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Role of plasminogen/plasmin system components and matrix metalloproteases in retinal artery occlusion following cardiac surgery

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Purpose: *To assess the impact of plasminogen/plasmin system components and matrix metalloproteases (MMPs) on the development of retinal artery occlusion (RAO) after cardiac surgery for valvular heart disease (VHD).*

Material and Methods: *Seventy three patients who underwent cardiac surgery for VHD were included in the study. Of these, 23 patients older than 60 years who had conventional-access mechanical prosthetic valve replacement with cardiopulmonary bypass were stratified into the group of high risk (HR) for RAO. Ten HR patients developed branch or central RAO. The low-risk (LR) group comprised 50 patients younger than 60 years who had valvuloplasty via a minimally invasive approach or the femoral artery using a biological implant. The control group comprised 15 patients who had no cardiac disease. Coagulogram indices and D-dimer were measured. Enzyme-linked immunosorbent assay kits were used to determine plasma plasminogen activator inhibitor (PAI)-1, plasmin-α2-antiplasmin complex (PAP), and tissue inhibitor of MMP-3 (TIMP-3) levels. MMP levels were determined by enzyme gelatin zymography.*

Results: *The PAI-1 levels were three times higher in HR groups than in controls. Mean PAI-1 levels were almost identical in patients who developed RAO and patients who did not develop RAO. The level of PAP in HR patients was 4-6 times lower than in the control group. MMP-9 activity was 2.5-fold lower in HR group patients who did not develop RAO than in those who developed RAO. In the latter patients, the level of plasma TIMP-3 was 2.3-fold lower than in controls. Mean plasma TIMP-3 level was almost identical in patients who did not develop RAO and LR patients. The mean plasma D-dimer level was the highest in patients who developed RAO; this was 16-fold higher than in the control group, and 11-fold higher than in patients who did not develop RAO.*

Conclusion: *High functional activity of plasmin and proteolytic MMP-9 activity are the major factors of the fibrinolytic system which cause the development of RAO in patients undergoing cardiac surgery. Regulators of plasminogen/plasmin activation (such as PAI-1 and PAP) and TIMP-3 do not play a substantial role in the development of complications in the form of RAO.*

Keywords:

retina, retinal artery occlusion, valvular heart disease, cardiosurgical interventions, plasminogen/plasmin system, MMP-9

Introduction

Valvular heart disease (VHD) encompasses a number of cardiovascular conditions and is a rapidly growing cause of global cardiovascular morbidity and mortality [1, 2]. Because there is no medication treatment for this disease, it accounts for about 20% of all cardiac surgical procedures in the United States and western countries [2, 3]. With advent of advanced surgical valvular interventions, transcatheter interventions for valvuloplasty and valve replacement have become routine and safe cardiosurgical procedures with stable outcomes and contribute to higher survival rates. Complications, however, can occur during and after surgery for VHD. The reported rates of perioperative visual loss range from 0.06% to 4.5%, depending on the procedure [3].

The rate of retinal artery (RAO) occlusion after surgery for VHD for patients aged 50 to 70 years has been reported to be three times higher than for patients younger than 50 and half as that for patients older than 70 years [4]. Complications are twice as common after valvular prosthetic procedures than after interventions for valvuloplasty. The rate of RAO after mechanical valve implantation is higher than after bioprosthetic valve implantation. In addition, the rate of retinal vein occlusion after conventional-access valvular heart surgery (VHS) is 35% higher than after minimally invasive VHS and 11 times higher than after surgery with femoral artery access.

Atherosclerotic plaque failure or maladaptive extracellular matrix (ECM) remodeling due to activation of the fibrinolytic system is an important potential pathogenetic cause for vascular occlusion. The matrix metalloprotease (MMP) system, which may be activated via the plasminogen/plasmin system, is claimed to have a role in matrix degradation and smooth muscle migration because it is involved in pericellular proteolysis [5]. Plasmin is an active initiator of this process because it directly breaks down fibrin and the matrix and activates other enzymes (like pro-MMP and heparanases) that break down the matrix [6-8].

The proteolytic system, however, has a series of controllers. The fibrinolytic system may be inhibited by specific plasminogen activator inhibitors (PAI, mainly PAI-1) or specific plasmin inhibitors (through the formation of plasmin-α2-antiplasmin complexes (PAP)) [6-10].

