and Harsfalvi, J., ***Differential Platelet Deposition Onto Collagen In Cone-And-Plate And Parallel Plate Flow Chambers.*** Platelets, 2006; 17(3): 185-90. If: 1,679

A pályázatban tervezzük az áramlási rendszerünk továbbfejlesztését, mely a felszíni folyamatok valós idejű megfigyelését tenné lehetővé, azonban ennek a fejlesztésnek a pénzigénye meghaladta a pályázat kereteit (eszközbeszerzés és munkaerő). Időközben, a fejlesztő cég ingyen kihelyezte a munkacsoportunkhoz az Impact–R síkon forgó kúp renszerű adhéziós eszközt, amely egyszerre négy kamrában végzi az adhéziót, így a beruházásra tervezett pénz csak kis hányadát költöttük el. Ezzel az eszközzel a vénás és artériás körülményeket modellezve vizsgáltunk különböző vaszkuláris hematológiai betegségek patomechanizmusát. Egy esettanulmányban igazoltuk thrombocyták fokozott kötődését VWF felszínhez.

Közlemény 9: Toth J, Kappelmayer J, Udvardy ML, Szanto T, Szarvas M, Rejto L, Soltesz P, Udvardy M, Harsfalvi J***. Increased platelet glucoprotein Ib number, enhanced platelet adhesion and severe ischaemia in a patient with polycythaemia vera.*** Platelets. 2009; 20 (4): 282-287 2009, If: 1,915

A thrombuskepződést jelentősen befolyásolta a áramlási körülményeket modellező kísérleti rendszerünkben a thrombin aktivtás gátlásása, mind kollagén, mind HMEC mátrixon. Ebben a munkában a vaszkuláris történéseket jelentősen befolyásoló inhibítor, egy módosított aptamer hatsának a vizsgálatát írjuk le.

Közlemény 10: Mendelboum, S.R., Horváth, A., Aradi, J., Bagoly, Z., Fazakas, F., Batta, Z., Muszbek, L., Hársfalvi, J., ***4-Thio-Deoxyuridylate Modified Thrombin Aptamer And Its Inhibitory Effect On Fibrin Clot Formation, Platelet Aggregation And Thrombus Growth On Subendothelial Matrix.*** J Thromb Haemost 2008; 6:1764-71, If: 5,94

A thromboemboliás szövődmények kockázati tényezői között számos faktor szerepel. Ezek közé tartoznak a műtéti beavatkozások is. E miatt van kiemelt jelentősége a mindennapi gyakorlat során jól használható evidenciákon alapuló protokolloknak. Az urológiai műtéteken áteső betegek thrombosis prophylaxisa csak a műtétes társszakmákból (elsősorban sebészet) átvett ajánlások alapján történik. Ez figyelmen kívül hagyja az urológiai műtétekre specifikusan jellemző sajátságokat. Az Európai Urológus Társaságnak, illetve a hazai Urológiai Szakmai Kollégiumnak nincs határozott ajánlása az urológiai műtétek thrombosis prophylaxisára vonatkozóan. Eredményeink arra utalnak, hogy a thrombusképződést befolyásoló tényezők közül a VWF és a trombin képződésének a mértéke jelentősen emelkedik a műtéti eljárások során, valamint az LMWH alkalmazása a prostata műtétek során felülvizsgálandó. Ennek a munkának a folytatása másik pályázatban és további munkatervünkben szerepel. Előkísérleteinket összefoglaltuk.

Közlemény összeállítás alatt (Melléklet 3.) Matyas Benyo, Tibor Flasko, Zoltan Batta, Zsuzsanna Szabo and Jolan *Harsfalvi* ***Increase in Thrombin Generation and von Willebrand Factor Levels after Laparoscopic Radical Prostatectomy.*** Journal of Thrombosis and Haemostasis

A VWF acut fázis fehérje. Nagy kliniki anyagon vizsgáljuk, hogy a gyulladásos betegségekben a VWF szintje és aktivitása hogyan változik, és a változás jellemzi-e a betegség progresszióját. Az alábbi közleményben azt a beteganyagot jellemezzük, amelyen jelenleg a VWF vizsgálatokat végezzük.

Közlemény 11: Papp, M Lakatos, PL Harsfalvi, J Gyula, F Karoly, P Udvardy, M Molnar, T Farkas, K Ferenc, N Veres, G Laszlo, L Kovacs, A Dinya, T Kocsis, AK Janos, P Istvan, A: ***Mannose-binding lectin level and deficiency is not associated with inflammatory bowel diseases, disease phenotype, serology profile, and NOD2/CARD15 genotype in a large Hungarian cohort***, Human Immunology 71;407-13, 2010

Melléklet 1. (szakmai és nyelvi lektorálás folyamatban)

Journal: **Thrombosis and Haemostasis**
Category of the communication: REGULAR ARTICLE
BASIC STUDY ON PREPARATION COLLAGEN MATRIX FOR VWF-PLATELET BINDING, A CONTRUTION TO DIAGNOSTIC TOOLS

Title of the article
Effect of coating conditions on collagen matrix formation: Von Willebrand factor and platelet binding

Authors
Shlomit Mendelboum Raviv[1], Katalin Szekeres-Csiki[1], Attila Jenei[2], Janos Nagy[2], Boris Shenkman[3], Naphtali Savion[4] and Jolan Harsfalvi[1]

[1]Clinical Research Center and [2]Department of Biophysics and Cell Biology, Medical and Health Science Center, University Debrecen, Hungary; [3]Amalia Biron Research Institute of Thrombosis and Hemostasis, Sheba Medical Center, Tel-Hashomer, [4]Eye Research Institute, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

A word count of the text: 3,678

Corresponding author:
Jolán Hársfalvi, Clinical Research Center, Medical and Health Science Center, University of Debrecen, 98 Nagyerdei krt., Debrecen, H-4012, Hungary.
Tel: +36 52 431 956; fax: + 36 52 340 011; e-mail: harsfalv@med.unideb.hu

Part of the work was presented at the ISTH meeting, Boston US, 10 July - 16 July 2009

Summary

Introduction and Aim Uniform collagen matrix is needed to study Von Willebrand factor (VWF) and platelet binding. We aimed to test the variation in collagen quantity, structure, capacity to bind VWF and platelets in matrices prepared by different collagen types and ways.

Materials and Methods Human type I and III collagen matrices were prepared by adsorption on 96 well plates or Thermanox coverslips. The effect of pH, salt concentration, ligand concentration and binding time were tested. Surface bound collagen and VWF were measured by antibody against collagen and VWF, respectively. Collagen was visualized by atomic force microscope (AFM) too. Platelet adhesion was tested under flow conditions at a shear rate of 1800 s^{-1} for 2 min.
Results Maximal binding of Human collagens type I and III was observed at

physiological pH in the presence of salt after 8 hours incubation and was 10 and 4 times greater in comparison to acidic conditions, respectively. The extent of VWF binding was parallel to the surface bound collagen in both collagen types and platelet adhesion expressed as % surface coverage was higher by 10-fold in physiological pH compared to acidic pH and with more organized and denser aggregates. Collagens fibrils structure was identical.

Conclusions Human type I and III collagens hardly bind to surfaces from acid solution, however, optimal collagen matrices are formed at physiological pH and salt concentration. The high result variation of *in vitro* collagen-VWF binding or platelet adhesion studies may well be due to the different conditions used for the preparation of collagen matrices.

Keywords: collagen, von Willebrand factor, platelet, thrombosis

Abbreviations
AFM Atomic Force Microscope
ELISA enzyme-linked immunosorbent assay
HMW high molecular weight (VWF)
IMW intermediate molecular weight (VWF)
LMW low molecular weight (VWF)
OD optical density
PBS phosphate buffer saline (0.02 M Na_2HPO_4-NaH_2PO_4, 0.15 M NaCl, pH 7.4)
rOD relative optical density
VWD von Willebrand disorder/disease
VWF von Willebrand factor
VWF:Ag von Willebrand factor antigen
VWF:CB von Willebrand factor collagen binding

Introduction

Platelet adhesion to exposed subendothelial matrices at sites of damaged vessel wall is the first step in haemostasis both at physiological and pathological conditions. Among components of the subendothelial matrices, the collagen and its role in haemostasis and thrombosis have been extensively investigated.
Several collagen types occur in the vessel wall, of which fibrillar collagen types I and III are considered to be the most important in supporting platelet adhesion to the damage vasculature (1), both of which are also highly concentrated in fibrous atherosclerotic plaque (2). Three polypeptide chains form the fundamental structure of the collagen molecule, which is characterized by the presence of one or more triple-helical monomers. Within this monomer, the three α-chains, wind around one another in a characteristic left handed triple helix. The triple-helical monomers self-associate to form typical highly ordered tissue collagen fibers. This conformation of collagen is

of crucial importance, since the three-dimensional structure is needed for recognition of collagen by its ligands (3).
One of the ligands is the von Willebrand factor (VWF). At arterial shear rates of flow collagen binds VWF from circulating blood, and then platelets are captured and tethered to the collagen and VWF complex through the platelet surface receptor GPIb-IX-V, initiating thrombus formation. This is followed by platelet activation mediated by the binding of one of the other ligands, the GPVI to collagen, leading to inside-out stimulation of integrin 2β1 and IIβ3 and platelet thrombus formation (4).
VWF Collagen-binding (VWF:CB) assay is recommended as a method for determining VWF adhesive activity (5). The assay is based on measurement of the quantity of VWF molecules bound to collagen, similar to the procedure for an enzyme-linked immunosorbent assay. In the last years, several VWF:CB assays have been developed and their results varied according to the different collagen preparation using to coat microplates, like tissue source of the collagen types, fibrillar collagen or acid-soluble counterpart, the buffer to dilute collagen and the range of the concentrations, incubation time etc. (see Supplemet).
The studies on collagen platelet interaction varying, not only in the VWF:CB conditions, but also in the way of the preparation of collagen surfaces (see Supplement). Like spraying versus absorption from buffer- or acid solution, which may result in very different nature of collagen matrices. The triple-helical structure of the collagen can be destroyed by denaturation, yielding random-coiled α-chains during isolation and preparation of collagen used to make matrices. The degree of renaturation can be different.
Studies on platelet thrombus formation needs a uniform collagen matrix, that is why we aimed to test the matrices of human collagen type I and III prepared at different conditions and their ability to bind VWF and support platelet adhesion.

