SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Cellular FXIII in platelets and in macrophage derived foam cells

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1. INTRODUCTION

Blood coagulation factor XIII (FXIII) is a special coagulation factor in several respects. With the exception of FXIII, all coagulation factors with potential enzymatic activity are zymogen precursors of proteolytic enzymes, which are transformed into active coagulation factors and cleave peptide bonds during the activation of the blood coagulation cascade. FXIII, on the other hand, is a precursor of a transglutaminase enzyme (TG), which in its activated form connects the glutamine and lysine side chains of proteins with isopeptide bonds. Protease precursor coagulation factors are plasma proteins, FXIII, on the other hand, is present both in the plasma (pFXIII) and in intracellular localization (cFXIII). While the proteolytic precursor plasma factors are monomers, FXIII in plasma is of tetrameric structure (FXIII-A_2B_2) composed of two types of subunits, two potentially active A (FXIII-A) and two inhibitory/stabilizing B subunits (FXIII-B). It becomes activated by the combined action of thrombin and Ca^{2+}. Thrombin cleaves off an activation peptide from FXIII-A, then, in the presence of Ca^{2+}, FXIII-B dissociates, and active transglutaminase is formed. The main function of activated plasma FXIII is the covalent connection of fibrin α- and γ-chains and the attachment of the α_2-plasmin inhibitor to fibrin, thereby mechanically stabilizing the fibrin network formed in the terminal phase of blood coagulation and protecting it from fibrinolytic degradation.

cFXIII is a dimer consisting of two A subunits (FXIII-A_2) and, so far, its presence has been detected in 7 different cell types (with the highest concentration in platelets and monocytes/macrophages). In non-activated "resting" cells, cFXIII is located in the cytosol, but during cell activation it can be exposed to the cell surface or bind to intracellular organelles. It might exert both intra- and extracellular functions. The presence of cFXIII in platelets has been known for a long time, but a detailed understanding of its function is still to be explored. We already know that as a result of strong receptor-mediated platelet activation (the combined activation of glycoprotein (GP) VI collagen receptor and protease activated PAR1 and PAR4 receptors) at least a part of cFXIII can be brought to the platelet surface, however, the mechanism has not yet been revealed. One of the key questions is whether such surface exposure also occurs as a result of non-receptor mediated activation, when the intracellular Ca^{2+} level also increases, but mechanisms independent of the increase in Ca^{2+} level are missing. One of the important steps of platelet activation is the production of microparticles. The question is whether cFXIII is brought to the surface of the microparticles during receptor-mediated and

non-receptor-mediated platelet activation. We thought that a deeper biochemical and immuno-electron microscopic examination of the above questions could provide us with further details on platelet activation.

The cFXIII content of monocytes and macrophages was first described in our laboratory. Today we also know that cFXIII can be transferred to the surface of these cells during the activation of macrophages. Macrophages and smooth muscle cells are the major cell populations in atherosclerotic plaques. Both cell types can transform into so-called foam cells by taking in modified low-density lipoprotein (LDL) under in vitro conditions, which can significantly affect the protein composition of the cells. Macrophages contain cFXIII, whereas vascular smooth muscle cells do not. The question is whether the transformation into foam cells affects the cFXIII content of macrophages, and whether vascular smooth muscle cells become cFXIII-expressing cells as a result of their transformation into foam cells. Another question was whether cFXIII-expressing foam cells appear in atherosclerotic plaques, and if so, whether cFXIII gets out into the extracellular compartment and exerts its protein cross-linking activity there.

Our knowledge on the structure and function of FXIII is discussed in several detailed reviews, however addressing the above questions would significantly contribute to a better understanding of the effect of cFXIII and to exploring its physio-pathological role.

2. REVIEW OF LITERATURE

2.1 Structure, activation and function of coagulation factor XIII

FXIII is a blood coagulation factor responsible for the mechanical stability of the fibrin network and one of the main regulators of fibrinolysis. FXIII is a pro-transglutaminase, the active form of which (FXIIIa) belongs to the family of TGs. Beside FXIIIa, we know seven additional TGs, which are designated by Arabic numbers (TG1-TG7). TGs, including FXIIIa, catalyze an acyl transfer reaction, in which a primary amine binds to a peptide-bound glutamine residue. In the first step, a peptide-bound glutamine substrate forms thioester with the active site cysteine at position 314 of the core domain, and ammonia is released. Then, in the presence of primary amine substrate, the acyl group is transferred to an acyl acceptor amine. The amine becomes attached to the γ-glutamyl residue via isopeptide bond, and the active-site cysteine becomes deacylated. If the amine substrate is provided by an ε-amino group of a peptide-bound lysine residue, polypeptide chains become cross-linked by Nε-(γ-L-glutamyl)-L-lysyl isopeptide bond catalyzed by TG (FXIIIa). The most commonly used FXIII activity determination is based on the measurement of ammonia released during the acyl transfer reaction.

FXIII occurs in two forms: it is found in plasma and intracellularly in some cells. pFXIII has a heterotetrameric structure, a complex of two potentially active FXIII-A subunits and two carrier/inhibitory FXIII-B subunits. The molecular weight of pFXIII is 326 kDa, its concentration in plasma is 14-28 mg/L.

Unlike other TGs, FXIII-A is of dimeric structure consisting of two FXIII-A monomers (FXIII-A_2). The molecular weight of FXIII-A is 83 kDa. FXIII-A consists of four structural domens: β-sandwich, catalytic core, β-barrel 1 and β-barrel 2. An activation peptide (AP-FXIII) consisting of 37 amino acids is attached to the N-terminal end of the β-sandwich. The FXIII-A monomers have a high-affinity Ca^{2+} binding site with a prominent role in the formation of the active form. Most likely, lower affinity Ca^{2+} binding sites can also be found on the surface of subunit A, but further studies are required to clarify their functional importance.

FXIII-A is found in several cells: platelets, monocytes, macrophages, osteoblast, osteocytes, chondrocytes, preadipocytes and corneal keratocytes. FXIII-A is mainly expressed by cells of bone marrow origin, the source of the A-subunit in plasma is controversial. The gene encoding the A-subunit, F13A1, was identified in these cells, but it lacks an endoplasmic reticulum (ER) signal sequence required for secretion and is excluded from the classical ER-

Golgi pathway in nucleated cells. Platelets were proposed to be the source of the A-subunit in plasma, but this was ruled out, as levels were unchanged in thrombocytopenic mice, where FXIII-A levels in the plasma did not change despite a reduced platelet count. Recent observations - also in mice - have now identified resident tissue macrophages as the cellular source of plasma A-subunit.

FXIII-B, a glycoprotein, is synthesized and secreted by hepatocytes. It contains 8.5% carbohydrate. According to the latest research, the carbohydrate content of the protein is responsible for the long half-life of FXIII-B and FXIII-A_2B_2 in plasma. It is characterized by a mosaic structure, made up of 10 repeating so-called "sushi" domains. Each sushi domain is held together by a pair of internal disulfide bonds. The task of B-subunit is to prevent the spontaneous activation and elimination of the catalytic subunit (FXIII-A) in plasma conditions, thus increasing the lifetime of FXIII-A.

The FXIII-A_2B_2 complex is formed in the plasma. Majority of FXIII-A is present in the plasma in complexed form, only 1% of it circulates in free form. In contrast, FXIII-B is in excess and 50% of it is in free form within the plasma. While the dimeric structure of FXIII-A is supported by unequivocal data, no reliable information on the structure of free FXIII-B is available. It is still uncertain, whether it is present as a dimer or a monomer. FXIII-A_2B_2 occurs bound to fibrinogen in the plasma through B-subunit.

The activation of pFXIII occurs in the final phase of the clotting cascade by thrombin and Ca^{2+}. In the initial step of the reaction thrombin cleaves off AP-FXIII from the N-terminus of FXIII-A. Then, in the presence of Ca^{2+}, the B subunits dissociate, and the resulting truncated FXIII-A dimer assumes an enzymatically active conformation. It is possible that the formed FXIII-A dimer detaches into active monomers. The conformational change requires Ca^{2+}. The polymerizing fibrin enhances the activation by 100-fold, the newly formed fibrin provides a surface for the activation process. Although, in physiological conditions zymogen pFXIII is generally activated by a protease (thrombin) and Ca^{2+} into active TG (FXIIIa), an extremely high Ca^{2+} concentrations (>100 mM) can initiate the dissociation of subunits and thus, producing non-proteolytic activation of pFXIII.