The purpose of this study was to assess the impact of plasminogen/plasmin system components and matrix metalloproteases on the development of retinal artery occlusion (RAO) after cardiac surgery for VHD.

Material and Methods

The study followed ethical standards as outlined in the European Convention on Human Rights and Biomedicine and the 1964 Declaration of Helsinki of the World Medical Association (and its later amendments including the amendments of 2000), complied with existing regulations of Ukraine, and was approved by the Bioethics Committee of the Shupyk National University of Healthcare of Ukraine (committee meeting minutes of November 1, 2021). Informed consent was obtained from all study subjects.

Seventy three patients who underwent cardiac surgical interventions in VHD were included in the study. Of these, 23 patients older than 60 years who had conventional-access mechanical prosthetic valve replacement with cardiopulmonary bypass were stratified into the group of high risk (HR) for RAO. Group 1 (HR_OC) comprised 10 HR patients who developed branch or central RAO with preserved cilioretinal artery, and group 2 comprised the 13 HR patients who did not develop RAO (HR_no-OC). Group 3 comprised 50 low-risk (LR) patients younger than 60 years who had valvuloplasty via a minimally invasive approach or the femoral artery using a biological implant.

The control group comprised 15 patients aged 45-65 years who had no cardiac disease, attended the ophthalmologist to have a spectacle prescription and agreed to have their blood samples collected. The proportion of males in each group ranged from 75% to 80%.

Prior to cardiac surgery and postoperatively, patients underwent eye examination including visual acuity assessment, static Humphrey perimetry, refractometry, tonometry, biomicroscopy, gonioscopy, and ophthalmoscopy using an aspheric Volk super-field NC lens and a three-mirror Goldmann contact lens. They also received spectral-domain optical coherence tomography (SD-OCT; Copernicus REVO, Optopol technology Sp, zo.o, Zaqiercie, Poland; scan programs, Retina 3 D and Rerina Reaster) and SD-OCT angiography (Copernicus REVO; scan program, Retina Angio wide 6x6 mm).

Venous blood samples were collected into 3.8% citrate (light blue cap) collection tubes, and plasma was separated by centrifugation. Thereafter, prothrombin test (PT) was performed and activated partial thromboplastin time (APTT), fibrinogen, D-dimer and international normalized ratio were measured. The rest of plasma was collected into EppendorfTM tubes and stored at -20ºC.

Fine Test Biotech Inc (Nanjing, China) SERPINE1, PAP/PIC, and TIMP-3 enzyme-linked immunosorbent assay (ELISA) kits (Cat Nos., EH0538, EH3419 and EH0296, respectively) were used to determine plasma PAI-1, PAP, and tissue inhibitor of MMP-3 (TIMP-3) levels, respectively. Measurements were performed using a HiPo MPP-96 microplate photometer (BioSan, Riga, Latvia), with plates incubated with shaking on a Biosan thermoshaker (PST-60HL-4) and washing performed on a Biosan plate washer (Inteliwasher 3D-IW8).

QuantAssay 0.8.2.6 software was used for calculations.

MMP levels were determined by enzyme phoresis (gelatin zymography) [11]. Plasma total protein levels were measured spectrophotometrically at 260 nm, 280 and 320 nm. Plasma was mixed with Laemmly buffer prior to application on electrophoresis. Electrophoretic separation of protein samples was conducted using sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE), with polyacrylamide and gelatin copolymer at a final concentration of 1 mg/m in Tris/glycine buffer, pH 8.6, at 70 V. The amount of total protein was 100 µg/track. After electrophoresis, the gels were taken out of the plates and washed in cold 2.5% Triton X-100 to remove SDS. The gels were incubated for 20 h at 37º C and stained in Coomassie R-250 solution. After destaining, zymograms were scanned for densitometric quantitation with TotalLab software (TL120, Nonlinear Inc., San Marcos, CA). Relative MMP activity was expressed in arbitrary units.

Statistical analysis was conducted using IBM SPSS Statistics 23 and MedStat. The distribution of data was assessed by the normality test (Shapiro-Wilk test), and because most data was normally distributed, it was expressed as the mean with standard deviation (SD). One-way analysis of variance (ANOVA) was used with Bonferroni correction for post-hoc analysis. P values $\leq$ 0.05 were considered significant. Results are presented in bar graphs with 95% confidence intervals (95% CI).