Materials and Methods

Materials

Human placenta pepsin-digested collagen type III and I were purchased from Sigma (St Louis, MO, USA). Anti human collagen type III was obtained from Chemicon (Temecula, California, USA). Purified VWF was from Haemate P (CSL Behring, Marburg, Germany). Horse-radish-peroxidase (HRP)-conjugated rabbit antihuman VWF polyclonal antibodies and goat anti rabbit IgG-HRP were purchased from Dackocytomation (Glostrup, Denmark). 96 well plates were obtained from three different sources: Grenier (Nurtingen, Germany), Nunc (Wiesbaden, Germany) and ENZYME-PLATE, Propilen E.C. (Pecs, Hungary).

Preparation of Collagen Matrices (Coating)

Human collagen type III or I were dissolved in 50 mM acetic acid to obtain 2 mg/mL stock solutions. For binding studies stock solutions were treated differently: (1) further diluted with 50 mM acetic acid; (2) with phosphate buffered saline (PBS); (3) with 0.05M acetic acid and neutralized locally adding Na_2HPO_4; (4) dialyzed against

PBS for 2 days at 4°C. Passive adsorption of the collagen was allowed at the following conditions.
The differently treated collagen solutions were added into the wells at final concentrations of 2, 1, 0.5, 0.25 μg/100 μL/well. Acetic acid and PBS diluted solutions were added into parallel wells as vehicle controls. All the six series were incubated overnight at 4°C. After incubation the plates proceeded for measurement of collagen antibody- or VWF binding. Parallel series were transferred into new wells and the acetic acid solution was neutralized with Na_2HPO_4. The remained emptied wells filled with PBS. All the series were incubated overnight again and proceeded as before.
To study the effect of pH on collagen binding to the surface, collagen types I and III stock solutions were diluted with different buffers: (1) sodium acetate 20 mM, pH 4.0; (2) sodium phosphate 20 mM, pH 6.4; (3) pH 7.4; and (4) pH 8; (5) sodium carbonate 20 mM, pH 9.0; and (6) acetic acid 20 mM, pH~3. To study the effect of osmotic balance, the concentration of the NaCl was varied in the buffers between 0-600 mM.

Collagen Antibody and VWF Binding to the Collagen Matrix
Collagen coated surfaces were blocked for 30 min at room temperature with 3% (w/v) casein in PBS, washed 3 time with PBS containing 0.1% Tween-20 (PBS-T). The wells were then incubated with anti human collagen type III (100 μL/well, diluted to 1:1000 in PBS-T) or alternatively with purified VWF (0.1 U/100 μL/well) for 1 h at room temperature followed by rabbit anti human VWF (diluted 1:3000 or 1:2000 in PBS-T, incubated for 1 h at room temperature). (The antibody reacts with native and heat denatured human collagen type III, and gives 10% cross-reactivity with human collagen type I). Bound antibodies were detected with HRP-conjugated goat anti rabbit IgG, after intensive washing o-phenylnenediamin (OPD), the HRP substrate was given and the color developed between 10-20 min was measured by Infinite 200M reader (Tecan Trading AG, Männedorf, Switzerland).

AFM Study
To check the morphology of human collagen type I and type III binding to glass coverslip from acid or physiological solutions we used Atomic Force Microscope (AFM). Collagen type III and I were diluted in acetic acid solution (0.05mM) or in PBS at pH 7.4 to a final concentration of 20 μg/mL. 10 μL of the samples were placed on glass coverslips and kept in humid box for 8 h at 4 ^{o}C. Imaging was performed with a costume-made stand-alone-type AFM at the Institute of Biophysics and Cell Biology (University of Debrecen, Hungary) from parts purchased from the University of Twente (Enschede, The Netherlands) and combined with a Zeiss Axiovert microscope (Carl Zeiss, München, Germany) in tapping mode with samples under water. Cantilevers with Si3N4 pyramidal tips (Park Scientific Instruments, FWMS-06AU, Sunnyvale, CA) and with diameters between 10 and 30 nm were used with an average spring constant of approximately 0.06 N/m. In this study, 4x4 μm (x,y) and 4

μm (z) surface dimensions were scanned. Images of 512x512 pixels were collected and processed with software including plane fitting and x-y flattening. Images were evaluated and taken with scale bar and color scale by SPIP (Image Metrology A/S, Lyngby, Denmark) software.

Blood collection

Blood was collected from antecubital vein of healthy adult donors. The syringe contained as an anticoagulant, low molecular weight heparin, Clexane (20 U/mL final concentration, enoxaparin sodium, Aventis, Germany). All donors claimed to have abstained from taking aspirin, or other drugs know to affect platelet function, in the preceding 10 days. Written informed consent was obtained from all subjects in accordance with the Declaration of Helsinki and our Institutional Review Board approved the study. The blood was used in a 2 h time period.

Platelet adhesion to collagen coated onto coverslips.

Human collagen, diluted to 100 μg/mL in PBS or 50 mM acetic acid, evenly layered over glass coverslip (13 mm in diameter, Deckgläser) and incubated in humid box overnight at 4°C. Excess, unbound collagen was removed by rinsing with PBS. The coverslips were blocked with 3% casein in PBS for 30 minutes followed by three times washing with PBS. The coated coverslips were mounted into the Impact-R well (Matis Medical, Brussels, Belgium) and anticoagulated blood was circulated at a shear rate of 1800/s for 2 min. Then the coverslips were washed with water, stained by May-Grüenwald and analyzed by light microscope connected to a camera of the Impact-R image analyzsis system. The surface coverage (SC; [%]) and average size (AS; [μm^2]) of the objects on the well surface were determined.

SEM immages of platelet aggregates on surface collagene SEM images demonstrating much more "developed" aggregate with fully activated and merging platelets in the collagen coated surface comparing to adhering, spreading platelets with filopodia.

The surface coverage of the acidic collagen coated slides was so low (about 1%) indicate no binding and therefore no rational to take it for SEM.

Results

Collagen surfaces, prepared to study VWF and platelet binding, do not only differ in the collagen types but also in the binding/coating conditions. In order to be able to make a standardized matrix, we tested surfaces of collagen prepared under various coating time, pH and the salt concentration of the collagen solutions used for making matrices. Human type III and type I collagen matrices were prepared by passive adsorption technique on surface of 96 well plate or glass microscope coverslips. Surface bound collagen and VWF captured to it from purified VWF preparation were measured by antibody against collagen and VWF, respectively. Platelet adhesion to these surfaces was tested under flow conditions.

Binding of the Collagen to Different Surfaces

Using 96 well plates from different materials: polystyrene and polyvinyl chloride with low-, medium- and high binding capacities, we tested the binding of the collagen diluted in PBS. Testing the quantity of collagen adsorbed to the surface by specific antibody, demonstrated that the maximum binding was comparable in all different plate materials. As it was expected, binding was 1.5-fold higher to the high binding capacity plates (results not shown). Therefore, the high (600 ng/mm^2 protein) binding capacity plates were used for further experiments.

Influence of Coating Time

In order to study the duration of coating and then VWF capturing by the immobilized collagen, we coated collagen from acetic acid and from PBS solutions for 2 and 4 h at 37°C, one or two overnights at 4°C. Duration of coating time did not influence the quantity of the collagen bound to the surface from PBS solution and there was hardly any binding from acid solution. However, the variation within plate was high at 2 and 4 h coating and low at one or two overnights, when 20 wells were coated with 100 μL of 5 μg/mL PBS collagen solutions. The variation coefficient was 58% and 25% to 13% and 11%, respectively, when the captured VWF was measured by peroxidase labelled specific antibody.

Influence of Ionic Strength at Constant pH

In order to test the effect of the ionic strength on collagen coating and VWF binding to the coated collagen, we added increasing concentrations of NaCl to solutions of human collagen type I and III. The collagens were diluted sequentially from 20 mg/mL in 10 mM or 20 mM phosphate buffer (pH 7.4). It can be seen on Fig. 1 that collagen binding increased in response to increasing salt concentration, either in the case of type I collagen (Fig. 1A) or type III (Fig. 1B), and the curves show saturation around 0.15-0.2 M salt concentration, as the phosphate buffer concentration was 20 or 10 mM (Kd for 10 mM was 0.0189 ± 0.00148, 0.0124 ± 0.119 for type I and III, and for 20 mM 0.0067 ± 0.0121, 0.0735 ± 0.0188 for type I and III, respectively).

Influence of pH at Constant Ionic Strength

We chose 3 different buffers presenting 6 different pH values in order to understand the effect of pH on binding capacity of collagen representing possible structural change that enable better binding of the collagen to the surface and/or VWF binding to the collagen. As we summarized in the Table 1, the best results were observed when a serial dilution of the collagens for coating was in physiological phosphate buffer (pH 7.4). The coating was less effective from all other buffers. However, addition of 150 mM NaCl to each of the solutions improved the coating capacity from all of the solutions. Y values at the Kd of the phosphate buffer diluted collagens presented in Table 1, which were calculated using the fitted curve of the corresponding series. Relative OD-s in response to the collagen concentration was used for curve fittings. The calculation was done both for the results of anti-collagen

antibody or VWF binding to the coated collagen as detected by anti-VWF antibody.

Collagen binding characteristics prepared from Acid and PBS Solutions

Many studies on collagen VWF or platelet binding are using collagen solubilized in acetic acid or dialyzed against PBS for coating the surface. Our results show that collagen coated from acid solution has lower ability for binding VWF than collagen from PBS solution.

In addition when we dialyzed the acetic acid solution of the collagen against PBS, we did not find different binding ability compare to collagen directly diluted in PBS. However, it is noteworthy that almost half of the collagen lost during the dialysis procedure.

In order to understand the reason, if collagen did not have the right structure to bind VWF or do not bind to the wells we designed a series of experiments shown on Fig. 2. We applied into the 96 wells collagens diluted to 20, 10, 5, 2.5 μμg/mL with PBS (●) or with 50 mM acetic acid (▲). Binding of collagens type I (Fig. 2 A, C) and type III (Fig. 2B, D) showed saturation curve from PBS solutions either when measured using anti-collagen specific antibody binding (Fig 2. A, B) or when measured following VWF binding to the coated collagen using anti-VWF antibody (Fig 2. C, D). The rOD-s were low when the collagens were coated from acid solution. When Na_2HPO_4 was added to one series of acid diluted collagen wells to neutralize acid and kept at 4°C for one night coating (Δ) the rOD-s and the characteristics of the fitted curve were comparable to the curve of the PBS diluted collagen. So was with another series of acid diluted collagens, which after the overnight coating transferred into new wells, where the acid solution was neutralized (■). Furthermore, emptied acid wells were filled with 100 μL PBS (□) and left 4°C for coating as before, but hardly any collagen or VWF binding was detected.

