Short Thesis for the degree of doctor of philosophy (PhD)

Effect of invasive therapeutic interventions on endothelial- and platelet activation in patients with chronic total coronary occlusion

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 27th of August, 2024 at 10:00.

Introduction and literature review

1. Chronic total coronary occlusion

Chronic total occlusion (CTO) is a manifestation of coronary heart disease (CHD) and generally considered to be a complete blockage of the coronary arteries with Thrombolysis In Myocardial Infarction (TIMI) 0 flow with a duration of more than 3 months. CTO is identified in approximately 20% of patients undergoing diagnostic coronary angiography. CHD basically means the reversible breakdown of the oxygen demand and supply of the myocardium. The pathology of CHD is originated from atherosclerotic processes. This entity is characterized by the accumulation of atherosclerotic plaques and intimal lesions in the lumen of the vessel, which narrows the lumen and thus obstructs blood flow. The most characteristic symptom of that is retrosternal chest pain, which at first only develops upon physical exertion, but is often accompanied by sweating, shortness of breath, dyspnea, epigastric pain, and nausea and vomiting.

2. Treatment of chronic total occlusion

Since CTO is a manifestation of CHD, its therapy includes the same elements as the treatment of CHD. Making lifestyle changes is essential. In the case of co-morbidities, the control of risk factors is also important. After lifestyle changes, the second step is the administration of optimal medical therapy, of which the group of platelet aggregation inhibitors should be highlighted. These agents are favorable from the aspect of prognosis with secunder prevention, since in case of a coronary patient, there is a greater chance that platelets contact with a thrombogenic surface, thereby being activated and forming a thrombus. If the possibility of CHD arises, a diagnostic coronary angiography can be performed after optimal medical therapy has been built up. The staining of the coronary system with contrast material reveals, how much atherosclerosis involves the coronary arteries. In case of significant stenosis, depending on their

number and extention, revascularization is the following step: that can be performed in two ways: percutaneous coronary intervention (PCI) or coronary artery bypass graft surgery (CABG). When choosing the most appropriate method of revascularization, the SYNTAX score can help us: this helps the decision taking into account the anatomical features of the lesions. According to the currently valid guideline for myocardial revascularization issued by the European Society of Cardiology (ESC) in 2018, percutaneous recanalization of the CTO is recommended based on class IIa, type B evidence.

3. Percutaneous coronary intervention in case of chronic total occlusion

This procedure means a percutaneous catheter coronary intervention. The approach is usually through the radial artery, although during the recanalization of CTOs, the femoral artery is usually also punctured. After that, a plastic sheath is inserted using the Seldinger technique. After positioning the catheter to the ostium of the coronary artery, a special guidewire is introduced into the lumen of the artery, passing through the stenosis to be dilated. As a rule, a metal structure called a stent is implanted after a balloon predilatation. In connection with the revascularization of CTOs, the steps of the intervention are also built from these, but there are some important differences, which are mainly due to the anatomical characteristics of chronic occlusion. Since the occluded segment is biologically active, the recanalization, neovascularization and various inflammatory processes ongoing inside the occlusion result in lesions with diverse composition and properties. Depending on the time of the formation of the blockage, the properties of the affected vessel segment may change: a not so old occlusion is predominantly soft, has a higher lipid content, is less fibrotic and calcified, and may contain microchannels, while an older occlusion of more than a year may contain dense, fibrotic, highly calcified areas with fewer microchannels. The length of the occluded segment is one of the most significant factors regarding the outcome of CTO-PCI: a lesion longer than 20 mm proved to be a strong predictor of unsuccessful passage of the occlusion. Due to all these anatomical and

histological peculiarities, the success rate of percutaneous interventions performed in connection with CTO is lower than the success rate of PCI in case of a non-occlusive stenoses. For the reasons detailed above, guidewires used in non-occlusive PCI are not suitable. For CTO-PCI, the special, stiffer, sharper guide wires and the microcatheters that support them were developed, with the help of them it is possible to get through the blockage more effectively.

In case of CTO-PCI, several techniques can be used, which can be divided into two large groups. One of the most frequently used procedures is the approach from the anterograde direction, from the proximal cap, while the other group includes the retrograde technique, during which the distal part of the chronically occluded segment is reached from a collateral vessel. Both procedures are feasible in two ways. One is the wire escalation (WE) technique, during which the lesion is resolved within the lumen of the vessel. The other group includes the subintimal dissection and reentry procedures, when the occlusion is bypassed by entering the subintimal space of the vessel wal and then the wire is led back into the lumen of the vessel. During this, balloon dilation and stent deposition also take place in the subintimal space. In case of the retrograde technique, the reverse controlled antegrade and retrograde tracking (rCART) technique is mostly used, during which the attempt to pass through the CTO body begins from both the antegrade and retrograde direction.