Results

The PAI-1 levels were three times higher in groups with a high risk for RAO than in the control group (1.13 ng/ml), and this difference was significant ($p \leq 0.05$, Fig. 1). Mean PAI-1 levels were almost identical in patients who developed RAO (3.27 ± 1.2 ng/ml) and patients who did not develop RAO (3.27 ± 1.2 ng/ml). In the group of patients with a low risk for RAO, the mean PAI-1 level was 1.73 ± 0.7 ng/ml, which was 1.5 times higher ($p \leq$

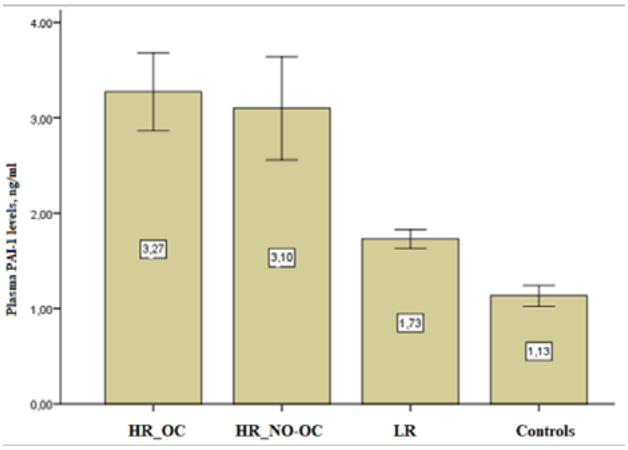

Fig. 1. Plasma plasminogen activator inhibitor (PAI)-1 levels in patients versus controls

Note: HR_NO-OC, the group of high-risk patients who did not develop RAO; LR, the group of patients with a low risk for complications; RAO, retinal artery occlusion; *, significant difference from controls; **, significant difference from LR patients; #, significant difference from HR_NO-OC; @, significant difference from HR_OC

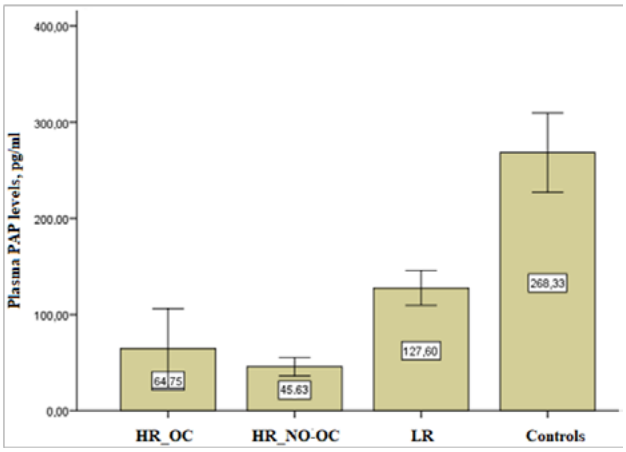

Fig. 2. Plasma plasmin-α2-antiplasmin complexes (PAP) levels in patients versus controls

Note: HR_OC, the group of high-risk patients who developed RAO; HR_NO-OC, the group of high-risk patients who did not develop RAO; LR, the group of patients with a low risk for complications; RAO, retinal artery occlusion; *, significant difference from controls; **, significant difference from LR patients; #, significant difference from HR_NO-OC; @, significant difference from HR_OC

0.05) than in the control group, and 1.8 times lower than in high-risk patients.

The PAP levels were significantly lower in groups with a high risk for RAO than in the control group (268.33 ± 12.3 pg/ml) (Fig. 2). The mean PAP level in high-risk patients who developed RAO (64.75 ± 17.4 pg/ml) was 4 times lower ($p \leq 0.05$), whereas in patients who did not develop RAO (45.63 ± 23.6 pg/ml), 6 times lower ($p \leq 0.05$) than in the control group. The mean PAP level in the group of patients with a low risk for RAO (127.6 ± 9.6 pg/ml) was 2.0-2.8 times higher ($p \leq 0.05$) than in high-risk patients, and half of that ($p \leq 0.05$) in the control group.

There was a significant difference in the plasma MMP level among the group of patients with a high risk for RAO (Fig. 3). MMP-9 activity was found in all patients who developed RAO. Densitometric analysis showed that the level of MMP-9 activity was 2.5-fold lower in high-risk group patients who did not develop RAO than in those who did develop RAO ($p \leq 0.05$).