In extracellular conditions, cFXIII can be activated by thrombin and Ca^{2+} similarly to pFXIII, but in the absence of FXIII-B there is no dissociation step. In an intracellular environment, cFXIII does not require proteolytic cleavage to assume the active structure. However, it is unclear whether this structure remains a dimeric form (FXIII-A_2°) after activation or dissociates into monomers (FXIII-A°). It was shown that no proteolytic modification of FXIII-A occurs during platelet activation, but the intracellular Ca^{2+} concentration increases,

which is sufficient to create the enzymatically active configuration of cFXIII. Activation of the cellular form by a non-proteolytic mechanism is considered physiological in platelets and probably also in monocytes. The intracellular Ca^{2+} concentration increases in platelets, e.g., in response to stimulation induced by thrombin or Ca^{2+}-ionophore.

The primary protein substrates of FXIIIa are fibrin α and γ chains and α_2-antiplasmin. By cross-linking the fibrin chains, γ chain dimers and α chain polymers are formed, with this and the binding of α_2-antiplasmin to the α chain, a stable clot resistant to mechanical effects and fibrinolysis is created. In recent years, another 23 FXIIIa substrates have been identified: blood coagulation factors (e.g. factor V), fibrinolytic proteins (e.g. plasminogen, lipoprotein A), adhesion and extracellular matrix proteins (e.g. thrombospondin, fibronectin), cytoskeletal proteins (e.g. actin and myosin). The importance of these proteins as physiological FXIIIa substrates awaits further proof.

The main role of FXIIIa is to stabilize the clot, but many other functions are also known. It is essential for maintaining pregnancy and proper wound healing, and FXIIIa is also involved in angiogenesis. FXIIIa has also been reported to reduce vascular permeability. The protective effect of FXIIIa against spontaneous cardiac rupture was demonstrated in mice, and in vitro experiments suggest that FXIIIa participates in the formation, stabilization and mineralization of the extracellular matrix.

FXIII-A deficiency is associated with a severe bleeding disorder. Inherited (autosomal recessive) and acquired forms are also known. The most common symptom of inherited FXIII-A deficiency is umbilical cord bleeding in infancy. In the absence of FXIII-A, various types of bleeding may occur throughout the body (e.g. subcutaneous hemorrhages, intracranial bleeding, intramuscular hematomas). FXIII deficiency might be associated with recurrent miscarriage and prolonged wound healing.

2.2 FXIII-A in platelets

The presence of FXIII-A in platelets was first demonstrated in the 1950s. These cells represent a huge reservoir of FXIII-A in the cytoplasm. A single platelet contains approximately 60±10 fg FXIII-A, which corresponds to 3% of total protein content. This concentration is 100-150-fold higher than in plasma. It is still not clear how FXIII-A can be released from cells and exert its extracellular effects. Mitchell et al. has shown that strong agonist stimulation, such as convulxin (CVX) and thrombin, induces the translocation of FXIII-A to the membrane and it appears on the surface of platelets. Platelets possess balloon-like structure due to the expansion

of the platelet membrane, as a result of rapid influx of water and sodium and chloride ions ("ballooning"). FXIII-A was concentrated in the protruding cap. This cap binds a number of other hemostatic proteins, including fibrinogen, plasminogen or plasminogen activator inhibitor-1, and it is probably composed of organelle remnants. It was also described that translocated cFXIII showed TG activity in experiments with α_2-antiplasmin or α_2-antiplasmin-derived peptide, suggesting that platelet cFXIII also exerts its effect extracellularly. Therefore, platelet cFXIII can participate in clot stabilization in addition to pFXIII, when the level of plasma FXIII-A_2B_2 complex is below 20%, the antifibrinolytic effect of platelet FXIII-A becomes apparent.

CVX and thrombin induce platelet activation via binding to their receptors. CVX stimulates the collagen receptor GPVI/FcRγ generating a downstream signal igniting platelet activation process while thrombin stimulation involves the protease-activated receptor-1 (PAR1), protease-activated receptor-4 (PAR4) and GPIb. Stimulation of these receptors by CVX+thrombin represents a robust signal leading to the activation of a number of signaling pathways involving protein phosphorylations and a strong elevation of intracellular Ca^{2+} concentration due to its release from intracellular pools. These signaling events result in aggregation of platelets, secretion of their granular content. In resting platelets, phosphatidylserine (PS) is located on the intracellular side of the phospholipid bilayer, but after activation it is translocated to the outer membrane layer. The negatively charged, procoagulant PS provides a surface for blood coagulation factors and promotes clot formation, primarily the proper functioning of the tenase and prothrombinase complexes. Influx of extracellular Ca^{2+} also contributes to these events. Platelet activation can also be carried out by non-receptor mediated mechanisms. The Ca^{2+}-ionophore calcimycin (A23187) is a receptor-independent activation agent that elevates Ca^{2+} concentration in the platelet cytoplasm and ignites Ca^{2+}-dependent biochemical pathways of the activation machinery. Due to the increased Ca^{2+} level in platelets, cFXIII is non-proteolytically activated. Intracellularly activated FXIII

1./ cross-links certain proteins (e.g. actin) in the cytoplasm,

2./ contributes to clot retraction, although the involvement of cFXIII to the clot retraction is still controversial. Kasahara et al. demonstrated that clot retraction was significantly impaired in FXIII-A knockout mice, in contrast, Kattula et al. found that platelet FXIII-A did not alter the process of clot retraction, changes were found only in the case of reduced pFXIII level,

3./ plays a role in the platelet spreading phase following cell activation.

In addition to the processes mentioned above, platelet activation also involves the production of microparticles. Microparticles are vesicles with a diameter of 0.1-1 μm that are

detached from the membrane and formed during the activation or apoptosis of various cells (e.g. platelets, monocytes, red blood cells). They can also be found in the circulation under physiological conditions, but their number could increase significantly in various pathologies associated with cell activation, such as cardiovascular diseases, sepsis or cancer. Microparticles mainly carry properties specific to the cell from which they originate. Similarly, to activated platelets, PS appears on the surface of procoagulant microparticles, derived from other cells.

2.3 FXIII-A in monocytes and macrophages

In the eighties we reported for the first time that monocytes and peritoneal macrophages express FXIII-A but not FXIII-B. This finding was confirmed in McDonagh's laboratory. The transglutaminase activity in monocytes from FXIII-A-deficient patients was below the limit of detection and only traces of tissue transglutaminase (TG2) could be detected in freshly prepared, non-stimulated monocytes. However, the TG2 content rapidly increased during culturing or stimulation of the cells. Monocyte/macrophage cFXIII might exert both intracellular, and if becomes externalized, extracellular function. Intracellular FXIIIa activity supports phagocytosis mediated by the Fc region of IgG and complement receptor. cFXIII, lacking signal peptide, is not secreted by the usual secretory pathway, however, it could become externalized through alternative mechanisms. An unorthodox secretory mechanism is suggested by the finding that in monocytes and macrophages, in association with Golgi vesicles, FXIIIa is directed to the plasma membrane. Most recently it was shown that FXIII-A becomes externalized and accumulated on the membrane of human monocytes in response to stimulation by interleukin-4 or interleukin-10 and becomes capable of exerting extracellular functions.

2.4 Atherosclerotic plaques and foam cells

Atherosclerosis, a chronic inflammatory disease, is a major cause of cardiovascular disease worldwide. The development of this multifactorial disease is influenced by many factors: stress, smoking, age, high blood pressure, physical inactivity and the accumulation of lipoproteins. It mainly affects arteries and remains asymptomatic for many years. The exact mechanism of the formation of atherosclerotic plaques is not known. There have been several theories about this process: (a) infiltration theory (endothelial damage increases its permeability and lipids and macromolecules move from the circulation to the subendothelial space, thus initiating atherosclerosis), (b) thrombogenic theory (a thrombus forms in the blood vessels, the

endothelium covers it and the thrombus becomes part of the subendothelial space, where its structure changes under the influence of macrophages), (c) theory of response to injury (result of an inflammatory process due to viral infection), (d) theory of lipid metabolism disorder (lipids and cholesterol levels rise and are then deposited on the walls of blood vessels).