In order to quantify the results, we fitted the rOD-s with equation and calculated the ligand binding capacity (table 2). No significant differences were observed between collagen type III and I when collagen specific antibody or VWF binding was measured. We found that coating resulted in the highest binding of collagen to the surface or VWF binding to the surface immobilized collagen when the acid stock solution of the collagen was diluted in PBS, and the lowest when it was diluted in acetic acid. However, when we neutralized the acetic acid collagen solution in the wells, there was no difference in binding compared to collagen coating from PBS solution.

AFM

To test if the quantity was different and not the structure of collagens bound to the surface from acetic acid or physiological solutions, we used AFM study. 10 μL of 20 μg/mL collagen solutions, were dropped onto glass coverslips and after 8 hours setting at 4 ^{o}C AFM analysis were done. Images in Fig. 3, 10x10 μm (x,y) surface and 20 μm (z) dimension show some quantitative differences between collagens bound from PBS (A,B) or acid solution (C,D), but no any structural differences can be seen.

It has to be noted, that for AFM study we applied 10-fold more concentrated acid collagen solutions to be able to see any object on the surface. The structure of the collagen type III fibrils (A,C) are similar to type I on (B,D) Although a significant differtence in the size and organization of the fibers can be seen. The average height and width of the fibrils is 20 nm on each sample. Some collagen fibrils were separate from the others and more fibrils were associated in large bundles.

Correlation of the Collagen Coated and the VWF Captured

When we analyzed the result obtained by measuring the collagen specific antibody binding and the VWF binding to coated collagen we found high correlation with linear regression both for type III and I collagen: r^2=0.902, y=(1.02±0.0206)x+0.0773±0.0102, n=268; and r^2=0.937, y=(0.938±0.0233)x+0.0651±0.012, n=112 respectively; where x is the rOD of the collagen and y is the rOD of the VWF. In other word as much collagen bound to the wells as much VWF bound to collagen in case of both type of collagens.

Effect of collagen coating conditions on platelet adhesion

Platelet adhesion was maximal on collagen surfaces coated from physiological PBS solution. The total amount of platelets adhering to the collagen surface was determined as the percent of surface covered by platelet aggregates and it was 12±4% and 1±1% in surfaces coated by collagen in PBS and acid solution, respectively. The platelet thrombi size, characterized as the average size of aggregates on the well surface was 90 ± 31 μm^2 (n=30) and 44 ± 9 μm^2 (n=14) in surfaces coated by collagen in PBS and acid solution, respectively.

[Shall we add 2 pictures on platelet adhesion to collagen/PBS coated surface vs no collagen???]

Discussion

The importance of the triple helical (tertiary) or polymeric, cross-linked (quaternary) structure of the collagen in thrombosis and haemostasis are well documented in recent studies (6).

Random attachment of different type of collagens, denaturation, and uncontrolled formation of collagen fibrils during immobilization by passive adsorption lead to a large variation of collagen matrices used by different studies in collagen VWF and platelet interaction. Although much progress has been made in elucidating the structure of collagen triple helices and the physicochemical basis for their stability and a rapidly emerging understanding of the mechanical and structural properties of native collagen fibrils guides further development of artificial collagenous materials for medical research and diagnostic tools (7-9). VWF and platelet binding to collagen mainly depending on the source and preparation of collagens used for coating and have important experimental implication, *see Supplement*.

In the present study, we used human fibrillar collagens type I and III at varying conditions to make collagen surfaces for VWF and platelet binding. We varied the pH

and ionic strength of the buffer used for the dilution of collagens, the different binding capacity plates, the binding time and the temperature. We measured VWF binding, parallel with collagen specific antibody binding, which had not been tested before. Our results indicated that collagen coating differs significantly so do the ability to bind VWF and consequently platelets.
We first tested the effect of coating collagen to different surfaces using 96 well plates from different materials that are commonly used. We found that high binding capacity plate has the higher collagen binding capacity too and therefore fitted for studying the collagen VWF interaction. Alternatively, as Fischer et al published, covalent immobilization of collagen onto microtiter plates is more suitable for the determination of collagen VWF binding (7). However, probable because the covalent binding allow the use of low concentrations of collagen for coating, the binding of VWF saturating at low VWF concentration, so the measurement range is very little.
Second we tested the effect of incubation or coating time on collagen binding to the wells and VWF binding to coated collagen, which is very varied in the literature, see Supplement. Some incubated the collagen in well for few hours while the other incubated the collagen overnight and even for 48 hours. Our results indicate that the error of the VWF binding to the collagen is increasing with the shortening the collagen binding time to less than 12 hours at 4 ^{o}C. The increase of this time over this period results in no improvement of collagen antibody and VWF binding. Our finding is corroborated with previous published data (8, 9).
Third we tested the effect of salt concentration on collagen binding to the surface. We found collagen binding dependency on increase slat concentration, and it reach to saturations at approximately 0.15 M, which is the physiological concentration. Further we added salt to the buffer with different pH and observed higher binding with those buffers that contain the salt.

Therefore optimal conditions that will form collagen fibrils with high binding ability is similar to those observed in vivo are: a solvent containing 20 mM phosphate, and NaCl to give an ionic strength of 0.13-0.25 at pH 7.4; temperatures between 20 and 30°C; and collagen concentrations between 0.02 and 1.3 mg/ml.

Phosphate is required to obtain well ordered fibrils (10, 11).
Fourth we tested the effect of buffer pH in which to dilute collagen for the purpose of coating ELISA plate. Many studies are using collagen solublized in acetic acid and then dialyzed against PBS for 48 hours before coating collagen surface (8, 12, 13).Our results show that collagen coated from acid solution has lower ability for binding to surfaces than collagen from PBS solution, and dialyzed collagen has the same binding capacity as collagen diluted in PBS. Hence diluting collagen directly in PBS is relatively short procedure with high binding ability of collagen binding to the surface. Further investigation of whether buffer pH used for dilute collagen influence on collagen binding to the surface. The best results were observed when buffer pH was close to 7.4 as in PBS. These results are similar to other studies (9-11, 14) which show that collagen binds better to the surface under neutralized condition and when

the buffer pH change to 7.4 collagen fibrils may form (10, 15, 16). Under acid condition collagen has a triple helical conformation, but the individual triple helices do not aggregate to form collagen fibers and not bind to the surface (8). In addition VWF may preferentially bind to fibrillar collagen(13, 17).
The collagen platelet interaction supports a two-step mechanism under flow conditions. The first step involves adhesion via both the indirect interaction of platelet glycoprotein (GP) Ib with collagen mediated by VWF binding to specific VWF-recognition sites in collagen and the direct interaction between platelet $\alpha 2\beta 1$ and specific $\alpha 2\beta 1$-recognition sites in collagen. This suffices to hold platelets at the collagen surface. The second step occurs via another collagen receptor (thought to be GPVI) that binds to simple collagen sequences, required essentially to delineate the collagen triple helix. Recognition of the triple helix leads to strengthening of attachment and platelet activation(18).

Fifth we tested the optimal collagen concentration for coating Kd= for PBS solution in consistent with published data (9) and in controversy with other studies which used higher collagen concentration, however those studies followed concentration historically used and haven't tested lower concentration, see Supplement.
We used AFM to probe the different properties of the centre and the surface of the fibril to compare the morphological investigations with tapping in liquid AFM mode. Nerlich et al. measured reduction of binding of human collagen Type I and III in acetic acid solution to mica. Their results showed 30% decrease in binding of collagens in acetic acid solution compared to physiological. In our study we used acetic acid as acid and PBS as physiological solution. AFM images show particular morphological and quantitative differences between acid and PBS solutions in both of two collagen Types. The binding of collagens decreased 50% in acetic acid solution. Collagen molecules, forming the fibril consist of an uninterrupted right handed triple helix called tropocollagen. The fibril has a binding pattern of 78 nm, a height of 30 nm and a width of 270 nm. Kadler et al. investigated the structure of human collagens. Between collagen Type I and III fibrils there was no difference in the structure. The diameter was in the range of 20-70 nm and the collagens produced very long fibril structures after binding. During our AFM measurements the size of collagen Type I and III fibrils were consistent. The height of the collagen fibrils was 25 nm and the width was 200 nm on average. These obtained size parameters are comparable to any other published results.

We found high correlation with VWF bound to the collagen and collagen bound to the surface. In other words the amount of VWF bound to collagen is dependent on the amount of collagen bound to the surface. In addition there is no different between collagen Type I and III on collagen binding to the surface and VWF binding to collagen.
The assembly of collagen molecules into fibrils is an entropy driven process, similar to that occurring in other protein selfassembly systems, such as microtubules, actin

filaments and flagella. These processes are driven by the loss of solvent molecules from the surface of protein molecules and result in assemblies with a circular cross-section, which minimizes the surface area/volume ratio of the final assembly. Although the broad principles of collagen fibril self-assembly are generally accepted, less is known about the molecular mechanisms of the assembly process. (Karl E. KADLER Biochem. J. (1996) *316*,)

Acknowledgements Ms. Andrea Bezi, Ms. Gizella Haramura, Dr. Miklos L Udvardy, Dr. Judit Toth acknowledged for their assistance.
This work was supported by Grants HPRN-CT-2002-00253 (European Commission), OTKA K62317, OTKA-NKTH NI 69238 (Hungarian Scientific Research Fund).

Conflict of interest statement
N. Savion is a founder of the Company developing the Impact-R. All other authors disclose any financial and personal relationships with other people or organisations that could inappropriately influence (bias) their work.

References

1. Farndale RW, Sixma JJ, Barnes MJ, et al. The role of collagen in thrombosis and hemostasis. J Thromb Haemost 2004 Apr;2(4):561-73.
2. Barnes MJ, Farndale RW. Collagens and atherosclerosis. Exp Gerontol 1999 Jul;34(4):513-25.
3. Farndale RW. Collagen-induced platelet activation. Blood Cells Mol Dis 2006 Mar-Apr;36(2):162-5.
4. Varga-Szabo D, Pleines I, Nieswandt B. Cell adhesion mechanisms in platelets. Arterioscler Thromb Vasc Biol 2008 Mar;28(3):403-12.
5. Favaloro EJ. An update on the von Willebrand factor collagen binding assay: 21 years of age and beyond adolescence but not yet a mature adult. Semin Thromb Hemost 2007 Nov;33(8):727-44.
6. Herr AB, Farndale RW. Structural insights into the interactions between platelet receptors and fibrillar collagen. J Biol Chem 2009 Jul 24;284(30):19781-5.
7. Fischer BE, Thomas KB, Dorner F. Collagen covalently immobilized onto plastic surfaces simplifies measurement of von Willebrand factor-collagen binding activity. Ann Hematol 1998 Mar-Apr;76(3-4):159-66.
8. Vanhoorelbeke K, Cauwenberghs N, Vauterin S, et al. A reliable and reproducible ELISA method to measure ristocetin cofactor activity of von Willebrand factor. Thromb Haemost 2000 Jan;83(1):107-13.
9. Paczuski R. Determination of von Willebrand factor activity with collagen-binding assay and diagnosis of von Willebrand disease: effect of collagen source and coating conditions. J Lab Clin Med 2002 Oct;140(4):250-4.
10. Williams BR, Gelman RA, Poppke DC, et al. Collagen fibril formation. Optimal in vitro conditions and preliminary kinetic results. J Biol Chem 1978 Sep 25;253(18):6578-85.