Because TIMP-3 is produced by perycites, it is believed to be the most specific for the control of tissue proteolysis that results in degradation of the matrix and migration of smooth-muscle cells. We, however, found that, in patients who developed RAO, the level of plasma TIMP-3 was 2.3-fold lower than in the control group ($p \leq 0.05$; Fig. 4).

The mean plasma D-dimer level was the highest (1.84 ± 0.23 mg/l) in patients who developed RAO; this was 16-fold higher ($p \leq 0.05$) than in the control group (0.11 ± 0.3 mg/l), and 11-fold higher ($p \leq 0.05$) than in high-risk patients who did not develop RAO (0.16 ± 0.3 mg/l), and 5.5-fold higher than in low-risk patients (0.33 ± 0.4 mg/l) (Fig. 5).

A similar picture was seen with regard to plasma fibrinogen levels (Fig. 6): the mean plasma fibrinogen level was the highest (5.49 ± 1.2 g/l) in patients who developed RAO; this was 2.5-fold higher ($p \leq 0.05$) than in the control group (2.16 ± 0.6 g/l). The level of plasma fibrinogen in high-risk patients who did not develop RAO (3.45 ± 0.6 g/l) was 1.6-fold lower than in those who developed RAO ($p \leq 0.05$) and 1.6-fold higher than in controls ($p \leq 0.05$). The mean plasma fibrinogen level in the group of patients with a low risk for RAO (3.99 ± 0.1 g/l) was 1.8-fold higher than in controls ($p \leq 0.05$), and 2.5-fold lower than in patients who developed RAO ($p \leq 0.05$).

Discussion

Hemostasis is an important physiological process for maintaining vascular integrity and securing a sufficient blood flow throughout the circulatory system. Therefore, it requires a dynamic interplay between the vascular system, platelets, the coagulatory system and the fibrinolytic system. Plasmin is primarily involved in fibrinolysis as it degrades the insoluble fibrin meshwork that constitutes blood clots. It is produced from plasminogen by urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA), both of which are synthesized by endothelial cells. PAI-1 is an important component of the homeostasis system which inhibits the activation of plasminogen by tPA and uPA, is synthesized by

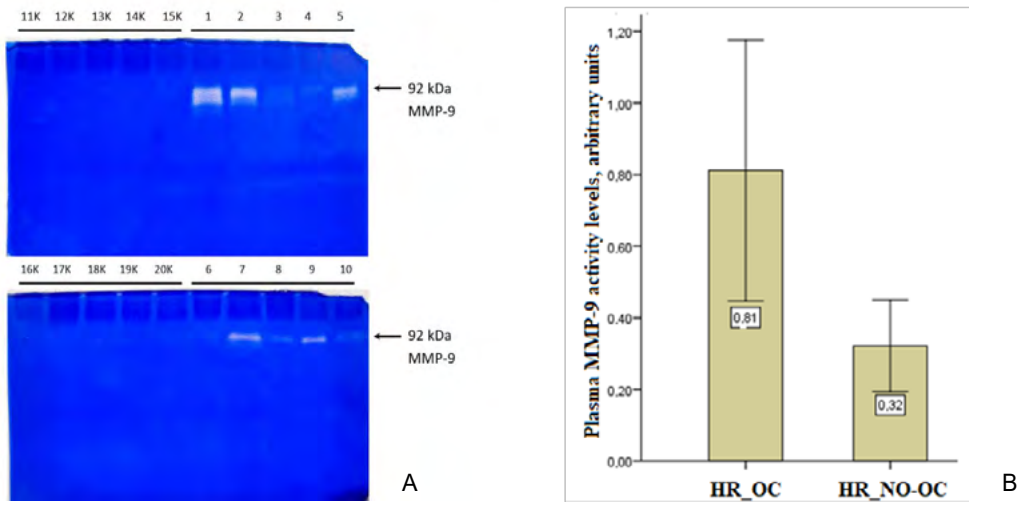

Fig. 3. Plasma matrix metalloprotease (MMP)-9 activity levels in patients versus controls. (A) Typical zymography image of MMP-9 activity. (B) A graph showing quantification of MMP-9 by densitometry analysis of zymogram

Note: HR_OC, the group of high-risk patients who developed RAO; HR_NO-OC, the group of high-risk patients who did not develop RAO