The pathology of atherosclerosis can be divided into six stages: intimal thickening, lipid-streak stage, pathologic intimal thickening, fibroatheromas, vulnerable plaque, ruptured plaque followed by thrombosis. Increased plasma cholesterol is associated with a change in the permeability of the endothelial layers, therefore different types of lipids, especially LDL, accumulate in the intima. Due to oxidative stress, LDL particles are oxidized (oxidized LDL; oxLDL), which induces endothelial dysfunction. As part of the inflammatory process, adhesion molecules are activated (intercellular adhesion molecule-1, vascular cell adhesion molecule-1) and chemokines (macrophage colony stimulating factor, monocyte chemoattractant protein-1) are released. OxLDL is taken up via CD36, scavenger receptor-A1 or lectin-like oxLDL receptor-1. In the stage of the fibrous plaque, vascular smooth muscle cells from the media layer of the arteries also migrate to the intima, and in the presence of collagen, the fibrotic cap is formed at the atherosclerotic plaque. This thick fibrotic cap contributes to the formation of a stable plaque. As the atherosclerotic process progresses, further cells migrate into the vessel wall, cell activity increases, and the level of hydrolytic enzymes increases (e.g. elastase). The excessive accumulation leads to apoptotic cell death and the cell remnants promote the formation of the lipid-rich, core region of the plaque. In the advanced lesion and thrombosis stage, the necrotic core increases, the interstitial material decreases, and the fibrotic cap thins. Such unstable plaques can easily rupture, causing components of the necrotic core to interact with clotting factors and blood cells, leading to thrombosis.

The atherosclerotic plaque has many acellular and cellular components. The acellular components include lipids (cholesterol, cholesterol ester, and phospholipids) and extracellular matrix components (fibronectin, collagen, and proteoglycans). The cellular components include macrophages, smooth muscle cells, dendritic cells, mesenchymal stem cells, endothelial cells, and T cells. In addition to macrophages and smooth muscle cells, dendritic cells, stem cells and endothelial cells are also capable of forming foam cells. However, the vast majority of the foam cell population formed during the atherosclerosis process is of macrophage and smooth muscle cell origin. The mechanism of lipid uptake by smooth muscle cells and macrophages is different. Macrophages easily take up the oxidized, acetylated or even the native form of LDL by scavenger receptor-dependent endocytosis. Despite the fact that smooth muscle cells also express CD36 receptor and can take up oxLDL, this cannot be the main pathway of their

transformation into foam cells. The uptake of the enzyme-modified form of LDL thrombinough macropinocytosis is more common. Such a modification can be induced by the combined effect of cholesterol ester hydrolase and proteolytic enzymes which are present in high concentration in atherosclerotic plaque. As mentioned earlier, both monocytes and macrophages contain FXIII-A, but prior to our work, there was no data on whether foam cells have FXIII-A content.

3. AIMS

The aim of our research was to investigate the presence of FXIII-A on the surface of platelets and platelet-derived microparticles, as well as in macrophage-derived foam cells using quantitative and qualitative methods.

We addressed the following questions:

- Can the surface transposition of cFXIII during receptor-mediated platelet activation, previously detected by immunofluorescence, be confirmed by immuno-electron microscopy?

- Does cFXIII appear on the surface of microparticles derived from activated platelets?

- Does the surface translocation of FXIII-A also occur if platelet activation is induced by non-receptor mediated activation?

- Is there a connection between the appearance of PS and FXIII-A on the surface of the membrane?

- Does the mechanism responsible for FXIII-A translocation require Ca^{2+} release from intracellular stores?

- How does the transformation of macrophages into foam cells affect cFXIII content?

- Do smooth muscle derived foam cells express cFXIII?

- What is the distribution of FXIII-A in atherosclerotic plaques?

- Can FXIIIa induced cross-linked protein structures be detected in atherosclerotic plaques?

4. MATERIALS AND METHODS

4.1 Ethical approvement

The collection and use of human peripheral blood samples, blood preparations (buffy coat) and tissue sections were carried in accordance with the Declaration of Helsinki developed by World Medical Association. Blood sampling was approved by Ethics Review Board of the University of Debrecen, Faculty of Medicine. Study on tissue sections was approved by the Institutional Review Board of County Emergency Clinical Hospital of Targu Mures, Romania (no. 29496/2019). Written informed consent was obtained from each patient involved in the study.

4.2 Preparation and activation of gel-filtered platelets

Blood samples were drawn from healthy volunteers who had not taken any antiplatelet medications in the previous 14 days. Peripheral blood was collected into vacutainer tubes containing 0.109 M sodium citrate anticoagulant (Vacutainer®; Becton Dickinson, Plymouth, UK), then it was diluted in 1:1 ratio with modified 4-(2-hydroxy-ethyl)-1-piperazine-ethane-sulfonic acid (HEPES) buffer (10 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% glucose, pH 7.45). Platelet-rich plasma was obtained from diluted blood samples by centrifugation at 170 *g* for 10 min at 25°C. Platelets were isolated from plasma by gel filtration on Sepharose CL-2B column (Sigma-Aldrich, St. Louis, MO, USA) equilibrated with modified HEPES buffer. During the passage through the chromatography column, the platelets are deformed, thus, gel-filtered platelets (GFPs) were incubated at 37 °C for 30 min to recover their original shape.

GFPs were supplemented with 2 mM $CaCl_2$ and 0.1% bovine serum albumin (BSA) before activation. Platelet activation was induced by 125 ng/ml CVX (Pentapharm, Basel, Switzerland) and/or 0.5 U/ml thrombin (Sigma-Aldrich, St. Louis, MO, USA) for 15, 30, or 45 min at 37 °C. In other experiments platelets were activated by various concentrations (0.1-1 μM) of calcimycin (Ca^{2+}-ionophore, Sigma-Aldrich, St. Louis, MO, USA). In certain experiments, immediately prior to activation by CVX and thrombin, platelets were pretreated by Rhosin-hydrochloride (10-100 μM; Tocris Bioscience, Bristol, UK) or 1,3,4,5-tertramethyl-2-imidazolium chloride (T101; 10-200 μM; Zedira, Darmstadt, Germany) TG inhibitor.

4.3 Detection of surface FXIII-A and phosphatidylserine

4.3.1 Flow cytometric analysis

One of the most sensitive markers of platelet activation is P-selectin expression, so in our case, the degree of cell activation was also determined based on this. P-selectin expression on platelets activated by receptor and non-receptor mediated pathways was detected by staining with phycoerythrin (PE)-labeled anti-CD62 Ab (1:35 dilution, Becton Dickinson, Plymouth, UK). PE-labeled mouse IgG_1 (1:35 dilution, Becton Dickinson, Plymouth, UK) was used as isotype control.

Staining for FXIII-A, for CD41a, and PS detection were performed in a triple immunofluorescent labeling system. For FXIII-A labeling, we used fluorescein-isothiocyanate (FITC)-conjugated anti-human FXIII-A antibody (2 μg/ml) produced in our laboratory. PE-conjugated annexin V protein (1:35 dilution; Becton Dickinson, Plymouth, UK) was used for the detection of surface PS, and PE-cyanine5 (PECy5)-conjugated anti-CD41a antibody (1:35 dilution; Becton Dickinson, Plymouth, UK) for the detection of CD41a (GPIIb/IIIa complex). In this case FITC-labeled mouse IgG_{2a} (1:35) served as isotype control. The antibodies were present during the respective platelet activation, after the incubation times indicated in paragraph 1.2, samples were diluted 12-fold with annexin V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4, Becton Dickinson, Plymouth, UK) and analyzed on a Beckman Coulter FC500 flow cytometer (Beckman Coulter, Pasadena, CA, USA, $\lambda_{excitation}$ = 488 nm, fluorescence channels: $\lambda_{emission}$ = 525/40 for FITC, $\lambda_{emission}$ = 675/30 for PE, $\lambda_{emission}$ = 695/30 for PECy5) equipped with CXP Analysis software (Beckman Coulter, Pasadena, CA, USA). Platelets were gated based on their labeling for CD41a, aggregates were excluded on the basis of characteristic forward versus side scatter pattern. Platelet-derived MPs were identified as CD41a positive events with the size below 1 μm. Reference beads of 0.6, 1.1, and 3 μm (Sigma-Aldrich, St. Louis, MO, USA) were used for calibration. Results were expressed as mean percentage of FXIII-A positive platelets or MPs.

4.3.2 Immunofluorescent analysis

GFPs in modified HEPES buffer (with 2 mM $CaCl_2$ and 0.1% BSA) were incubated at 37 °C for 15 min in the absence or presence of 125 ng/ml CVX plus 0.5 U/ml thrombin or 0.7 μM calcimycin. Samples were diluted 10-fold with HEPES buffer to stop cell activation.