11. Pareti FI, Niiya K, McPherson JM, et al. Isolation and characterization of two domains of human von Willebrand factor that interact with fibrillar collagen types I and III. J Biol Chem 1987 Oct 5;262(28):13835-41.
12. Lankhof H, van Hoeij M, Schiphorst ME, et al. A3 domain is essential for interaction of von Willebrand factor with collagen type III. Thromb Haemost 1996 Jun;75(6):950-8.
13. van der Plas RM, Gomes L, Marquart JA, et al. Binding of von Willebrand factor to collagen type III: role of specific amino acids in the collagen binding domain of vWF and effects of neighboring domains. Thromb Haemost 2000 Dec;84(6):1005-11.
14. Shoulders MD, Raines RT. Collagen structure and stability. Annu Rev Biochem 2009;78:929-58.
15. Wood GC, Keech MK. The formation of fibrils from collagen solutions. 1. The effect of experimental conditions: kinetic and electron-microscope studies. Biochem J 1960 Jun;75:588-98.
16. Scott DM, Griffin B, Pepper DS, et al. The binding of purified factor VIII/von Willebrand factor to collagens of differing type and form. Thromb Res 1981 Dec 1-15;24(5-6):467-72.
17. Mazzucato M, Spessotto P, Masotti A, et al. Identification of domains responsible for von Willebrand factor type VI collagen interaction mediating platelet adhesion under high flow. J Biol Chem 1999 Jan 29;274(5):3033-41.
18. Cruz MA, Yuan H, Lee JR, et al. Interaction of the von Willebrand factor (vWF) with collagen. Localization of the primary collagen-binding site by analysis of recombinant vWF a domain polypeptides. J Biol Chem 1995 May 5;270(18):10822-7.
19. Bernardo A, Bergeron AL, Sun CW, et al. Von Willebrand factor present in fibrillar collagen enhances platelet adhesion to collagen and collagen-induced platelet aggregation. J Thromb Haemost 2004 Apr;2(4):660-9.
20. Houdijk WP, Sakariassen KS, Nievelstein PF, et al. Role of factor VIII-von Willebrand factor and fibronectin in the interaction of platelets in flowing blood with monomeric and fibrillar human collagen types I and III. J Clin Invest 1985 Feb;75(2):531-40.
21. Zijenah LS, Barnes MJ. Platelet-reactive sites in human collagens I and III: evidence for cell-recognition sites in collagen unrelated to RGD and like sequences. Thromb Res 1990 Aug 1;59(3):553-66.
22. Morton LF, Peachey AR, Barnes MJ. Platelet-reactive sites in collagens type I and type III. Evidence for separate adhesion and aggregatory sites. Biochem J 1989 Feb 15;258(1):157-63.

Figures and Legends

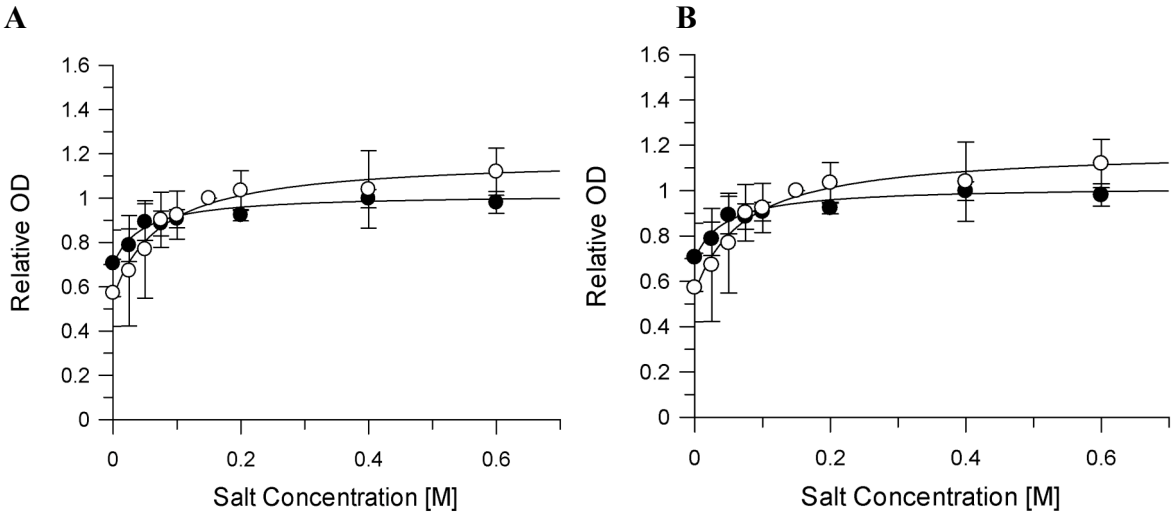
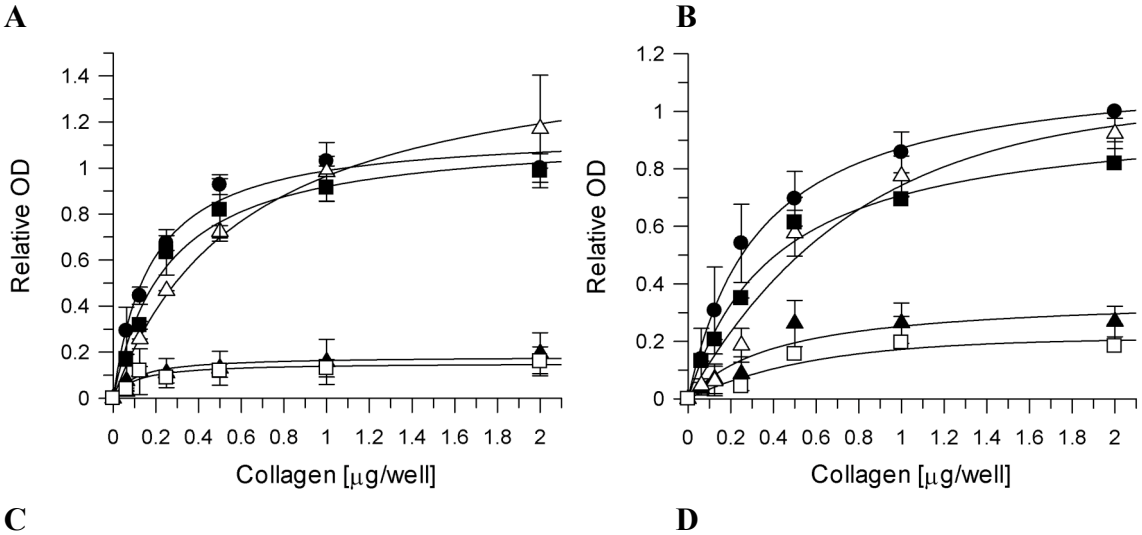


Fig 1. Effect of salt concentration on collagen coating.
Human collagen type I (A) and Human collagen type III (B) were dilute to a final concentration of 0.020 mg/mL in phosphate buffer (pH 7.4) at concentrations of 10 (●) and 20 (○) [please check the propreate position of the symbols] mM with increasing concentration of NaCl and immobilized to ELISA plate. VWF binding to immobilized collagens were detected as described in the methods. Relative OD (rOD) was calculated by dividing the absolute OD obtained from the each salt concentration with absolute OD at 0.15 M NaCl concentration. Values represent mean ± standard deviation of three independent experiments.



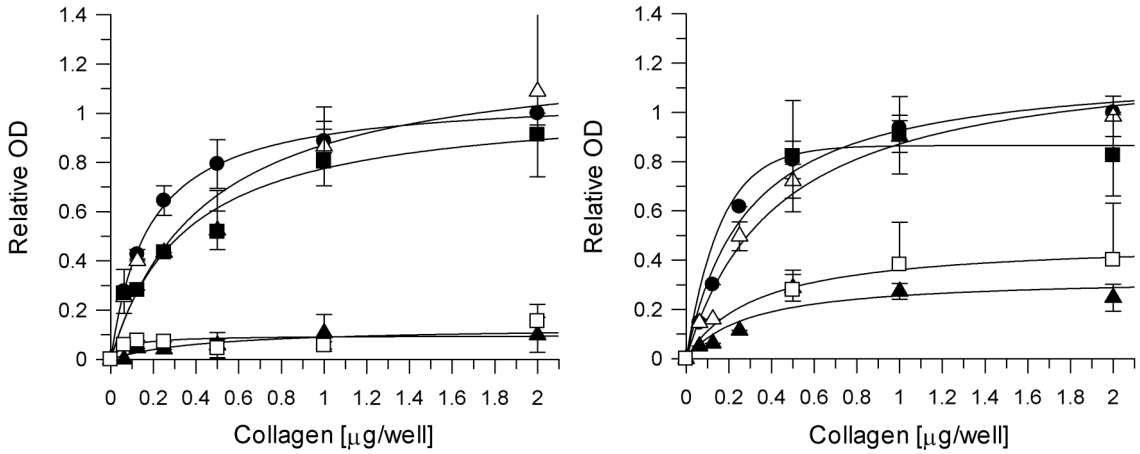
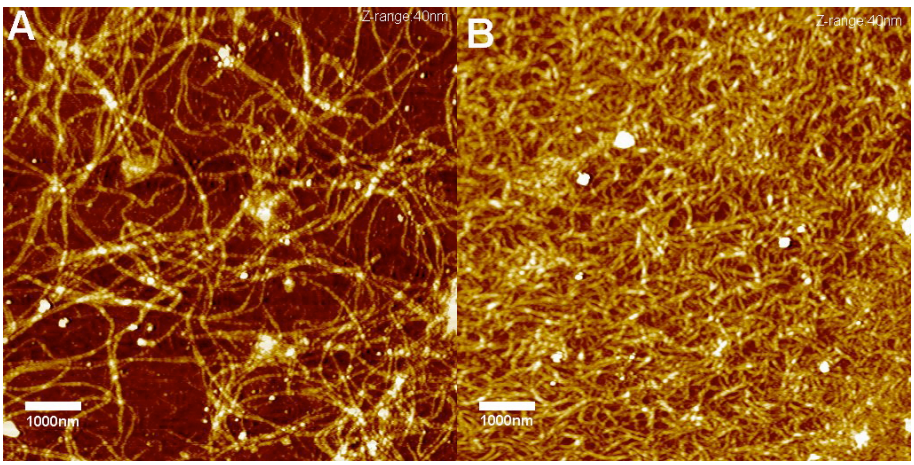