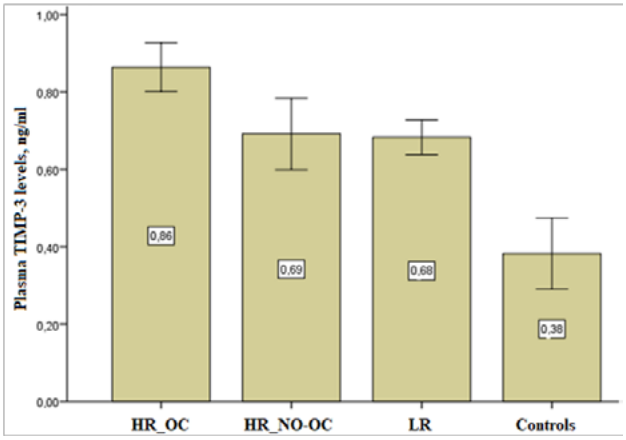

Fig. 4. Plasma tissue inhibitor of matrix metalloprotease-3 (TIMP-3) levels in patients versus controls

Note: HR_OC, the group of high-risk patients who developed RAO; HR_NO-OC, the group of high-risk patients who did not develop RAO; LR, the group of patients with a low risk for complications; RAO, retinal artery occlusion; *, significant difference from controls; **, significant difference from LR patients; #, significant difference from HR_NO-OC; @, significant difference from HR_OC

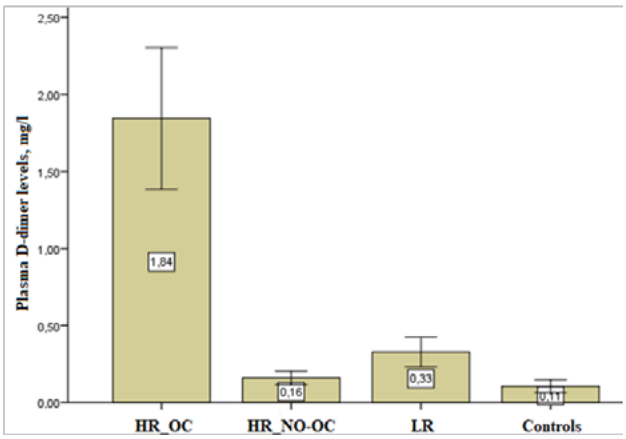

Fig. 5. Plasma D-dimer levels in patients versus controls

Note: HR_OC, the group of high-risk patients who developed RAO; HR_NO-OC, the group of high-risk patients who did not develop RAO; LR, the group of patients with a low risk for complications; RAO, retinal artery occlusion; *, significant difference from controls; **, significant difference from LR patients; #, significant difference from HR_NO-OC; @, significant difference from HR_OC

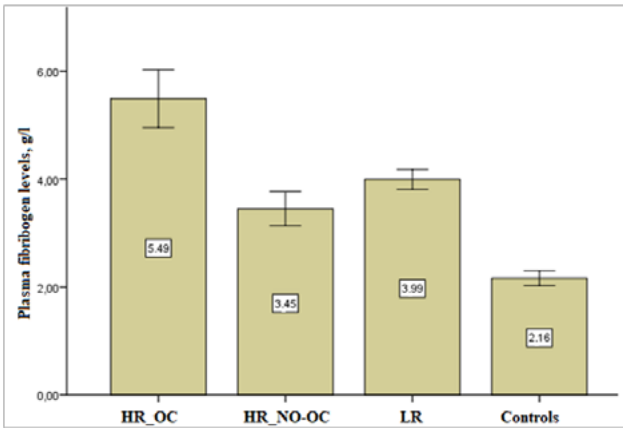

Fig. 6. Plasma fibrinogen in patients versus controls

Note: HR_OC, the group of high-risk patients who developed RAO; HR_NO-OC, the group of high-risk patients who did not develop RAO; LR, the group of patients with a low risk for complications; RAO, retinal artery occlusion; *, significant difference from controls; **, significant difference from LR patients; #, significant difference from HR_NO-OC; @, significant difference from HR_OC

megakaryocytes, and is accumulated in platelet α-granules. Impaired fibrinolysis resulting from high plasma PAI-1 can lead to excessive fibrin accumulation within vessels and tissue damage, resulting in a prothrombotic condition that can contribute to the development of cardiovascular disorders [12, 13].