The cells and the formed MPs were stained with rabbit anti-human FXIII-A antibody produced in our laboratory (1:200 dilution), mouse anti-human CD41a antibody (1:25 dilution; Thermo Fisher Scientific, Waltham, MA, USA), and Alexa-fluor 568 annexin V conjugate (1:20 dilution, Thermo Fisher Scientific, Waltham, MA, USA) for 60 min. Then, platelets and vesicles were immobilized onto glass slides (4×10^4 cells/slide) by Cytospin 3 cytocentrifuge (Shandon, Pittsburg, UK) at 72 × *g* for 4 min and slides were air-dried. Some samples were fixed with acetone for 15 min for permeabilization and then blocked with BSA for another 15 min. Immune labeling was visualized by incubation with DyLight 405-labeled goat anti-mouse (1:100 dilution, Thermo Fisher Scientific, Waltham, MA, USA) and DyLight 488-labeled horse anti-rabbit (1:100 dilution, Thermo Fisher Scientific, Waltham, MA, USA) secondary antibodies for 45 min at room temperature, in the dark. Subsequently, slides were washed and covered with Vectashield® antifade mounting medium (Vector Laboratories, Burlingame, CA, USA). Phosphate-buffered saline (PBS) was used for washing and for the dilution of antibodies. Staining steps were performed at room temperature in the dark. Images were taken by ZEISS LSM 700 confocal microscope (Zeiss, Oberkochen, Germany) through Plan-Apochromat 63x/1.40 oil immersion objective and analyzed by Zen software.

4.3.3 Immuno-electron microscopic investigation

GFPs were processed for electron microscopic investigation as described for immunofluorescent analysis. Equal volumes of activated and non-activated GFPs and 8% paraformaldehyde solution dissolved in 0.01 M PBS were carefully mixed and stored overnight at 4°C. Five microliters of the mixture was placed onto a carbon coated nickel 200 mesh grid (Electron Microscopy Sciences, Hatfield, PA, USA). After waiting 10 min for the deposition of platelets and extracellular vesicles onto the carbon layer, the rest of the liquid was removed by blotting. The grids were washed and incubated in a blocking solution containing 1% ovalbumin in 0.01 M PBS for 30 min. Platelets and extracellular vesicles on the grids were reacted with a mixture of rabbit anti-FXIII-A (1:5 dilution) and mouse anti-CD41a (1:20 dilution) for 90 min. After several washes, the samples were incubated with a mixture of goat anti-rabbit IgG conjugated to 15 nm gold particles (1:20 dilution; BBI Solutions, Cardiff, UK) and goat anti-mouse IgG conjugated to 10 nm gold particles (1:20 dilution; BBI Solutions, Cardiff, UK) for 120 min. Both the primary and secondary antibodies were dissolved in 0.01 M PBS containing 1% normal goat serum (Vector Laboratories, Burlingame, CA, USA) and the incubations were performed at room temperature. After several washes, the samples were

counterstained with uranyl acetate (Electron Microscopy Sciences, Hatfield, PA, USA) for 30 min and lead citrate (VWR International Ltd., Radnor, PA, USA) for 5 min. Platelets and extracellular vesicles were investigated in a JEOL 1010 transmission electron microscope (JEOL, Tokyo, Japan) and photographed with an in-column Olympus Valeta CCD camera.

4.4 Platelet aggregation induced by the Ca^{2+}-ionophore, calcimycin

GFPs in HEPES buffer containing 2 mM $CaCl_2$ and 0.1% BSA were stimulated by various concentration of calcimycin (0.1-1 μM). The aggregation was detected in Chrono-Log 700 lumiaggregometer (Chrono-Log Corp., Havertown, PA, USA). For negative control, the activation agent was replaced by HEPES buffer.

4.5 Intracellular Ca^{2+} measurement

In the first step, platelet count was adjusted to 30,000/μL with HEPES-Ca^{2+} buffer (2 mM $CaCl_2$) and Fluo-4-acetoxy-methylester (Fluo-4-AM; Thermo Fisher Scientific, Waltham, MA, USA) was added to a final concentration of 3 mM. The cells were gently mixed and incubated for 15 min at room temperature in the dark. Afterward, PECy5 anti-CD41a (final dilution 1:20) was added and the cells were incubated for an additional 15 min. The platelet count was then adjusted to 6,000/μL with HEPES-Ca^{2+} buffer. Intracellular Ca^{2+} level was detected in Novocyte 3000 RYB flow cytometer (Agilent Technologies, Santa Clara, CA, USA; $\lambda_{excitation}$ = 488 nm; fluorescence channels: $\lambda_{emission}$ = 530/30 for Fluo-4-AM and $\lambda_{emission}$ = 660/20 for PECy5). In order to establish the baseline, events were acquired for 60 s and acquisition was interrupted when activating the platelets with 125 ng/ml CVX + 0.5 U/ml thrombin or after adding 0.7 μM calcimycin and continued for at least 7.5 min. The technical time gap between the baseline and the acquisition following activation was 91 s. The results were analyzed by FlowJo software (Becton Dickinson, Plymouth, UK).

4.6 Culturing of macrophages and induction of foam cell formation

Human buffy coat from healthy donors was obtained from the Hungarian National Blood Transfusion Service. In order to separate monocytes, buffy coat was diluted with an equal volume of PBS containing 5 mM EDTA (PBS-EDTA). Thirty milliliters of the cell suspension was layered onto 15 mL of Histopaque-1077® (Sigma-Aldrich, St. Louis, MO, USA) and

centrifuged at 400× *g* for 30 min at 25 °C. After density gradient centrifugation, peripheral blood mononuclear cells above the Ficoll layer were aspirated and washed twice with PBS-EDTA. Monocytes were isolated by negative selection-based magnetic cell sorting (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) according to the manufacturer's protocol. To differentiate into macrophages, isolated cells (10^6 cells/ml) were cultured in RPMI 1640 medium containing 2 mM L-glutamine and 25 mM HEPES (Life Technologies, Waltham, MA, USA), 10 µg/mL gentamycin (Krka, d. d., Novo mesto, Novo mesto, Slovenia), 10% fetal bovine serum (FBS; Life Technologies, Grand Island, NY, USA), and 50 ng/mL granulocyte-macrophage colony-stimulating factor (Life Technologies, Carlsbad, CA, USA). Foam cells were then generated from macrophages by the addition of 50 µg/mL oxLDL (Life Technologies, Eugene, OR, USA). In both cases cells were incubated for 3 days at 37 °C in 5% CO_2 humidified air. This cell suspension was used in the detection/measurement of cFXIII by enzyme-linked immunosorbent assay (ELISA) and Western blotting techniques. Cells were cultured in Teflon dishes to keep cells in suspension. Isolation of monocytes and culture of cells was performed under sterile conditions.

4.7 Immunofluorescent analysis of macrophage derived foam cells

For immunofluorescent studies, cells were seeded onto non-treated, 8-well Lab-Tek™ chamber slides (Nunc™, Thermo Fisher Scientific, Rochester, NY, USA) at 5×10^5 cells/well density. RPMI 1640 medium was removed and cells were washed three times with PBS. Adherent macrophages and foam cells were fixed in 3.7% paraformaldehyde for 30 min, followed by rinsing with PBS. Non-specific IgG binding was blocked by normal human serum (EMD Millipore Corporation, Burlington, MA, USA) for 15 min. Foam cells were stained with rabbit anti-human FXIII-A antibody (1:200 dilution) for 60 min and DyLight 488-labeled goat anti-rabbit antibody (1:100 dilution; Vector Laboratories, Burlingame, CA, USA) was used as a secondary antibody for 45 min. In the next step, non-binding antibodies were removed by washing with PBS. Then, the samples were incubated for 15 min with diluted oil red O (ORO; Sigma-Aldrich, St. Louis, MO, USA) solution (6:4 dilution in distilled water), a specific marker of lipid accumulation. Finally, after washing with distilled water, the samples were covered with Vectashield® mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA), to visualize the nuclei. Slides were investigated with Zeiss LSM 700 confocal microscope and solid-state diode lasers (405 nm for DAPI, 488 nm for FITC, and 555 nm for oil red O). Detection of the fluorescence signals was performed by

selective laser excitation coupled to efficient splitting of the emitted light using a variable secondary dichroic (VSD) beam-splitter.

4.8 Preparation of enzyme-modified LDL

A slight modification of the method described by Bhakdi et al. was used for the generation of enzyme-modified LDL. Briefly, human native LDL (density 4 mg/mL) from plasma of healthy donors was isolated by ultracentrifugation. LDL was diluted to 2 mg/mL in modified HEPES buffer (20 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.0). For enzymatic modification, LDL was digested with 4 µg/mL trypsin from bovine pancreas (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 6 h and with 24 µg/mL cholesterol esterase from Pseudomonas sp. (Sigma-Aldrich, St. Louis, MO, USA) for an additional 6 h at 37 °C. In the second step, another 4 µg/mL trypsin and 36 µg/mL cholesterol esterase were added, and the mixture was incubated at 37 °C for 24 h. Finally, trypsin activity was blocked by 10 µg/mL soybean trypsin inhibitor (Roche Diagnostics, Mannheim, Germany) for 60 min at 37 °C and modified LDL was dialyzed against PBS.