Fig 2. Coating collagen from different solutions.
Human collagen type I (A,C) and type III (B,D) were coated from different solutions and the binding of collagen specific antibody (A,B) or VWF and specific antibody (C,D) was used for detection.
Collagens diluted to 20, 10, 5, 2.5 μg/ml with PBS (●) or with 50 mM acetic acid (▲) were applied into the wells. Na_2HPO_4 was added to one series of acid diluted collagens to neutralize acid (Δ). All the wells were incubated overnight at 4°C. Next day liquid from wells containing acetic acid were transferred into the wells of a new plate and all the other wells were emptied and detection were proceeded by collagen specific antibody (A,B) or through VWF and its specific antibody binding.. Transferred acetic acid were neutralized by adding Na_2HPO_4 into the new wells (■) and series of collagens diluted with PBS or with 50mM acetic acid were applied into other wells of this new plate again. PBS was added into another series of wells, after the acid diluted collagen emptied and washed off (□). All the plates were incubated overnight at 4°C and next day the detection proceeded again.
Relative OD was calculated by dividing the absolute OD obtained from the measurement for each treatment and concentration with absolute OD of wells coated with 20 μg/ml collagen diluted in PBS. Values represent mean ± standard deviation of four independent experiments.



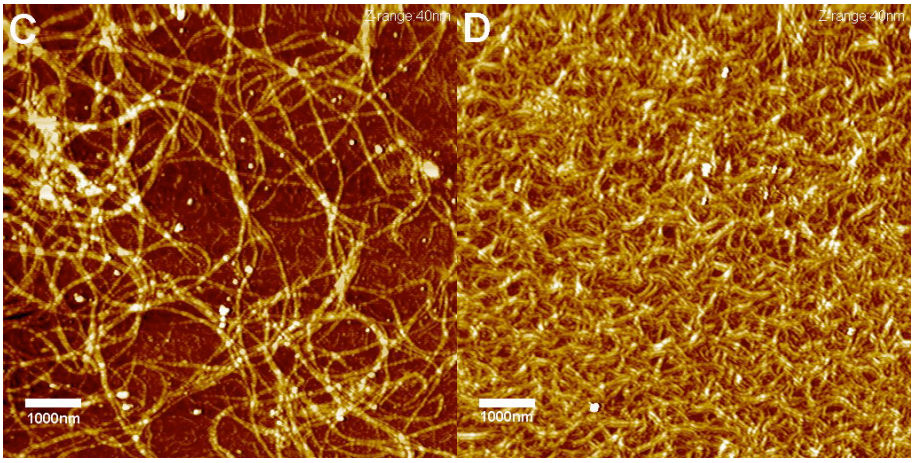


Fig 3. AFM images of collagen type I and III bound to glass from acid or PBS solutions.

Scanning was done by tapping mode in liquid (area 10 μm x 10 μ m, Z-range: 40nm, resolution: 512 x 512 pixels). Images of collagen bound from acetic acid (A, C) and from PBS solution (B, D), human collagen type I (A, B) type III (C, D) PBS solution.

Table 1. Collagen binding from acid solution or buffers with different pH and various salt concentrations.

A.

	Without salt		With salt	
Collagen binding conditions (type I)	Y±SE detection by Ab binding	Y±SE detection by VW binding	Y±SE detection by Ab binding	Y±SE detection by VW binding
acetic acid pH 3	0.170 ± 0.047	0.108 ± 0.027	0.277 ± 0.084	0.243 ± 0.028
Sodium acetate pH 4	0.371 ± 0.122	0.249 ± 0.036	0.434 ± 0.081	0.358 ± 0.084
Phosphate pH 6.4	0.257 ± 0.065	0.170 ± 0.064	0.456 ± 0.130	0.327 ± 0.061
pH 7.4	0.358 ± 0.041	0.273 ± 0.142	0.634 ± 0.074	0.510 ± 0.030
pH 8	0.300 ± 0.047	0.169 ± 0.053	0.561 ± 0.119	0.454 ± 0.025
Carbonate pH 9.2	0.213 ± 0.025	0.152 ± 0.062	0.425 ± 0.139	0.399 ± 0.103

B.

	Without salt		With salt	
Collagen binding conditions (type III)	Y±SE detection by Ab binding	Y±SE detection by VW binding	Y±SE detection by Ab binding	Y±SE detection by VW binding
acetic acid pH 3	0.177 ± 0.065	0.144 ± 0.054	0.241 ± 0.123	0.238 ± 0.065
Sodium acetate pH 4	0.347 ± 0.060	0.272 ± 0.081	0.359 ± 0.071	0.420 ± 0.086

Phosphate pH 6.4	0.270 ± 0.053	0.280 ± 0.081	0.398 ± 0.146	0.438 ± 0.087
pH 7.4	0.407 ± 0.071	0.413 ± 0.071	0.505 ± 0.088	0.584 ± 0.024
pH 8	0.315 ± 0.110	0.326 ± 0.095	0.383 ± 0.136	0.457 ± 0.137
Carbonate pH 9.2	0.199 ± 0.037	0.230 ± 0.110	0.382 ± 0.121	0.408 ± 0.121

Human collagen type I (A) and type III (B) were diluted with the following buffers (20 mM): Sodium acetate buffer, pH 4.0, phosphate buffer, pH 6.4, 7.4, and 8, carbonate buffer, pH 9.2 and acetic acid, pH~3 in the absence or presence of salt (sodium chloride) at a final concentration of 0.15 M.
Surface bound collagen was detected by specific antibodies against collagen (Ab binding) or via VWF binding and antibody against of VWF (VW binding), as describe in materials and methods.
Nonlinear fitting of the rOD-s in response to the concentration of the collagen resulted in saturation like curves. Y values were calculated from each of the curves at the Kd of the collagen diluted in PBS. (Optical densities related in each measurement to the OD value of the well, which was coated with 20 μg/ml collagen in PBS in order to obtain rOD).

Table 2. Collagen binding from acid solution or neutralized coating buffers

Collagen coating conditions	Human Collagen Type I		Human Collagen Type III	
	Y±SE detection by Ab binding	Y±SE detection by VW binding	Y±SE detection by Ab binding	Y±SE detection by VW binding
PBS	0.594 ± 0.017	0.572 ± 0.023	0.591 ± 0.025	0.570 ± 0.019
AA	0.124 ± 0.074	0.047 ± 0.037	0.168 ± 0.06	0.102 ± 0.057
AA+Na_2HPO_4	0.432 ± 0.086	0.420 ± 0.017	0.410 ± 0.145	0.513 ± 0.077
s(AA)+PBS	0.088 ± 0.006	0.027	0.024	0.224 ± 0.015
tAA+Na_2HPO_4	0.486 ± 0.054	0.371 ± 0.092	0.409 ± 0.065	0.596 ± 0.224

Human collagen type I and type III, 2 mg/mL stock solutions, were diluted with PBS or with 50mM acetic acid (AA). 100 μL of the solutions were applied into the high binding capacity ELISA plate wells and incubated overnight at 4°C. Na_2HPO_4 was added into some of the wells containing collagen diluted in 50 mM acetic acid to neutralize acid (AA+ Na_2HPO_4). Next day liquid from wells containing acetic acid diluted collagen were transferred into new wells. Na_2HPO_4 was added into these new wells to neutralize the acid and kept at 4°C overnight (tAA+ Na_2HPO_4). After the transfer of acetic acid solution, unfilled wells either were washed with PBS and proceeded for measurement of surface bound collagen or incubated with PBS overnight before proceeding (S(AA)+PBS). Collagen and VWF were detected as described in material and method. Calculation was done as it written in text of Table 1.

What is known on this topic?
The triple helical domain conformation of collagen is of crucial importance, since the three-dimensional structure is needed for recognition of collagen by its ligands.
Many different collagen surfaces/matrices are used to study VWF and platelet binding in the scientific literature
Stability of collagen solutions at neutral pH was better than acidic or basic pH
Polish article
Ruggeri

What does this paper add?
Presenting evidence that collagen coating of surfaces from acid solution for VWF binding and platelet adhesion studies are very uncertain, however after neutralization and in the presence physiological salt con the molecules remained in solution result in good coating AFM technique measurements using antibody against collagen, or VWF binding,
Salt concentration at (2x Kd) mM of collagen solutions facilitate coating, and the VWF binding to this surfaces is optimal

Melléklet 2. (közlemény angol nyelvű írása folyamatban)

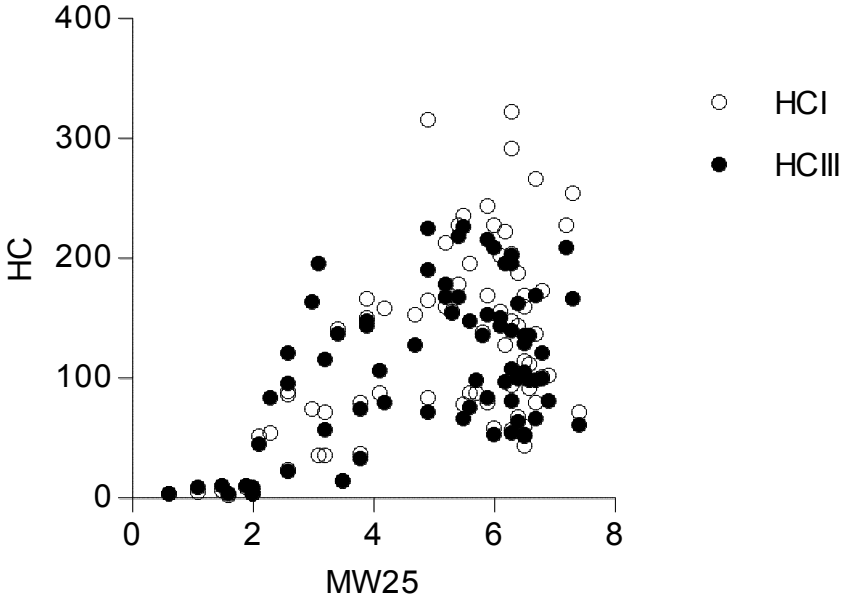
I-es és III-as típusú humán kollagén alkalmazása a von Willebrand faktor aktivitásának jellemzésére
VWF bindig activity of humn collagen Type I and III.
Szekeres-Csiki Katalin, Udvardy Miklós László, Bézi Andrea, Hársfalvi Jolán