We found that the plasma PAI-1 level in patients with a high risk for RAO was three-fold higher than in controls, which confirmed a hypofibrinolytic state and the threat of thrombus formation. Plasma PAI-1 expression was almost identical in both high-risk groups, indicating that PAI-1 has no significant role in thrombolysis in the development of vascular occlusion.

The fibrinolytic system may also be inhibited by specific plasmin inhibitors, mainly through the formation of covalent plasmin-α2-antiplasmin complexes (PAP), whose presence is a marker of plasmin generation in clinical states associated with plasminogen activation [14].

A 4-6-fold reduction in the level of PAP complexes in patients with a high risk for RAO compared to controls and low-risk patients confirmed a hypofibrinolytic state in these patients. This data, however, do not provide the basis for considering plasma PAP as a key marker of RAO, because there was no substantial difference in the level of plasma PAP between the group which developed RAO and the group which did not [15].

Plasmin is a broad-spectrum protease that is presumed to hydrolyze many extracellular proteins, the most notable of which is fibrin [16]. D-dimer is a product of fibrin degradation and a major marker of plasmin activity. We found the plasma D-dimer level in patients who developed RAO to be critically high; this was 16-fold higher than in the control group, and 11-fold higher than in patients who did not develop RAO. Consequently, it can be concluded that the functional activity of plasmin in these patients was high, despite identical levels of expression of PAI-1 and PAP, major system regulators whose action is directed at inhibiting plasmin formation.

We did not considered the insignificantly increased plasma fibrinogen level in patients who developed RAO as a significant factor in the formation of blood clots, because increased levels of acute-phase proteins such as fibrinogen are natural in patients after cardiac surgery.

Plasmin plays a key role in the final degradation of fibrin and ECM proteins in the fibrinolytic sequence [17]. Basement membrane degradation and migration and endothelial cell migration and ECM invasion are mediated by increased proteolysis by overactive plasmin and are closely associated with MMP activity [16,18,19]. MMPs are known to play an important role in basement membrane and ECM degradation and migration of vascular smooth myocytes [5]. MMP activity in turn is regulated by TIMPs.

We managed to identify MMP (to put it more precisely, MMP-9) in plasma by zymography only in patients who developed RAO. Trace plasma MMP-9 levels in high-risk patients who did not develop RAO were verified by densitometry. It is known that, under physiological conditions, MMP is not expressed in endothelial cells. Therefore, the level of MMP-9 activity was 2.5-fold higher in high-risk group patients who developed RAO than in those who did not develop RAO.

The plasma TIMP-3 levels in high-risk group patients who developed RAO were somewhat higher than in other groups, but the difference in the plasma TIMP-3 level between patients who did not develop RAO and low-risk patients was not statistically significant. Consequently, high MMP-9 activity is an important factor of the development of RAO.

In stroke, MMP-9 expression is associated with neutrophil infiltration in infracted and hemorrhagic areas of the brain [18, 19]. Fibrin stimulates neutrophil adherence; in turn neutrophils adhering to fibrin promote thrombin generation and fibrin deposition [20,21,22]. MMP-9 is also expressed by endothelial cells and to a lesser extent by astrocytes and neurons in ischemic brain. Moreover, it is a potential mediator of breaksown of the blood brain barrier, brain swelling and hemorrhage [14, 23].

Therefore, in the presence of a high MMP-9 activity, conditions develop for maladaptive ECM remodeling and/or atherosclerotic plaque failure. For clarity, we designed a scheme (Fig. 7) illustrating interactions of plasminogen/plasmin system components and MMPs in patients during cardiosurgical interventions and cardiac valvuloplasty procedures.

It is difficult to unambiguously determine whether such a substantial difference in MMP-9 activity among patients undergoing cardiosurgical procedures develops at the expense of a high plasmin activity or a low TIMP-3 activity. Studies on the regulation of MMPs and TIMPs in

