Thrombosis Research 129 (2012) e29–e35

Contents lists available at SciVerse ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Coating conditions matter to collagen matrix formation regarding von Willebrand factor and platelet binding☆

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ARTICLE INFO

Article history:
Received 19 May 2011
Received in revised form 30 August 2011
Accepted 25 September 2011
Available online 5 November 2011

Keywords:
Collagen matrix
Von Willebrand factor
Platelet adhesion
Thrombus formation

ABSTRACT

Introduction: Von Willebrand factor (VWF) and platelet binding needs a uniform collagen matrix therefore we aimed to find an optimal condition for the preparation of human type-I and type-III collagen matrices.
Method: The effects of pH, salt and ligand concentration and binding time were tested when collagen matrices were prepared by adsorption. Surface-bound collagen and collagen-bound VWF measured by specific antibodies. Platelet adhesion was tested under flow conditions at a shear rate of 1800 s^{-1} for 2 min. Matrices and platelets were visualized by atomic force and scanning electron microscope.
Results: The extent of human collagens type-I and III binding to the surface was 10 and 4 times greater and binding was maximal under 8–16 hours, when coated from physiological buffer solution versus acid solution. Collagen fibrils were more developed and platelet adhesion was higher, with more organized and denser aggregates. VWF binding was parallel to the surface bound collagen in both collagen types.
Conclusion: Collagen coating of surfaces for VWF binding and platelet adhesion studies is very variable from acid solution. Our experiments provide evidences that neutralizing the acid and adding NaCl in physiological concentration, thereby facilitating formation of collagen fibril molecules in solution, results in efficient coating of human type-I and type III collagens, which then bind normal VWF equally well.

Introduction

Platelet adhesion to exposed subendothelial matrices at the sites of damage to the vessel wall is the first step in haemostasis under both physiological and pathological conditions. Among components of the subendothelial matrix, collagen, and its role in haemostasis and thrombosis has 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 damaged vasculature [1]. Both types 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, these monomers, 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 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, collagen binds VWF from circulating blood and then platelets are captured and tethered on VWF immobilized by collagen, through the platelet surface receptor GPIb-IX-V, initiating thrombus formation. This is followed by platelet activation mediated by the binding of ligands like GPVI to collagen, leading to inside-out stimulation of integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ and maintaining platelet thrombus formation [4]. The adhesive activity of the VWF is crucial in this process.

Binding of VWF to collagen can be measured quantitatively and it is recommended as a method for determining VWF adhesive activity (VWF:CB) [5]. The assay is based on the measurement of VWF molecules bound to collagen by a VWF-specific antibody, similarly to the

Abbreviations: AFM, atomic force microscopy; SEM, scanning electron microscopy; OD, optical density; rOD, relative optical density; PBS, phosphate buffered saline; VWD, von Willebrand disorder/disease; VWF, von Willebrand factor; VWF:Ag, von Willebrand factor antigen; VWF:CB, von Willebrand factor collagen binding; GP, glycoprotein; HRP, horseradish peroxidase; SC, surface coverage; AS, average size; K_d, dissociation constant.

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

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doi:10.1016/j.thromres.2011.09.030

procedure for an enzyme-linked immunosorbent assay for VWF:Ag measurement. Several VWF:CB assays have been developed, and collagen binding activity of VWF is measured routinely when differential diagnosis of von Willebrand disease is needed. Differences between ways of preparing collagen solutions and differences between ways of using them to coat microplates may influence the result significantly. In a survey of articles reporting the use of collagen-coated surfaces for VWF binding or for platelet adhesion, we noted the collagen type and its source, the concentration of collagen, the type of solvents used for coating the surface, the pH, and the coating conditions. The findings are summarized in a table (Supplementary Table 1).

It is known that collagen solution varies according to the manner in which it is prepared [6,7]. Collagen precipitation *in vitro* models *in vivo* fibril formation. Different solutions result in differences in the degree of precipitate formation. The importance of the triple helical (tertiary) or polymeric, cross-linked (quaternary) structure of the collagen in thrombosis and haemostasis is also well documented in recent studies [8]. Much progress has been made in elucidating the structure of collagen triple helices and the physicochemical basis for their stability [9-11]. These results implicate that studies on collagen-VWF binding and platelet thrombus formation need a uniform collagen matrix.

Whether possible structural changes during the coating process enable better binding of the collagen to the surface and/or VWF binding to the collagen has not been studied systematically. To be able to make a uniform matrix, we aimed to test the matrices of human collagen type-I and type-III prepared under different conditions in order to improve their ability to bind VWF and to support platelet adhesion what matters to obtain comparable results.

Materials and methods

Pepsin-digested collagens from human placenta, Sigma (Collagen type III: Catalogue# C4407, Collagen type I: Catalogue# C7774; St Louis, MO, USA); polyclonal, rabbit anti-human collagen type-III with a 10% cross-reactivity against type I (Catalogue# AB747; Chemicon Temecula, California, USA); purified VWF, Haemate P (CSL Behring, Marburg, Germany); horseradish-peroxidase (HRP)-conjugated rabbit polyclonal anti-human VWF, goat anti-rabbit IgG-HRP, Dackocytomation (Glostrup, Denmark); 96-well plates, Nunc MaxiSorp, (polystyrene, catalogue#: 44-2404-21; Wiesbaden, Germany), Propilen E.C. (polypropylene; Pecs, Hungary), Grenier (polyvinyl chloride; medium and high binding capacity, Catalogue# 655080 and 655081 respectively; Nürtingen, Germany).