4.9 Human aortic smooth muscle cell derived foam cell formation

The human aortic smooth muscle cells (HAoSMCs) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Naive cells (donor: 38 years old healthy male) were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) containing 1 mM sodium pyruvate, 2 mM L-glutamine, 10% FBS, and 1% gentamycin in a T75 flask at 37 °C in 5% CO_2 humidified air. Cells were grown to 90% confluence and used at passage 8, then seeded onto non-treated 4-well chamber slides (Nunc™, Thermo Fisher Scientific, Rochester, NY, USA) under the same conditions for 24 h. At the end of the incubation, adherent HAoSMCs were washed three times with PBS and treated with 75 µg/mL eLDL or with native LDL (negative control) in DMEM medium for further 24 h.

4.10 Immunofluorescent analysis of human aortic smooth muscle cell derived foam

After a 24-h treatment by eLDL, DMEM medium was removed and slides were washed three times with PBS. Cells were fixed by a 58:2 mixture of methanol and acetic acid for 15

min. Cells were washed again and then blocking with normal human serum was performed for 15 min. HAoSMCs were stained by rabbit anti-human FXIII-A antibody (1:200 dilution) or mouse anti-human alpha-smooth muscle actin antibody (1:250 dilution, Thermo Fisher Scientific, Waltham, MA, USA) for 60 min. After washing with PBS, DyLight 488-labeled goat anti-rabbit (1:100 dilution) or DyLight 488-labeled goat anti-mouse (1:100 dilution) secondary antibodies were added for 45 min. To visualize eLDL uptake by HAoSMCs, ORO staining was carried out for 15 min, then cells were washed with distillated water. Finally, slides were mounted by Vectashield® mounting medium with DAPI to counterstain nuclei and cFXIII and lipid content of cells were investigated using ZEISS LSM 700 confocal microscope.

4.11 Quantification of FXIII-A by ELISA in macrophage derived foam cells

For the detection of FXIII-A, cells from macrophages and macrophage-derived foam cells cultures were removed every day and centrifuged at 200× *g* for 10 min at 25 °C. Pellets were resuspended in the mixture of PBS, 1% Triton, 120 µg/mL 2-methyl-4-isothiazolin-3-one (MIT, Sigma-Aldrich, St. Louis, MO, USA), and 1x SIGMAFAST™ Protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA). FXIII-A antigen concentration was determined by sandwich ELISA developed in our laboratory. Results were adjusted to 10^6 cells. Means represent the results of 5 measurements.

4.12 Western blotting

Cultured cells were collected daily and centrifuged at 200× *g* for 10 min at 25 °C. Cell pellets were resuspended in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (62,5 mM Tris, 2% SDS, 8 M urea, pH 6.8) then denatured in boiling water for 5 min. After reduction, by adding 5% 2-mercaptoethanol proteins of equal number, lysed cells were separated by SDS-PAGE (7.5% gel), followed by transfer to polyvinilidene difluoride membrane. Affinity purified sheep anti-FXIII-A antibody (1:3000 dilution, Affinity Biologicals, Ancaster, Hamilton, ON, Canada), biotinylated anti-sheep IgG (1:1000 dilution, Vector Laboratories, Burlingame, CA, USA), avidin-biotinylated peroxidase complex. Enhanced chemiluminescent (ECL) reagent (ECL Plus+, Amersham, Little Chalfont, UK) were used for the immune reaction detecting FXIII-A in the cell lysates. Results were compared to that obtained with 100 ng of recombinant FXIII-A (Novo Nordisk A/S, Bagsvaerd, Denmark).

Precision Plus Protein Standards, Dual Color (Bio-Rad, Hercules, CA, USA) was used as a molecular weight marker.

4.13 Investigation of atherosclerotic plaque by immunohistochemistry

Tissue fragments harvested by conventional transluminal angioplasty from 7 patients diagnosed with symptomatic carotid artery atherosclerosis were fixed in 4% formaldehyde and embedded in paraffin. Histological features of carotid plaques were examined in 5 μm sections stained with hematoxylin and eosin. Using the criteria of the American Heart Association, clinically relevant type IV plaques (known also as "atheroma") were selected for further investigations. Macrophages were visualized by immunohistochemistry using anti-CD68 mouse monoclonal antibody, clone KP1 (Immunologic, Duiven, The Netherlands). Anti-FXIII-A rabbit polyclonal antibody was used (Thermo Fisher Scientific, Fermont, CA, USA) for the detection of cellular and extracellular localization of FXIII-A. In parallel experiments, Nε-(γ-L-glutamyl)-L-lysyl isopeptide bonds were detected by an antibody purchased from Covalab (Villeurbanne, France). EnVision FLEX/HRP (Agilent, Dako Santa Clara, CA, USA) was used as a secondary antibody in combination with 3,3′-diaminobenzidine chromogen (DAB) substrate to give the reaction product a brown color. Nuclei were counterstained with hematoxylin. For negative control, normal serum was substituted for the primary antibody.

4.14 Lipids in FXIII-A positive cells of the atherosclerotic plaque

The combined presence of neutral lipids and cytoplasmic FXIII-A was detected in cryosections. Anti-FXIII-A primary antibody, in combination with ORO was used. Briefly, after fixation in isopropyl alcohol and endogenous peroxidase blocking, a polyclonal antibody against FXIII-A was added and incubated overnight in a thermostat at 56 °C. After subsequent washing, secondary antibody (EnVision FLEX/HRP) was added for 30 min, which was followed by immersion in ready to use ORO solution for 5 min. The slides were then rinsed with running tap water, and color development was carried out by PolyDetector HRP Green (Bio SB, Santa Barbara, CA, USA) following the manufacturer's instructions.

4.15 Statistical analysis

GraphPad Prism 8.01 (GraphPad Prism Inc., La Jolla, CA, USA) software was used for statistical analysis. The normality of the data was determined by the Kolmogorov-Smirnov test. Unpaired t-test or Mann-Whitney test, depending on normality, was used to compare the percentage of FXIII-A positive platelets and platelet-derived microparticles. The same tests were used for evaluating the effect of different activation agents. Comparison of the cFXIII content of macrophages and macrophage-derived foam cells determined by ELISA technique was performed using a paired t-test. If the p value did not exceed 0.05, the results were considered statistically significant.

5. RESULTS

5.1 Surface exposure of activation markers following platelet stimulation

Flow cytometry revealed that both receptor mediated platelet activation induced by the CVX+thrombin and non-receptor mediated platelet activation induced by Ca^{2+}-ionophore resulted in the surface exposure of the α–granular protein P-selectin. 0.3 μM calcimycin was sufficient for exposing P-selectin to the surface of more than 90% of platelets, comparable to stimulation with CVX+thrombin. Exposure of the anionic phospholipid, PS on the surface of activated platelets was quantified by binding of annexin V. As demonstrated by this technique, such transposition was detected in one third of CVX+thrombin activated platelets and in 61% of the platelets activated by 0.7 μM calcimycin. At lower calcimycin concentration the percentage of PS-positive microparticles was significantly higher than PS-positive platelets. A higher calcimycin concentration (0.7 μM) was required to induce close to maximal platelet aggregation.

5.2 Receptor-mediated transposition of cFXIII to the surface of activated platelets and platelet microparticles

Flow cytometric analysis of platelets and platelet microparticles for surface exposed cFXIII revealed that both CVX and thrombin alone were able to induce transposition of cFXIII in a significant percentage of activated platelets and microparticles. However, the dual stimulus through the GPVI/FcRγ and PAR1 plus PAR4 receptors was needed to elicit a robust effect. It seems that only the combination of CVX+thrombin was able to produce a distinct platelet population that emerges as the combination of two separate platelet populations representing functionally different entities. In this case, more than 60% of activated platelets and microparticles showed FXIII-A positivity. Such surface distribution is similar to what was observed with alpha granular factor V.

The surface appearance of CD41a, annexin V and cFXIII was detected by triple immunofluorescence staining of platelets stimulated with CVX+thrombin. CD41a was detected all over the surface of the procoagulant platelet. A strong signal for PS over the surface of the membrane is consistent with procoagulant platelet formation. PS was localized on activated platelets circularly on the membrane and in the region of the extension. cFXIII showing intensive staining was concentrated within the cap-like structure protruding from the cell.