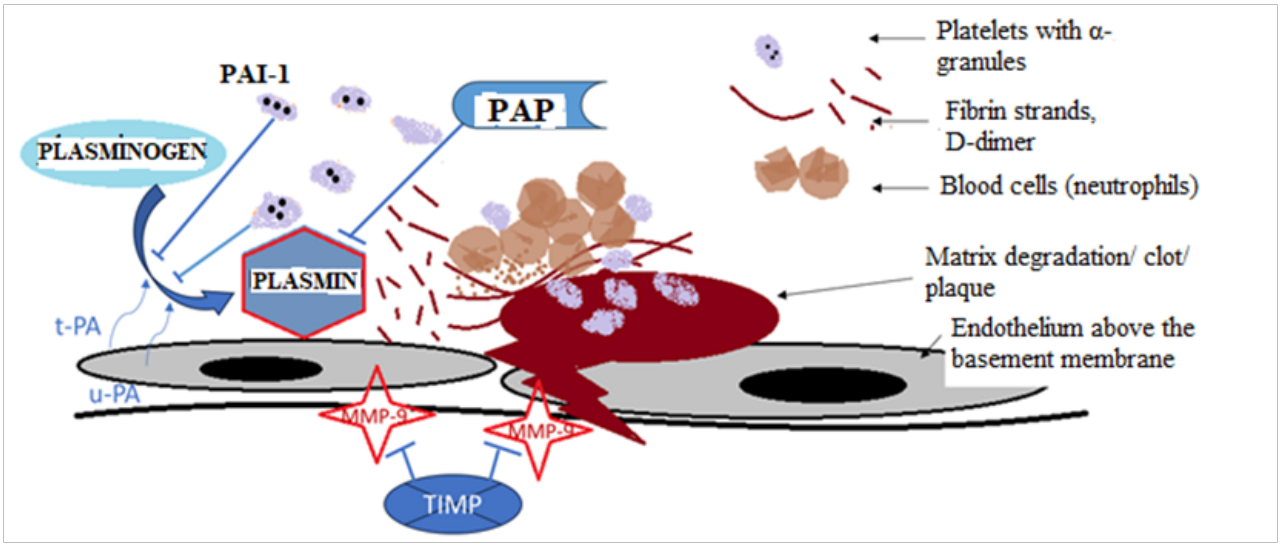

Fig. 7. A scheme illustrating interactions of plasminogen/plasmin system components and matrix metalloproteases in patients with retinal artery occlusion. Note: tPA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; PAI-1, plasma plasminogen activator inhibitor; PAP, plasmin-α2-antiplasmin complexes; TIMP, tissue inhibitor of matrix metalloprotease; MMP-9, matrix metalloprotease-9. A blue T-shaped line represents inhibiting effects, and a blue arrowhead line represents activating effects

endothelial cells by angiogenic cytokines have revealed a variable response that is in part cell-type dependent [16]. We, however, believe that it is a reduction in the activity of MMPs (as a key factor of proteolysis) that should be an essential component of the path towards the prevention of thrombotic occlusion following cardiogenic intervention.

Endothelial cells in normal tissues basally do not or slightly positively express MMP and TIMP. The expression of these molecules in endothelial cells (and sometimes, pericytes), however, increases in various physiological and pathological states. MMP-9 and TIMP-1 are homogeneously distributed on the cell surface and localized at the Golgi complex. Lafleur and colleagues [24] demonstrated that TIMP-2 and TIMP-3 from perivascular cells (smooth muscle cells and pericytes) can inhibit MMP-2 activity in endothelial cells, and this interplay may be important for maintaining basic endothelial homeostasis of regulating vascular growth during angiogenesis induction.

Therefore, we found that high functional activity of plasmin and proteolytic MMP-9 activity are the major factors of the fibrinolytic system which cause the development of RAO in patients undergoing cardiac surgery. Regulators of plasminogen/plasmin activation (such as PAI-1 and PAP) and TIMP-3 do not play a substantial role in the development of complications in the form of RAO. There is a need for further research on the mechanisms for the prevention of activation of the above-mentioned components of the fibrinolysis system and the development of measures for the prevention of postoperative loss of vision.

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Disclosures

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Ethical statement: *The study involved human subjects, was approved by the local ethics committee, and adhered to the tenets of the declaration of Helsinki. This study did not include animal experiments.*

Disclaimer: *The opinions expressed in this article are those of the authors and do not reflect the official position of the institution.*

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Abbreviations: *APTT, activated partial thromboplastin time; MMP activity level, matrix metalloprotease activity level; PAI-1, plasminogen activator inhibitor-1; PAP, plasmin-α2-antiplasmin complexes; PT, prothrombin test; TIMP-3, tissue inhibitor of MMP-3*