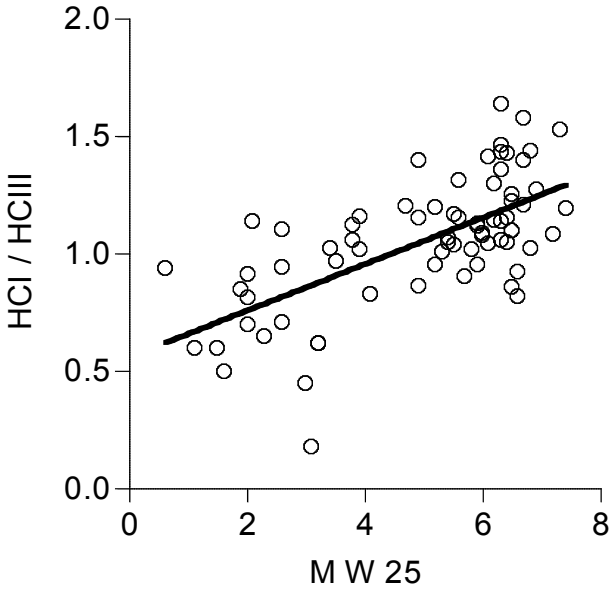
Journal of Thrombosis and Haemostasis

A von Willebrand faktor (VWF) egy nagy molekulatömegű azonos alegységekből felépülő glikoprotein, mely alegységenként kollagén és thrombocyták számára szolgáló kötőhellyel is rendelkezik. Érsérülések során a VWF a szabaddá váló szubendoteliális kollagénhez kötődik, majd a thrombocyták az immobilizált VWF molekulákhoz kapcsolódnak. A megfelelő hatékonyságú thrombocyta adhézióhoz szükséges a nagy molekulatömegű – feltehetőleg nagyobb aviditású – VWF multimerek (HMWM) jelenléte. A nagy multimerek mennyiségének egyik gyakorlati diagnosztikában használt módszere a VWF kollagén kötőképességének mérése (VWF kollagén kötő aktivitás - VWF:CB). Ez a módszer közvetett információt szolgáltat a nagy multimerek jelenlétéről, ugyanakkor kényegesen könnyebben kivitelezhető, mint a közvetlen információval szolgáló VWF SDS-elektroforézis. Több, különböző kollagén típust használó VWF:CB teszt is ismert. Egy hipotézis szerint a különböző kollagéntípusok és a VWF kölcsönhatásának molekuláris mechanizmusa eltér. Azonban közvetlen bizonyítékot szolgáltató, statisztikailag jól elemzett eredmények erre nincsenek. Ha a hipotézis igaz, akkor a különböző kollagént használó tesztek eredménye ilyen módon – a nagy multimerek tekintetében is – várhatóan eltérő.
Arra elég sok közlemény utal, hogy a VWF szint emelkedik a thrombotikus állapotokban. Mivel a VWF aktivitása a multimerizációjának foka között összefüggés van, célunk annak az összefüggésnek a vizsgálata, hogy a nagy multimerek többlete thrombosis rizikó jelzője lehet.

Ahhoz, hogy ezt a kérdést vizsgáljuk, egy megbízható VWF aktivitás módszerrel kell rendelkeznünk. Az alábbi **munka közvetlen célja volt** különböző típusú humán kollagének nagy VWF multimereket kötő képességének összehasonlítása.
A vizsgálat során I-es és III-as típusú humán kollagénekkel, arányaiban különböző mennyiségű, nagy VWF multimereket tartalamzó mintákkal mért VWF:CB eredményeket hasonlítottuk össze VWF elektroforézis és egy VWF funkcionális teszt (VWF risztocetin kofaktor aktivitás - VWF:RCo) eredményével.
Vizsgálatainkat normál multimer szerkezetű (n= 49) és kevesebb nagy multimert tartalmazó (n= 25) plazma minták felhasználásával végeztük, melyeket 3.2% Na-citráttal alvadásgátolt vénás vérből dupla centrifugálással (2000xg, 20 perc) nyertünk és -70 °C-on tároltunk. A méréshez 96 lyukú mikrotiter lemezt ecetsavban oldott (2mg/ml, 50mM ecetsav) majd PBS-ben (10μg/ml) hígított humán I-es vagy humán III-as típusú kollagénnel (C7774 és C4407 Sigma) fedtünk egy estén át 4 °C-on, majd mosás után a plazma minták két hígításával (400x és 800x PBS-T-ben) 1 órán át 37 °C-on inkubáltuk. Újabb mosást követően a kollagénhez kötött VWF-t HRPO-val jelzett poliklonális antitesttel (P0226, DAKO) és TMB szubsztráttal detektáltunk. Kalibrátorként a Siemens cég Standard Human Plasma készítményét használtuk. A von Willebrand antigén szintet (VWF:Ag) azonos elvű szendvics ELISA technikával határoztuk meg, de ekkor az mikrotiter lemezt poliklonális anti-VWF antitesttel (A0082, DAKO) fedtük. Risztocetin kofaktor aktivitás (VWF:RCo) mérést aggregációs módszerrel végeztük, a multimer szerkezet kvantitatív értékelését (MW_{25}) a jelentés más részében részletezett saját módszerünkkel elvégeztük.
Eredmények Egyes közlemények szerint még az azonos gyártási sorozatból származó kollagén preparátumok különböző oldatai is más mértékben képesek a kollagén kötésére. Ennek vizsgálatára humán I és III típusú kollagént három különböző napon feloldottunk és azonos minták felhasználásával (VWF:Ag 10-400%) vizsgáltuk a kollagén kötés mértékét, de statisztikailag szignifikáns eltérést nem találtunk. I-es és III-as típusú kollagénhez való kötődést összehasonlítva VWF:Ag 10-400% közötti mintákon (n=73) a Spearman korrelációs együttható r = 0,66 volt, az ortogonális regressziós egyenes (Demming) meredeksége 0,757± 0.0382, tengelymetszete 15.27 ± 5.617. Összehasonlítottuk a multimer méretet közvetlenül leíró MW_{25} paramétert és a hemosztatikus aktivitás jellemzésére használt VWF:CB \ VWF:Ag (n=73) paramétert, amelyet a kevesebb nagy multimert tartalmazó minták szűrésére is használnak a diagnosztikai laboratóriumokban. I-es típusú kollagén esetében a Spearman korrelációs együttható r = 0,66 volt, a regressziós egyenes meredeksége 0,214± 0.0234, míg III-as típusú kollagén esetében az értékek r = 0,5, meredeksége 0,127± 0.0250 adódtak. A két típusú kollagén nagy multimer kötésének összehasonlításához megvizsgáltuk ugyanazon minta két kollagénen mért VWF kötés hányadosának és az MW_{25} paraméter összefüggését is, ebben az esetben az r=0,57, meredekség 0,091±0,0150, tengelymetszet 0,625±0,0797 volt.
Értékelés A kollagén VWF kötő képességének vizsgálatát leggyakrabban két okból végzik el. Kézenfekvő a teszt elvégzése a VWF olyan – ritka – mutációinak gyanújakor, amelyek megváltoztatják a VWF kollagén kötő helyét, ezáltal okozva súlyos thrombocyta funkciós zavart. Sokkal gyakrabban a nagy multimerek jelenlétének igazolásához kerül sor a vizsgálatra, amely a nagy multimerek nagyobb mértékű kollagén kötése által szolgáltat erről közvetett információt. Ez a második alkalmazás azon az állati kollagének megfigyelésével

nyert elméleten alapul, hogy a nagyobb multimerek nemcsak a thrombocytákhoz, de a kollagénhez is hatékonyabban kötődnek, ugyanakkor ebből a szempontból az emberi kollagéneket még nem hasonlították össze. Először azt az irodalomban leírt állítást vizsgáltuk, hogy a különböző időben feloldott kollagén preparátumok, mégha azonos gyártási sorozatból is származnak, eltérést mutathatnak VWF kötő képességükben. Eredményeink szerint azonban a megfelelő gondossággal végzett előkészítés (friss ecetsav, oldási idő betartása, megfelelő puffer használata) esetén ilyen eltérések elkerülhetőek. Méréseink szerint mind a humán I mind a III típusú kollagén kötése széles tartományban arányosságot mutat a multimer szerkezettel, de a direkt összehasonlítás alapján (HCI/HCIII vs MW_{25}) az I típusú kollagén ebből a szempontból érzékenyebb.




2.0
1.5
1.0
0.5
0.0
HCI / HCIII
0
2
4
6
8
M W 25

Melléklet 3. (szakmai és nyelvi lektorálás folyamatban)

Journal of Thrombosis and Haemostasis

Category of the communication: REGULAR ARTICLE

Title of the article

Increase in Thrombin Generation and von Willebrand Factor Levels after Laparoscopic Radical Prostatectomy

Authors
Matyas Benyo[2], Tibor Flasko [2] Zoltan Batta[1] Zsuzsanna Szabo[1] and Jolan Harsfalvi[1]

[1]Clinical Research Center and [2]Department of Urology, Medical and Health Science Center, University Debrecen, Hungary

A word count of the text: 2869

Corresponding author
Jolán Hársfalvi, Clinical Research Center, Medical and Health Science Center, University of Debrecen, 98 Nagyerdei krt., Debrecen, H-4012, Hungary. Tel: +36 52 431 956; fax: + 36 52 340 011; e-mail: harsfalv@med.unideb.hu

Acknowledgements
This work was supported by OTKA K62317, OTKA-NKTH NI 69238 (Hungarian Scientific Research Funds).