4. Changes in endothelial cells in connection with vascular injury

The endothelium is formed by a monocellular layer of endothelial cells, lining the inner wall of blood vessels and lymphatic vessels. The endothelium produces and releases many substances and factors into the circulation. A substance produced by endothelial cells that has a vasodilating effect, e.g. nitric oxide (NO), prostacyclin, bradykinin, while endothelin, angiotensin II, thromboxane A2 (TXA2) have a vasoconstrictor effect. These factors also affect platelet activation, substances that cause vasodilation usually inhibit it, while factors that cause vasoconstriction stimulate platelet activation. Adhesion molecules produced by endothelial

cells help the adhesion and migration of inflammatory cells. Endothelium also contributes to the maintenance of normal haemostasis by the production of pro- and anticoagulant substances. Procoagulant factors are produced by the endothelium in connection with injury and act in the direction of promoting platelet activation, while anticoagulant factors inhibit the activation and aggregation of platelets and help fibrinolysis. Under physiological conditions, the production and degradation of factors with opposite effects are in balance, but as a result of physical, chemical, immunological, mechanical (e.g. injury of the vessel wall) and other stimuli, the endothelium is damaged, endothelial dysfunction may develop, which, with the aforementioned balance being upset, can cause increased risk of thrombosis.

5. Changes in platelets in connection with vascular injury

Thrombocytes are small fragments without nuclei derived from megakaryocytes. Their primary role is in hemostasis: by forming a blood clot, they block the site of the vascular injury and then provide a surface for the further processes of coagulation. In case of atherosclerotic plaque rupture and other vascular injury, platelets contact with extracellular matrix components and adhesion proteins, thereby starting a process that leads to the adhesion, activation and aggregation of platelets. Clot formation begins with the adhesion of platelets, while the activation of platelets begins, which is accompanied by the secretion of the contents of α- and δ-granules. The adhesion protein P-selectin (CD62P) is released from the α-granules onto the platelet membrane, as well as fibrinogen, fibronectin, V. and VIII. factors, platelet factor 4, platelet-derived growth factor get into the circulation. δ-granules contain adenosine diphosphate (ADP), adenosine triphosphate, adrenaline, serotonin, histamine, and ionized calcium. Of these, calcium and ADP are particularly important, since calcium is necessary for the activation of several coagulation factors, while ADP, which is connected to the $P2Y_{12}$ receptor, is an important activator of platelets. The activated platelets also synthesize TXA_2, which is also involved in the activation of the surrounding platelets. Parallel to these events, the third

component of the coagulation process, the coagulation cascade is also activated. At the end of the cascade, thrombin is generated, which activates the protease-activated receptor (PAR) on the surface of the platelets, causing further aggregation, and contributing to the stabilization of the blood clot by converting fibrinogen into fibrin.

6. The role of microRNAs

MicroRNAs (miRNAs) are short, non-coding RNA molecules, containing 18 to 25 nucleotides, which bind to the 3' untranslated region of target. The result of this connection is usually the inhibition of the synthesis of the protein, but degradation of the mRNA can also occur. They play a key role in the posttranscriptional regulation of gene expression and protein translation that is required for numerous physiological and pathological cell functions. One miRNA can participate in the regulation of hundreds of mRNAs, while one gene can be regulated by dozens of miRNAs. MiRNAs are expressed to varying degrees in different tissues, and their altered expression may play a role in the development of many diseases. For example, miRNA-223 can downregulate the expression of the $P2Y_{12}$ receptor, which plays an important role in the activation of platelets. MiRNA-126 also affects platelet activation, mainly by regulating the expression of surface proteins (including the $P2Y_{12}$ receptor), however it is expressed by endothelial cells, it was also identified in the processes of atherosclerosis. According to Harris et al.'s study, the increased level of miRNA-126 causes a decrease in the concentration of vascular cell adhesion molecule-1 (VCAM-1). MiRNA-181b, which is mainly expressed by endothelial cells, was associated with increased VCAM-1 and E-selectin levels after coronary stenting by Fejes et al., while its increased expression inhibited the thrombin-induced endothelial cell activation based on the results of another study. Circulating miRNAs have proven to be suitable biomarkers in the laboratory analysis of many diseases.

7. The justification of the study

CTO-PCI sometimes poses sometimes poses a considerable challenge to interventional cardiologists, since the penetration of the occlusion with the wire and the deployment of the stent are far from simple tasks. During the procedure, it may be necessary to replace the microcatheters and CTO wires. After passing the calcified lesion with the wire, it is often problematic to get the smallest, first dilating balloon into the correct localization. In case of a retrograde approach, getting through the collaterals and reaching the guiding position that provides adequate support are also time-consuming. In case of reentry technique, the return into the true lumen can also be lengthy. Because of all these, the intervention is often prolonged, and due to the manipulation with the devices, balloon dilation and stent implantation, a significant activation of platelets and the coagulation cascade can be expected, which can affect the short- and long-term effectiveness of the intervention. The incidence of early reocclusions and stent thrombosis (ST), as well as late restenosis may be proportional to the tendency for increased platelet aggregation and endothelial cell activation.