CD41a- and PS-positive microparticles of approximately 700–900 nm could also be observed. Consistent with the flow cytometry data these microparticles also showed surface staining for FXIII-A. The resolution of immunofluorescence is not sufficient to explore microparticles of smaller size, but on the slides prepared by cytocentrifuge we were able to detect clumped microparticles of up to 2 μm diameter, which showed intensive staining for FXIII-A.

We next studied FXIII-A localization using high resolution immune electron microscopy (IEM). Non-activated (resting) platelets fail to expose FXIII-A to their surface, while they are intensively labeled for the surface marker CD41a. Activation of platelets with CVX+thrombin reveals translocation of FXIII-A from the cytoplasm to the external leaflet of the stimulated membrane. Like on the immunofluorescent image, the accumulation of FXIII-A within the cap-like structure is observed by IEM, as well. Microparticles of various size from 200 to 800 nm labeled positively for FXIII-A. The larger microparticles still retained their membrane structure, as evidenced by CD41a labeling, but also co-stained for FXIII-A. Interestingly, microparticles of smaller size, which may represent extracellular cytoplasmic fragments, lack discernable membrane staining for CD41a, but they are heavily labeled for FXIII-A. Presumably, these can be extracellular cytoplasmic fragments that are held together by contractile proteins (e.g. actin).

5.3 Non-receptor mediated activation fails to transpose cellular FXIII to the surface of activated platelets and platelet microparticles

The flow cytometric data demonstrate that platelet activation by the Ca^{2+}-ionophore, calcimycin, that is, by directly elevating the intracellular free Ca^{2+} concentration, fails to switch on mechanisms responsible for the translocation of cFXIII to the surface of activated platelets and the formed microparticles. Extending the activation period did not change the situation considerably. Even after 45 min activation time only 6.6% ± 3% of platelets and 5.5% ± 2.2% of microparticles exposed cFXIII to the surface.

Platelets undergoing non-receptor mediated activation using Ca^{2+}-ionophore revealed staining for PS and FXIII-A after permeabilization, but FXIII-A was located in the cytoplasm of these platelets. In contrast, non-permeabilized platelets do not stain for FXIII-A despite an intensive strong staining for PS and CD41a. Similarly, no FXIII-A positive microparticles were detected by immunofluorescent staining following calcimycin-induced platelet activation.

IEM examination revealed two types of calcimycin-activated platelets. One type shows a platelet in the process of vesiculation, while the other type was characterized by intense pseudopod formation. Strong surface labeling for CD41a was present on both types of activated

platelets but no surface exposure of FXIII-A was evident. Microparticles of different size formed as the result of platelet activation by calcimycin. In this case the membrane and its labeling for CD41a remained intact, and no surface labeling for FXIII-A could be detected. Note that activation by calcimycin failed to induce the formation of membrane-free FXIII-A positive microparticles/extracellular cytoplasmic fragments observed when platelets were activated by CVX+thrombin.

5.4 Changes of cytosolic free Ca^{2+} concentration following platelet activation

The effect of CVX+thrombin or calcimycin treatment on the cytosolic free Ca^{2+} concentration of platelets was analyzed by flow cytometry. Platelets were loaded with the Ca^{2+} indicator Fluo-4-AM and were labeled with fluorescence-conjugated anti-CD41a antibody. The latter signal was used for the gating of the flow cytometric signal for platelets. The distribution of the fluorescent signal belonging to Fluo-4-AM of resting platelets in the HEPES-Ca^{2+} buffer was detected for 60 seconds. Data acquisition was then briefly interrupted for the application of the agonists and then resumed. The mean fluorescence of the Fluo-4-AM signal showed an about 5-fold increase upon the addition of the agonist cocktail CVX+thrombin, which corresponds to an increase in the cytosolic free calcium concentration. The response of the cells to CVX+thrombin was homogenous—most of the cells showed an immediate and marked increase in the Ca^{2+} concentration. The quantitative analysis of the median fluorescence intensity (MFI) for the 60-s-long bins shows that the increase in the cytosolic Ca^{2+} concentration was transient; the decline in the MFI as a function of time indicates this phenomenon. The same data acquisition protocol was applied when calcimycin was used as activating agent. The increase in the Ca^{2+} concentration upon calcimycin addition was immediate and homogenous among platelets. The increase in the Ca^{2+} concentration was sustained over the data acquisition duration of 600 s, and the magnitude of the fluorescence increase was significantly greater than the one induced by CVX+thrombin.

5.5 The effect of RhoA and transglutaminase inhibitor on CVX+thrombin induced cFXIII translocation

As calcimycin failed to induce cFXIII translocation, it was presumed that Ca^{2+} independent mechanisms are important in the surface exposure of cFXIII by

CVX+thrombin induced platelet activation. The small guanosine-triphosphate hydrolase enzyme (GTPase), RhoA, plays an important role in the Ca^{2+} independent part of G protein-coupled receptor signaling and integrin-mediated signaling. For this reason, we investigated the effect of RhoA inhibitor, Rhosin, on cFXIII translocation. The concentration-dependent inhibition of cFXIII translocation by Rhosin underlines the importance of RhoA mediated Ca^{2+} independent pathways in the surface exposure of cFXIII during platelet activation induced through collagen and PAR receptors.

A further point to be addressed was if the transformation of cFXIII into an active TG (FXIIIa) was required for its transposition across the platelet membrane. Platelet activation by thrombin has been shown to result in the non-proteolytic activation of part of cFXIII present in the intracellular compartment. T101 is a powerful inhibitor of TG2 and FXIIIa with an IC_{50} of about 0.25 μM. However, it cannot penetrate the membrane and is able to Inhibit only the active cFXIII fraction that was translocated to the outer membrane layer. We studied the effect of T101 on cFXIII externalization. The concentration dependent partial inhibition of cFXIII exposure by T101 suggests that the active form of cFXIII increases the translocation efficiency. However, based on these data one can hardly decide if the decreased translocation is related to the inhibition of surface-exposed cFXIIIa.

5.6 Investigation of macrophage and HAoSMC derived foam cells by immunofluorescence microscopy for FXIII-A expression and LDL ingestion

First, we investigated if FXIII-A is retained in macrophages undergoing transition into foam cells by immunohistochemistry. Monocytes isolated from human peripheral blood differentiated into macrophages were cultured and transformed into foam cells by ingesting oxLDL. Representative immunofluorescent images showed four macrophages, all of which were intensively stained for FXIII-A. These macrophages accumulated oxLDL to different extents. In cells with considerable accumulation of lipid particles, FXIII-A became marginalized in the cytoplasm. Evidently the ingested lipid particles occupied a significant part of the central cytoplasm, pushing other cytoplasmic constituents toward the sub-membranous region.

In addition to macrophages, several other cell types present in the atherosclerotic plaque might potentially accumulate lipids and could be transformed into foam cells. Vascular smooth muscle cells, other major constituents of the atherosclerotic plaque, are also capable of transformation into foam cells. oxLDL is a relatively poor inducer of such transformation, but

these cells can easily uptake enzyme-modified LDL that had been digested by trypsin plus cholesterol esterase. Human aortic smooth muscle cells were cultured and used for investigating the ingestion of eLDL. These cells showed intense staining for actin, however, their transformation into foam cells was not accompanied by the expression of FXIII-A.

5.7 Transformation of macrophages into foam cells results in elevated expression of cellular FXIII

The next question we addressed was if the ingestion of oxLDL particles influences the FXIII-A content of the macrophages. Using FXIII-A ELISA, it was shown that 24 h after a single dose of oxLDL, the formed foam cells exhibited more than double FXIII-A as compared to their non-transformed counterparts. The elevated FXIII-A content only slightly decreased during the following 48 h. These results were confirmed by Western blotting technique, as well.

5.8 Macrophages and FXIII-A in the atherosclerotic plaque

After demonstrating that macrophage-derived foam cells contain a considerable amount of cellular FXIII, we explored if FXIII-A is present in the atherosclerotic plaque, and if yes, it is of intracytoplasmic and/or extracellular localization. CD68-positive macrophages surrounding the lipid core are clearly visible in type IV carotid artery plaque. In another plaque with a similar structure, beside cells expressing FXIII-A staining of extracellular component can also be observed.