Summary
Malignant neoplasms and long time surgical procedures came on increased risk of thrombotic events, like pulmonary embolism or deep vein thrombosis. The authors' aim was to determine the potential thrombotic risk associated with laparoscopic radical prostatectomy in cases of prostate cancer.
10 patients were selected, who were studied and followed up after laparoscopic radical prostatectomy. 1 day prior, 1 hour, 6 days, 1 month and 10 months after radical prostatectomy blood tests were done regarding haematology, haemostatic and thrombin potential by thrombin generation assay (TGA). The last one is a global test representing the peak thrombin generated by the addition of recombinant tissue factor and phospholipid to citrated plasma. It provides information on the balance of humoral factors and their inhibitors. Von Willebrand factor parameters were also tested as a main counter player of the cellular factors.
Baseline thrombin generation level was slightly elevated, as compared with to controls. The majority of the patients had a 20% increase in thrombin generation immediately after the surgery and had the highest peak thrombin value 6 days after the procedure (peak thrombin >400 nM). The preoperative values were high in two of the cases because of the previous cardiovascular disease. Peak thrombin level increased immediately after the surgery in one of the cases, when the duration of the operation was longer and in other case delayed increase in peak thrombin was experienced because of long hospitalization time. Peak thrombin levels generated in the plasmas of all patients returned to below the preoperative value after ten months. In addition, peak thrombin generation was not influenced by LMWH administered once a day. The von Willebrand factor level elevated 2 to 3-fold, in the plasma obtained right

after the operation, remained high at 6 days and reduced to 50% only by one month. The VWF levels were in the reference range at the one-year check up.
According to our results TGA show that the balance of the plasma haemostatic system shifted to an increased thrombin generation potential during and after laparoscopic radical prostatectomy, focusing on (1) the risk of thrombotic events and allow (2) the follow up of the anticoagulation. VWF levels were also elevated and declined more slowly, indicating a risk of thrombotic events originated at arterial or capillary sites.
The authors' plan is to extend the number of data to perform a RCT (randomized controlled trial), which compares the different methods of thromboprophylaxis (LMWH, PCB – pneumatic compression boots etc.) and the different techniques of radical prostatectomy. The final purpose is to complete the guideline for thromboprophylaxis regarding the research data and findings of international studies in different prostate cancer treatment options.

Introduction

Cancer of the prostate (PCa) is now recognized as one of the most important medical problems facing of the male population. PCa is the most common solid neoplasm, with an incidence rate of 214 cases per 1000 men, outnumbering lung and colorectal cancer [1]. Furthermore, PCa is currently the second most common cause of cancer death in men [2] Since effective screening programs, finding of localized PCa patients and setting up early diagnosis are available, treatment is more effective. Currently, radical prostatectomy is the only treatment for localized PCa that has shown a cancer-specific survival benefit when compared to conservative management in a prospective, randomized trial [3]. Surgical expertise has decreased the complication rates and improved cancer cure [4].
Pelvic surgery – like radical prostatectomy – is associated with an increased risk of thrombosis, leading to the development of deep venous thrombosis (DVT) and pulmonary embolus (PE). During surgery, there are major disturbances in the coagulation and inflammatory systems because of the hemorrhage/hemodilution, blood transfusion, and surgical stresses. Procoagulant activity and inflammation are increased postoperatively; thus, antithrombotic therapy is required to prevent perioperative thrombotic complications. However, a limitation of current treatment is that conventional clotting tests do not reflect the entire physiological processes of coagulation making optimal pharmacologic therapy difficult [5]. Measurement of thrombin generation from plasma identifies patient at risk for thrombotic complications and provide information about antithrombotic treatment [6,7]. Peak thrombin generation levels were positively correlated with levels of other hemostatic factors. Specifically, in the ARIC study, there was a positive association between peak thrombin generation levels and FVIII and VWF *irodalom*.
Most reports regarding the risk of thrombotic events in the peri-operative period -especially after radical prostatectomy- are based on case-related studies or clinical symptoms [8 9 10]
Prospective, preferably laboratory-based, randomized trials are needed to properly evaluate the nature of the hypercoagulable state after these surgical procedures.
There have been significant advances in the management of perioperative hemostasis and thrombosis because of the introduction of novel hemostatic and antithrombotic drugs.
Estimation of an individual's potential to generate thrombin may correlate more closely with a hypercoagulable state, compared to traditional routine coagulation tests.
Our aims were to determine the potential risk of thrombotic events after laparoscopic radical prostatectomy, following the plasma thrombin generation capacity or thrombin potential during and after this surgical procedure, and to test the effectiveness of LMWH (Low Molecular Weight Heparin) administration.

Materials and methods

10 patients were selected and followed up who had laparoscopic radical prostatectomy (in 9 cases extraperitoneal, and 1 transperitoneal way). Selection of the patients was performed according to the following. Patients who had myocardial infarct, deep vein thrombosis, stroke with residual symptoms, any other kind of malignancy or who were treated by hormontherapy were excluded from the study.

The mean age was 63 years (range 50 to 70). One patient had minor stroke without residual symptoms and cardiac rhythm disorder (patient # 2), and one had diabetes (# 4) in their cardiovascular history. These persons were rated in a higher risk group of complication is anesthesia (American Society of Anesthesiologist -ASA IIIversus ASA II. All patients were overall in good condition, and the preoperative staging predicrted localized PCa. The results of Gleason Scoring were below 7 in 9 cases (range 3 to 8) after prostate biopsies, and the average PSA (prostate specific antigen) level were 9.28 ng/ml (range 4.37 – 14.1). The mean operation time was 191 minutes (range 120-260), and the mean narcosis time was 250 minutes (range 190-330).

The thromboprohylaxis was used by the guide of the surgical guidelines. LMWH was administered (patients 2 and 4 6000 IU/ml/day, the others 4000 IU/ml/day enoxaparine) every evening during the hospital stay and continued in the first month after the laparoscopic procedure. The blood sampling was performed in every case at least 12 hours after drug administration. 1 day prior (A sample), 1 hour (B sample), 6 days (C sample), 28 days (D sample) and 10 months (E sample) after radical prostatectomy blood was collected from the antecubital vein into the appropriate Vacutaine tubes (Beckdon Dickinson, England).

Routine laboratory measurements like total prostate specific antigen (tPSA), haematology parameters like red blood cell (RBC), white blood cell (WBC), platelet count (PLT), prothtombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), fibrinogen (Fng), D-Dimer (D-D), antithrombin III (ATIII) and low molecular weight heparin (LMWH) were carried out according to the protocols of the Department of Pathobiochemistry and Molecular Biology.

For thrombin generation assay (TGA) 9 parts of venous blood collected to 1 part of sodium citrate solution (0.11 mol/L) was centrifuged at 20 °C for 15 minutes at 2.500 x g,. Plasma separated into a new tube was centrifuged again, and aliquots of the supernatant were stored at -70°C. Analysis was performed within three weeks. Individual aliquots were sampled into black 96 well plates (Greiner GmbH, Germany) immediately after they were taken from the deep freezer, defrosted in a 37°C water bath by titling for 15 minutes, and vortex mixed for 5 seconds, and, TGA were carried out using TECHNOTHROMBIN® TGA kit with reagent C (RC) and BIOTEK Flx800 reader with KC4 TGA software (Technoclone GmbH, Austria).

VWF:Ag was measured by ELISA(Cejka 1982). VWF:CB was measured as Ag, but Type III collagen (Sigma, St Louis, MO, USA) was used for coating. Optical density reading was carried out using Infiniti 200M (Tecan Trading AG, Switzerland), standard curve fitting (four-parameter Marquit) and the calculations were carried out using the Magellan software of the instrument.

Statistical tests were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com

Results

To determine the potential risk of thrombotic events after laparoscopic radical prostatectomy, following the plasma thrombin generation capacity or thrombin potential during and after this surgical procedure along with the VWF level, and to test the effectiveness of the conventional LMWH (Low Molecular Weight Heparin) administration we present here the results of 10 selected patients.

There were not severe intraoperative complications. The histology results were between pT2a and pT2c without lymph node metastasis, only patient's #9 result showed pT3b PCa.In this case postoperative irradiation and hormontherapy were administered. All of the patients were mobilized on the first, and most of them (except of patient #1 and #10) were emitted on the 6th postoperative day. In one case lymph leakage occurred in the early postoperative period, which recovered in 2 weeks (patient # 10). Except two persons the patients were continent (1 pads per day) two months after operation. After 1 year follow-up 80% still had mild erectile problems. There were no clinical symptoms of thrombosis among the patients after one-year follow-up.

Table 1. presents the essential results of the blood tests. Elevated tPSA levels decreased and remained zero during the follow-up period after prostatectomy. There were no unexpected changes in basic hematology and hemostasis parameters like RBC-s, WBC-s, platelet count, and PT, APTI, TT. The increase of the WBC is physiological reaction right after the procedure. Fibrinogen level almost doubled and D-dimer level increased 4-fold by the 6th postoperative day. As other parameters of thrombin generation [prothrombin fragments 1.2 (F1.2), thrombin-antithrombin complex (TAT)] were expected to change paralell with fibrinogen and D-dimer, they were not tested.

Table I. Routine tests' results

Parameter	Reference value	A	B	C	D	E
tPSA [ng/mL]	<4.1	11 5.9-14	10 6.6-20	1.8 1.1-3.1	0.0 0.0-0.04	0.0 0.0-0.0
RBC [T/L]	4.7-6.1	6.0 4.0-8.0	6.4 4.7-8.1	4.8 4.0-5.0	4.3 3.9-4.9	4.3 3.4-4.7
WBC [G/L]	4.5-10.8	6.6 3.9-2.3	16 10-18	7.5 4.5-11	6.0 4-8	6.4 4.7-8.1
PLT [G/L]	150-400	208 127-239	175 135-252	251 172-304	198 148-370	229 179-331
PT [sec]	8.8	8.1 7.9-8.8	8.8 8.3-9.1	7.7 7.3-8.2	8.3 7.8-9.2	8.0 7.8-8.1
APTT [sec]	28.1	28 27-30	25 24-33	27 25-31	27 26-31	29 28-32
TT [sec]	16.2	17 16-18	18 17-18	16 15-18	17 17-18	18 18-19
Fng [g/L]	1.5 - 4	3.9 2.6-4.1	2.8 2.4-3.3	6.2 4.9-6.9	3.9 3.2-5.7	ND
D-D [μg/L]	<0.5	0.26 0.25-0.26	0.80 0.51-1.70	1.10 0.86-1.30	ND	ND
TGA [nM]	229 122-297	267 221-319	261 190-376	422 267-649	268 240-377	264 24-279
LMWH [IU/mL]	0.5-1.2	ND	ND	0.05 0-0.23	ND	ND
ATIII [%]	106±18	119 106-145	102 91-147	116 90-140	104 88-141	95 73-140

Blood was collected1 day prior (A), and 1 hour (B), 6 days (C), 28 days (D) and 10 months (E) after radical prostatectomy, at least 12 hours after LMWH administration.

Routine laboratory measurements like total prostate specific antigen (tPSA), haematology parameters like white blood cell (WBC), red blood cell (RBC), platelet count (PLT) and haemostasis parameters, like prothtombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT),

fibrinogen (Fng), D-Dimer (D-D), antithrombin III (ATIII) were tested according to the routine laboratory tests (http://www.kbmpi.hu).