Preparation of collagen matrices and measuring binding efficacy

Human collagen type-III or I was dissolved in 0.05 M acetic acid (pH 2.8) to obtain 2 mg/mL stock solutions. The stock solutions were treated differently: (1) further diluted with 0.05 M acetic acid; (2) diluted with phosphate buffered saline (PBS); (3) diluted with 0.05 M acetic acid and neutralized locally by adding Na_2HPO_4; (4) dialyzed against PBS for 2 days at 4 °C. The solutions were added into the wells (100 μL/well) at final concentration of 20, 10, 5, 2.5 μg/mL, and were incubated overnight at 4 °C.

To study the effect of pH, collagens were coated from 6 different solutions: sodium acetate 0.02 M, pH 4.0 (1); sodium phosphate 0.02 M, pH 6.4 (2), pH 7.4 (3) and pH 8.0 (4); sodium carbonate 0.02 M, pH 9.0 (5) and acetic acid 0.05 M, pH 2.8 (6); with varying concentration of NaCl. Collagen coated wells were blocked with 3% (w/v) casein in PBS for 30 minutes at room temperature. Each step followed by washing with PBS contained 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 with purified VWF (0.1 U/100 μL/well) for one hour at room temperature. The bound collagen and VWF were detected with anti-rabbit IgG-HRP or rabbit anti-human VWF-HRP (100 μL/well diluted to 1:3000 or 1:2000 in PBS-T) and the incubation continued for one hour. Then peroxidase substrate was given and the reaction was stopped with 2 M H_2SO_4, the optical density (OD) was measured by Infinite 200 M reader (Tecan TradingAG, Männedorf, Switzerland), at optimal colour development of the substrate reaction, when the collagen was coated from PBS.

Atomic force microscope (AFM)

Drops of 10 μL solutions were put onto glass coverslips and kept in a humid box for 48 h at 4 °C. The final concentrations of the collagens were 200 μg/mL in acid and 20 μg/mL in PBS solutions. Imaging was performed with a custom-made stand-alone-type AFM (Department of Biophysics and Cell Biology, University of Debrecen, Hungary; University of Twente, Enschede, The Netherlands; Zeiss Axiovert microscope, Carl Zeiss, Jena, Germany) in tapping mode with samples under water. Cantilevers with Si_3N_4 pyramidal tips (Park Scientific Instruments, FWMS-06 AU, Sunnyvale, CA) and with diameters between 10 and 30 nm were used with an average spring constant of approximately 0.06 N/m. 10×10 μm (x,y) surfaces were scanned. Images of 512×512 pixels were collected and processed with software including plane fitting and x-y flattening. Images were analyzed and processed by SPIP software (Image Metrology A/S, Lyngby, Denmark).

Blood collection

Blood was collected from healthy donors. The syringe contained low molecular weight heparin, as anticoagulant (20 U/mL final concentration, enoxaparin sodium, Aventis, Germany). All donors claimed to have abstained from taking aspirin, or other drugs known to affect platelet function, in the preceding 10 days. Written informed consent was obtained from all subjects in accordance with the Declaration of Helsinki.

Platelet adhesion

Human collagen, diluted to 100 μg/mL in PBS or 0.05 M acetic acid, was layered over thermanox coverslips (13 mm in diameter, Deckgläser) and incubated in a humid box overnight at 4 °C. The coverslips were blocked with 3% casein in PBS for 30 minutes followed by washing with PBS. The coated coverslips were mounted in the wells of an Impact-R, *in vitro* flow chamber (Matis Medical, Brussels, Belgium) and anticoagulated blood was circulated at a shear rate of 1800 s^{-1} for 2 min. Then the coverslips were washed with water, stained by May-Grünwald and analyzed by light microscope connected to a camera of the Impact-R image analysis system. The surface coverage (SC; [%]) and average size (AS; [μm^2]) of the objects on the well surface were determined.

Scanning electron microscope (SEM)

Morphology of the platelets, which had adhered after shear stress onto collagen matrix was studied by SEM. The coverslips of the platelet adhesion experiments were washed with water and then fixed with 2.5% phosphate buffered glutaraldehyde (pH 7.2), washed in the same buffer, and post-fixed by 2% osmium tetroxide, then by 2% tannic acid-guanidine hydrochloride. After dehydration in graded alcohol solution the alcohol was exchanged to Freon 112 (Du Pont Company, Wilmington, Delaware). The coverslips were air-dried, gold coated, and examined by a Jeol 840 scanning electron microscope (Jeol USA Inc, Peabodt. Mass) at the Faculty of Life Science (Bar Ilan University, Ramat Gan, Israel).

Calculations

The OD-s of different measurements were normalized by calculating the relative OD in each series. Relative OD was calculated by