5.9 Visualization of FXIII-A and isopeptide cross-links within the atherosclerotic plaque by immunohistochemistry

The immunohistochemical localization of FXIII-A in the atherosclerotic plaque showed that, FXIII-A is present in numerous macrophage-like cells underneath the lipid core. In many of these cells, the empty non-stained part of the cytoplasm indicates that lipids that had been ingested by the cells were solubilized and removed by solvents used for the fixation/staining procedure. The intense extracellular staining of the lipid containing core of FXIII-A suggests that FXIII-A derived from the plasma and/or released from apoptotic/necrotic macrophages are bound to core constituents. The possibility of non-specific binding of primary or secondary

antibodies used for the visualization of FXIII-A was excluded by the lack of staining in experiments with negative controls.

A further question was if FXIII present in the atherosclerotic plaque was active and, as an active transglutaminase, if it was involved in cross-linking proteins. Using a specific antibody that detects Nε-(γ-glutamyl)-L-lysyl bonds, it was shown that the non-cellular part of the plaque is loaded by cross-linked protein structures. This result clearly indicates that FXIII is not just present in the atherosclerotic plaque, but it actively contributes to its structurization.

5.10 FXIII-A-containing foam cells within the atherosclerotic plaque

After establishing the presence of FXIII-A-containing cells in the atherosclerotic plaque and its cross-linked protein products, it was attempted to show that part of the FXIII-A-containing macrophages housing the plaque were transformed into foam cells. In cryosections ORO stained droplets, both in the intracellular and extracellular compartments are observed. Several cells show co-staining for ORO and an anti-FXIII-A antibody. This finding shows that FXIII-A-containing foam cells are not only in vitro experimental products, but also exist in vivo in the atherosclerotic plaque.

6. DISCUSSION

6.1 FXIII-A on the surface of platelets and platelet-derived microparticles

Platelets stimulated by receptor mediated activation using CVX+thrombin and non-receptor mediated activation with Ca^{2+}-ionophore share some common features including externalization of PS to the outer leaflet of the membrane and degranulation, observed as surface expression of the α-granule protein P-selectin. However, the morphology of platelets activated by the receptor mediated pathways and non-receptor mediated mechanism were profoundly different. Following CVX+thrombin stimulus approximately two-thirds of the platelets assumed a balloon-like morphology with a single cap protruding from the platelet body. In contrast, platelets stimulated with Ca^{2+}-ionophore formed vacuolized or pseudopod-based structures. Similarly, the localization of cFXIII in platelets activated by receptor mediated and non-receptor mediated stimuli is also different. In the absence of receptor stimulation cFXIII did not translocate to the surface of activated platelets and was not exposed on platelet microparticles. In theory, such a major difference might be due to a different extent of intracellular Ca^{2+} release induced through the receptor mediated and non-receptor mediated activation pathways. However, the elevation of intracellular Ca^{2+} concentration due to calcimycin induced activation was even higher than the one induced by CVX+thrombin. The results suggest it is not the elevation of Ca^{2+} in the cytosol that directs cFXIII to the outer membrane surface but rather the complex intracellular signaling mechanisms that accompany receptor stimulation. It is to be noted that Mattheij et al., using another Ca^{2+}-ionophore, ionomycin, observed surface exposure of transglutaminase activity by a limited number of platelets. However, the high ionomycin concentration in their experiments, more than 10-fold higher than that of the calcimycin in ours, and their long time of platelet stimulation might have induced less specific increased permeability of the platelet membrane.

A robust response resulting in the translocation of cFXIII requires coinciding signalization through collagen receptor and PARs. Both signalization pathways involve a sequence of Ca^{2+}-independent biochemical steps/mechanisms. Activation through the collagen receptor, GPVI, occurs via phosphotyrosine signaling cascade, which eventually leads to diacyl glycerol (DAG) production that activates Ser/Thrombin kinases of the protein kinase C family. At the cytoplasmic site PARs are bound to heterotrimeric G proteins, G_q and $G_{12/13}$. $G\alpha_q$ is involved in the activation of PLCβ and consequently in inositol triphosphate and DAG production, while $G\alpha_{12/13}$ exerts its effect through RhoA activation. The inhibition of cFXIII

translocation by the RhoA inhibitor, Rhosin, demonstrated its connection with a Ca^{2+}-independent part of signalization pathway.

PS translocated to the platelet surface shows circumferential location on the balloon-like part of activated platelets and there is an abundance of PS concentrated in the cap. Platelets from FXIII-A deficient patients are capable of formation of balloon-shaped platelets with associated "cap," indicating that cFXIII does not participate in these processes. Clotting factors responsible for thrombin generation, FIXa, FX/FXa, FVIII, FVa, prothrombin plus fibrinogen, and other adhesive proteins are associated with the negatively charged phospholipid surface primarily due to PS translocated to the outer membrane layer in such "coated" platelets. On such a procoagulant surface transformation of adhered FX and prothrombin into active clotting factors becomes highly accelerated. Most clotting factors involved in thrombin generation come from extracellular sources, while fibrinogen and part of FV are also released from α-granules. As a cytoplasmic protein cFXIII is not secreted through the classical secretory pathway or by degranulation of platelets. Still, its fate is reminiscent of the surface retention of alpha granular FV.

Mitchell et al. demonstrated by flow cytometric and immunofluorescent methods that on CVX+thrombin activated platelets FXIII-A becomes surface exposed and in procoagulant platelets it is associated to the cap-like structure. Here we confirmed these findings and used high-resolution IEM to visualize FXIII-A in the cap. Interestingly, most recently it was shown that monocytes stimulated by interleukin 4 and 10 also transpose cFXIII to the membrane. Cordell et al. reported that in monocyte-derived macrophages cFXIII becomes associated with Golgi proteins, which has been implicated in the delivery of non-classically secreted proteins to the plasma membrane. The latter pathway might be involved in the appearance of FXIII-A on the outer surface of activated platelets; however, further investigations are needed to explore the detailed mechanism of the trans-bilayer movement of cFXIII during receptor mediated platelet activation. A further question is how surface-exposed cFXIII becomes concentrated on the cap-like structure protruding from activated platelets. FXIII zymogen present in the plasma might bind to activated platelet; however, in our experimental set-up no extracellular FXIII was present.

Platelets activated by Thrombin/TRAP or collagen/CVX and particularly by strong dual-agonist stimulation release a wide range of extracellular vesicles that differ in size and morphology. Here, we investigated microvesicles in the range of 100–1000 nm for which the common terminology, microparticles, is used. cFXIII was documented in platelet microparticles some time ago; however, its surface exposure has not been investigated before.

Our data has unequivocally demonstrated by flow cytometry, confocal immunofluorescence microscopy, and IEM that the majority of microparticles, formed as the result of platelet activation by CVX+thrombin, expose cFXIII to their surface. It was also shown that FXIII-A positive microparticles may aggregate and appear as circular or other irregularly clumped structures. Detailed high-resolution IEM images revealed two types of FXIII-A labeled microparticles. The larger ones of 400–800 nm diameter retained CD41a positivity, which suggests an intact membrane layer. The smaller ones, up-to 200 nm, were CD41a negative but intensively labeled for FXIII-A, suggesting that they are of cytoplasmic origin. Neither in non-stimulated platelet preparations nor in calcimycin activated platelet preparations could such microparticles be detected. These results suggest that they derived from platelets undergoing robust receptor mediated activation.

The cross-linking of α_2-antiplasmin and α_2-antiplasmin derived peptide to fibrin by cFXIII exposed to the surface of CVX+thrombin activated platelets strongly indicates that this transglutaminase is in active form. In an earlier study the transglutaminase induced binding of serotonin to several substrate proteins also indicated the presence of active FXIII on the platelet membrane. FXIII has been shown to become activated non-proteolytically in the intracellular compartment during stimulation of platelets by thrombin. It is likely that this robust stimulation simultaneously translocated the activated form of cFXIII to the outer cell surface as a result of intracellular signaling. It is feasible that non-activated FXIII becomes exposed to the platelet surface and is subsequently proteolytically activated by thrombin when bound to the exterior of the platelet. However, without addition of exogenous thrombin, using TRAP-6 and collagen as stimuli similar transglutaminase activity was noted. Collectively, these data suggest that at least part of cFXIII is translocated to the surface of stimulated platelets in activated form. These events localize cFXIIIa in a region of the thrombus where the transglutaminase can promote cross-linking reactions to stabilize the forming platelet–fibrin aggregate. A further question was if activation of cFXIII is required for its transposition. The decreased surface availability of cFXIII in the presence of a transglutaminase inhibitor suggests some role of the active form.