Thrombin generation measured by TGA was evaluated by the peak thrombin (nM), the area under the curve (AUC, which is identical with endogen thrombin potential in CAT assay), the lag phase and V-index. The changes of peak thrombin levels and the area under the curve or the endogen thrombin potential were not different. We found the peak thrombin levels, what is presented on Fig. 1., are more comparable to published data. These results were grouped by the followings: Patients #2 and #4 had higher risk of complication by anesthesia risk stratification (ASA III.) Patient # 5 had longer operation and narcosis time because of adhesion due to previous abdominal surgery of perforated appendicitis. Patient #1 had mild subcutan abscess, its recovery took 20 days from the 10th postoperative day. All patients had at least one period of increased or high level of peak thrombin (>400 nM). Patients #3 and #6-10 had the maximum peak thrombin level one week after procedure. Peak thrombin levels of patients #2 and #4 were constant high. Patient # 5 had increased peak thrombin only right after the operation. In case of patient #1 the long time of hospitalization, because of inflammation resulted in delayed increase of peak thrombin level.

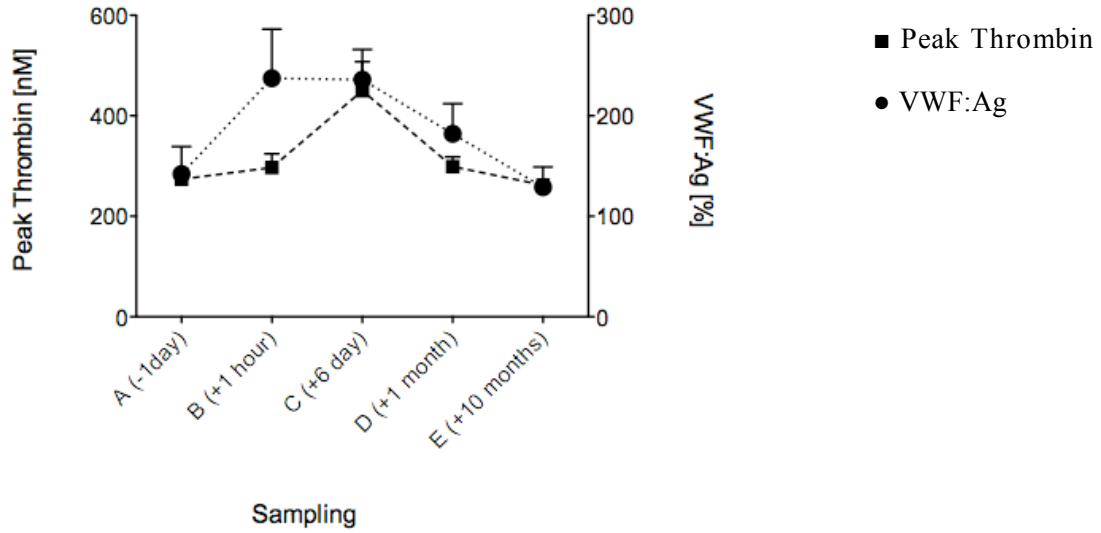


Fig 1. also shows the VWF levels parallel to peak thrombin. The VWF measured 1 hour after the operation increased 2 to 5-fold, and slowly declined

Discussion

Laparoscopic radical prostatectomy is safe, oncological efficient and the life quality changes are acceptable for the patients in the treatment of PCa[11],[12],[13]. Our study's first approach was to follow the potencial thrombotic risk during and after this procedure in order to reduce the postoperative morbidity.

Many clinical trials proved significance of the increased risk of thrombotic events after pelvic surgery – like radical prostatectomy. Venous thromboembolism was adjudicated as the most common cause of death (46.3%) after cancer surgery according to a prospective observational study in patients undergoing general, urologic, or gynecologic surgery[14], which led to compose guidelines on the major fields of medicine. The everyday prevention of these complications is still not unified, no consensus exists in the literature and the world lacks evidence based guideline on the ideal management of thromboprophylaxis in patients undergoing radical prostatectomy[9],[15] In European countries pharmacological agents are administered routinely, while in the USA non-pharmacological methods are favored[10]. In

European countries pharmacological agents are administered routinely, while in the USA non-pharmacological methods are favored.
In Europe's practice heparins, notably low-molecular-weight heparins (LMWHs), are given in standard doses.
Worldwide 52% (between-country range 35%–73%) of hospitalized patients are judged to be at risk of VTE [Cohen AT, ENDORSE study, Lancet 2008; 371: 387–94]. Hospitals that implement active, multifaceted quality improvement initiatives to support the introduction of prophylaxis protocols have seen improvements in the appropriateness of prophylaxis prescribing and better clinical outcomes (S . L . COHN JTH 2009).
Measurement of thrombin generation is a good indicator of heparin effect [16] The patients of our study were administered by the routine LMWH as described in the surgical guidelines, but LMWH effect could not be detected in these samples 12 hours after drug administration neither by the Xa activity measurement of LMWH, nor by the TGA. Because all measurements showed the heparin level below the therapic range, the effectiveness of this practice of LMWH administration is questionable.
The routine hemostatic and hematology parameters due to these results are not suitable for special analysis, these are not correlated with the peak thrombin activity, which is already proved by international findings.
The TGA is a sensitive marker for estimating the risk and predicting of thrombotic events and many reports present the benefits of this test on the field of cardiovascular diseases [6, 7]. The thrombotic risk of urological surgical procedures is still not identified by laboratory methods. Tissue factor-specific procoagulant activity of plasma microparticles measured by TGA contributes to intravascular coagulation activation in patients with early-stage prostate cancer and may represent a potential link between hypercoagulability, inflammation, and disease progression[17]. Our results corroborate this finding.
Due to the analysis of large population studies the majority of thrombotic events occur in the first postoperative week or 4 weeks after the surgical procedure [14]. We expected increases of peak thrombin level based on these data, that's why we collected the blood samples on the 6th (sample C) and the 28th (one month, sample D) postoperative day. These are the periods, when patients have the highest risk for thrombotic events. By the majority of the present cases (7 of 10) the increase in peak thrombin levels proved our predictions. All patients had at least one period of increased or high level of TGA (peak thrombin activity >400 nM). The cutoff value of 400 nM is from the publication of Hron et al. [18]. It is well known that cardiovascular disease in patient's history (thrombotic event, cardiac arrhythmia, diabetes) increases the possibility of malignant thrombin generation and activity [19]. Our results suggest that these precursors may have a preconditioning effect as well. By two examined patients the peak thrombin levels were constant high, but did not increase after the procedure. In one of our cases we experienced elevated peak thrombin levels after long time narcosis and hospitalization due to longed immobility.

Conclusion

It is common that cardiovascular diseases, malignant neoplasms and long time surgical procedures mean increased risk of thrombotic events. Due to the analysis of large number clinical studies the expected postoperative period, when patients have the highest risk for thrombotic events are 6 days or 1 month after operation. The authors' study supports these findings with laboratory results of thrombin generation and activity, which is a sensitive marker for prediction of thrombotic events. VWF parameters were also showed potential risk of thrombus formation in high shear vessels. Proper prevention and hemostatic follow up of patients after radical prostatectomy will decrease the number of thrombotic events, which can assist us reducing prostate cancer mortality.

References

1. Boyle P, Ferlay J. Cancer incidence and mortality in Europe, 2004. Ann Oncol. 2005;16:481-488.
2. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. CA Cancer J Clin. 2008;58:71-96.
3. Bill-Axelson A, Holmberg L, Filen F, et al. Radical prostatectomy versus watchful waiting in localized prostate cancer: the Scandinavian prostate cancer group-4 randomized trial. J Natl Cancer Inst. 2008;100:1144-1154.
4. Augustin H, Hammerer P, Graefen M, et al. Intraoperative and perioperative morbidity of contemporary radical retropubic prostatectomy in a consecutive series of 1243 patients: results of a single center between 1999 and 2002. Eur Urol. 2003;43:113-118.
5. Tanaka KA, Key NS, Levy JH. Blood coagulation: hemostasis and thrombin regulation. Anesth Analg. 2009;108:1433-1446.
6. Sumann G, Fries D, Griesmacher A, et al. Blood coagulation activation and fibrinolysis during a downhill marathon run. Blood Coagul Fibrinolysis. 2007;18:435-440.
7. Orbe J, Zudaire M, Serrano R, et al. Increased thrombin generation after acute versus chronic coronary disease as assessed by the thrombin generation test. Thromb Haemost. 2008;99:382-387.
8. Secin FP, Jiborn T, Bjartell AS, et al. Multi-institutional study of symptomatic deep venous thrombosis and pulmonary embolism in prostate cancer patients undergoing laparoscopic or robot-assisted laparoscopic radical prostatectomy. Eur Urol. 2008;53:134-145.
9. Galvin DJ, Mulvin D, Quinlan DM. Thromboprophylaxis for radical prostatectomy: a comparative analysis of present practice between the USA, the UK, and Ireland. Prostate. 2004;60:338-342.
10. Koya MP, Manoharan M, Kim SS, Soloway MS. Venous thromboembolism in radical prostatectomy: is heparinoid prophylaxis warranted? BJU Int. 2005;96:1019-1021.
11. Stolzenburg JU, Rabenalt R, Do M, et al. Endoscopic extraperitoneal radical prostatectomy: oncological and functional results after 700 procedures. J Urol. 2005;174:1271-1275; discussion 1275.
12. Rassweiler J, Stolzenburg J, Sulser T, et al. Laparoscopic radical prostatectomy--the experience of the German Laparoscopic Working Group. Eur Urol. 2006;49:113-119.
13. Menon M, Shrivastava A, Tewari A. Laparoscopic radical prostatectomy: conventional and robotic. Urology. 2005;66:101-104.
14. Agnelli G, Bolis G, Capussotti L, et al. A clinical outcome-based prospective study on venous thromboembolism after cancer surgery: the @RISTOS project. Ann Surg. 2006;243:89-95.
15. Inc. AUAEaR. Best practice policy statement for the prevention of deep vein thrombosis in patients undergoing urologic surgery. 2008:29.
16. al Dieri R, Alban S, Beguin S, Hemker HC. Thrombin generation for the control of heparin treatment, comparison with the activated partial thromboplastin time. J Thromb Haemost. 2004;2:1395-1401.
17. Haubold K, Rink M, Spath B, et al. Tissue factor procoagulant activity of plasma microparticles is increased in patients with early-stage prostate cancer. Thromb Haemost. 2009;101:1147-1155.
18. Hron G, Kollars M, Binder BR, Eichinger S, Kyrle PA. Identification of patients at low risk for recurrent venous thromboembolism by measuring thrombin generation. JAMA. 2006;296:397-402.

19. Granger CB, Miller JM, Bovill EG, et al. Rebound increase in thrombin generation and activity after cessation of intravenous heparin in patients with acute coronary syndromes. Circulation. 1995;91:1929-1935.