Although no direct evidence was provided, it is likely that FXIII exposed on microparticles also represent activated cFXIII and play a role in intra-thrombi fibrin stabilization. The small cytoplasmic fragments without surrounding membrane and intensively labeled for FXIII-A are of particular interest. Their formation and exact structure are still to be explored and further studies on their involvement in thrombus stabilization might reveal an intriguing new mechanism.

In summary, the results of our study, demonstrate the following new findings:

1./ Receptor mediated activation has a role in the translocation of cFXIII to the outer membrane layer of activated platelet and this process is not linked to the exposure of procoagulant PS.

2./ It has been shown for the first time that cFXIII is present on the surface of microparticles. Despite the elevated intracellular Ca^{2+}levels, the Ca^{2+}-ionophore, calcimycin, failed to induce cFXIII translocation.

3./ This finding reveals the importance of Ca^{2+}-independent signaling mechanism(s) in the transposition of this cytoplasmic protein to the membrane surface.

4./ The decreased surface availability of cFXIII in CVX+thrombin activated platelets pretreated by the RhoA inhibitor, Rhosin, also supports the importance of Ca^{2+}-independent mechanisms.

6.2 FXIII-A in human macrophage-derived foam cells

Macrophages are multipotent, multifunctional cells which may undergo considerable transformation in response to various inducers. In different environmental conditions, they may remain non-polarized, or, by different polarizing agents, they could be transformed into cells with pro-inflammatory M1 or anti-inflammatory M2 phenotypes. Recent studies distinguished three main clusters of macrophages. The resident-like macrophages, with a phenotype resembling the M2 subtype can infiltrate the plaque. They are affected by a number of factors in the vessel wall which may influence their actual dynamic state of polarization. Although the presence of FXIII in human atherosclerotic lesions has been described in 1998 by Romanic et al., only a few studies have been reported on this subject. FXIII expressed in alternatively activated macrophages have been detected within the aortic valve in patients with aortic stenosis and it has been suggested to be involved in the stenosis valve progression.

Macrophages are capable of up taking lipids and transforming into foam cells. Foam cells play a major role in the initiation and progression of atherosclerosis. Going through apoptosis, autophagy, necroptosis, and pyroptosis, they provide the major source of the necrotic core in the atherosclerotic plaque. In addition to macrophages, in certain conditions, vascular smooth muscle cells, stem/progenitor cells, and endothelial cells might also ingest lipids and become transformed into foam cells. Smooth muscle cell derived foam cells undergoing phenotypic transformation represent a considerable proportion, approximately 50% of this cell type in the plaque. In response to the modifications of the local environment, vascular smooth muscle cells

switch from a contractile to a secretory phenotype and may also display macrophagic marker expression and a macrophagic behavior. Expressing macrophagic markers and displaying macrophagic behavior, in theory, could involve the synthesis of FXIII-A. Both macrophage- and HAoSMC derived cells are capable of ingesting lipid particles. HAoSMCs do not express FXIII-A and its transformation into foam cells did not change the situation. This finding suggests that transformation into foam cells is not responsible for the additional FXIII-A acquired by macrophage-derived foam cells; it is not the characteristic of foam cell formation in general.

FXIII-A content of macrophages shows a drastic increase when stimulated by interleukin-4, while interferon γ fails to elicit such a change, i.e., FXIII-A content of the cells is drastically different in polarized M1 and M2 phenotypes. As the uptake of oxLDL also induces macrophage differentiation and activation toward M2 phenotype, we were interested in how the cellular FXIII-A content is influenced by the transformation of macrophages into foam cells. In our experiments, the FXIII-A level of the non-polarized macrophages became more than double following the ingestion of oxLDL particles. Although longer and more robust stimulation by interleukin-4 induced a considerably higher increase of cell-associated FXIII-A, it would be interesting to study how polarizing agents would influence the FXIII-A level in foam cells.

In tissue sections, a rather abundant cell population was stained for FXIII-A. Most of the FXIII-A+ cells appear as macrophage-derived foam cells; the empty part of the cells suggests the solubilization of lipid particles during fixation/staining. Indeed, the combination of staining for FXIII-A with the lipid stain ORO clearly demonstrated that FXIII-A and lipid droplets could be found within the same cell population. In the cryosections, a considerable non-cellular area of the atherosclerotic plaque also showed intensive staining for FXIII-A. FXIII-A in the sclerotic core is very likely derived from foam cells that lost their integrity and participate in building up the necrotic core. A further question was if FXIII present in the atherosclerotic plaque is in an active form, i.e., is it functional and does it cross-link substrate proteins. The finding that FXIII is upregulated on the surface of human monocytes in response to stimulation by IL-4 and IL-10 suggests that it might be present in a surface-associated form as an active transglutaminase in the plaque. It is also very likely that cFXIII becomes released from disintegrating cFXIII-containing cells in an active form. The main extracellular function of FXIII in tissues is the cross-linking of substrate proteins through iso-peptide bonds. Using an antibody that specifically detects iso-peptide bonds, we were able to detect cross-linked protein structures in the atherosclerotic plaque that show that FXIIIa exerts its TG activity in the

extracellular compartment. These findings are important for the mechanism of lipid core formation and for the structurization of the plaque.

In summary, the results of our study demonstrate the following new findings:

1./ The transformation of macrophages into foam cells increases their intracellular FXIII-A content, while similar transformation of HAoSMCs fails to produce intracellular FXIII-A.

2./ FXIII-A is abundant in the atherosclerotic plaque; it is present both in plaque macrophages and in the extracellular compartment. FXIII-A-containing macrophages can also be transformed into foam cells in the atherosclerotic plaque.

3./ The presence of protein structures cross-linked through iso-peptide bonds, the product of FXIII-A, suggests that at least part of FXIII-A is functioning in the plaque as an active transglutaminase.

7. SUMMARY

The A subunit of coagulation factor XIII (FXIII-A), a potential transglutaminase, which in addition to plasma, is also found in the cytoplasm of certain cells (cellular FXIII; cFXIII). Platelets represent a huge reservoir of cFXIII (3% of total protein content). As a result of convulxin (CVX)+thrombin activation, FXIII-A becomes translocated to the surface. Monocytes and macrophages present in atherosclerotic plaque, also contain FXIII-A. The aim of our study was to investigate the externalization of FXIII-A on platelets and platelet derived microparticles and evaluate the FXIII-A content of macrophage derived foam cells by quantitative and qualitative methods.

Receptor mediated activation by CVX+thrombin exposed cFXIII to the surface of more than 60% of platelets and platelet derived microparticles. Electron microscopy revealed microparticles with preserved membrane structure and microparticles devoid of labeling for membrane glycoprotein CD41a. cFXIII was observed on both types of vesicles but was more abundant in the absence of CD41a. Rhosin, a RhoA inhibitor, significantly decreased cFXIII translocation. Non-receptor mediated activation of platelets by Ca^{2+}-ionophore elevated intracellular Ca^{2+} concentration, induced the translocation of phosphatidylserine to the surface of platelets and vesicles, but failed to expose cFXIII. The elevation of intracellular Ca^{2+} concentration is sufficient for the translocation of phosphatidylserine from the internal layer of the membrane, while the translocation of cFXIII from the platelet cytoplasm requires additional receptor mediated mechanism(s).

Immunofluorescent studies on macrophage derived foam cells demonstrated that FXIII-A is retained during the transformation of macrophages into foam cells. ELISA and Western blotting techniques revealed that the transformation elevated the intracellular FXIII-A content. This phenomenon seems specific for macrophage derived foam cells, the transformation of vascular smooth muscle cells into foam cells fails to induce the expression of FXIII-A. FXIII-A containing macrophages are abundant in the atherosclerotic plaque and FXIII-A is also present in the extracellular compartment. Cells showing combined staining for FXIII-A and oxidized LDL in tissue sections demonstrated that FXIII-A-containing macrophages within the atherosclerotic plaque are also transformed into foam cells. The protein cross-linking activity of FXIII-A in the plaque was demonstrated using an antibody labeling the iso-peptide bonds. FXIII-A containing foam cells may contribute to the formation of lipid core and the plaque structurization.

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LIST OF PUBLICATIONS

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List of publications related to the dissertation

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   Int. J. Mol. Sci. 24 (5), 1-12, 2023.
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List of other publications

3. Mezei, Z. A., Katona, É., Kállai, J., Bereczky, Z., **Somodi, L.**, Molnár, É., Kovács, B., Miklós, T., Ajzner, É., Muszbek, L.: Factor XIII levels and factor XIII B subunit polymorphisms in patients with venous thromboembolism.
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