Aims

The aim of our studies is to measure and compare the platelet and endothelial cell activation in connection with CTO interventions performed with different techniques by determining different platelet and endothelial cell activation markers and the investigating the role of coagulation processes in early and late complications.

To achieve our objective, we divided our research into two main parts. In the first part, we wanted to compare the cell activation effect of the two basic CTO-PCI techniques (WE and DR) by quantifying endothelial cell activation markers and performing thrombin generation (TG) test. These measurments were supplemented with clinical follow-up data so that we could assess the safety of the interventions.

In the second part of our research, we analyzed the quantitative changes of activation markers related to the characteristics and technical parameters of the interventions. In order to assess these, we performed platelet activation tests and determined the relative expression of some selected microRNAs.

Patients and methods

1. The study population

Between January 2019 and September 2020, in total 59 patients who admitted to the Department of Cardiology and Cardiac Surgery, University of Debrecen for elective CTO-PCI, were enrolled. Enrollment criteria included the age over 18 years, CTO confirmed with coronarography, the presence of viable myocardial tissue distal from the occlusion, dual antiplatelet therapy prior to the intervention, and a written informed consent from the patient. Patients were excluded if the revascularization attempt was unsuccessful or severe life-threatening complication occurred during the intervention. Exclusion criteria also included ongoing acute coronary syndrome, active malignant disease, and pregnancy. Chronic total occlusion was diagnosed when it was confirmed with coronarography and persisted for at least 3 months (TIMI flow 0). Revascularization was considered successful when <30 % stenosis was left over in the treated vessel segment and TIMI flow 3 was restored.

2. Description of the study

First, the patients were informed and consent forms were signed. After that, patients' anamnestic data (including current medical therapy), clinical parameters, and blood groups were recorded. Blood samples were obtained in three different time points for quantitation of some cell activation dependent biomarkers. Using peripheral blood samples, platelet surface P-selectin expression, the concentration of soluble P-selectin, soluble VCAM-1, soluble intercellular adhesion molecule-1 (ICAM-1) and vWF antigen (Ag), as well as the relative expression of microRNA-223, -181b and -126 were measured. To assess the role of coagulation processes, thrombin generation test was used. The first blood sample was taken from a peripheral vein before the intervention, while the second and third samples were taken 48 hours after the intervention and 3-6 months later, also from a peripheral vein. The quantity of activation

markers determined from the second and third blood samples was always compared with the results measured in the initial samples for all patient groups. At the time of the third blood draw, which was 3-6 months later, during the patients' outpatient examination, patients' clinical condition and subjective complaints were evaluated. The incidence of the major adverse cardiovascular event (MACE) was also documented during the follow-up period, this clinical endpoint was composed of the incidence of stroke, myocardial infarction (MI), mortality and the target vessel revascularization (TVR). To explore the correlations between the characteristics of the interventions and the quantitative changes in cell activation markers associated with them, the patient population was divided into groups based on 4 criteria. Beyond the WE vs DR comparison, we divided our population based on the Japanese CTO score (J-CTO score), the length of the intervention, and the total length of the implanted stents. Patients were classified into low (J-CTO score 0 or 1, n=24) and high score (J-CTO score 2 or 3, n=26) groups. Based on the length of the intervention, shorter (40-117 minutes, n=24) and longer (118-255 minutes, n=20) interventions were distinguished, while according to the total stent length, shorter (16-53 mm, n=22) and longer (54-127 mm, n=28) groups were created.

3. Laboratory measurements

The surface P-selectin expression of platelets was determined from a whole blood sample, which was fixed with a 1% paraformaldehyde solution. CD42a FITC, CD62P and Platelet Control IgG1 antibodies (Becton Dickinson) were used to stain platelets. For each measurements, 10,000 platelets from each sample were analyzed using an FC 500 flow cytometer (Beckman Coulter, Brea, CA, USA).

The concentrations of soluble proteins were determined using an enzyme-linked immunosorbent assay (ELISA). After thawing, the frozen plasma samples were further centrifuged at 10,000 g for 1 minute at room temperature (RT). Quantikine ELISA Human P-selectin/CD62P Immunoassay ELISA kit (R&D Systems, Minneapolis, MN, USA) was used to

determine soluble P-selectin concentrations according to the manufacturer's instructions. Soluble VCAM-1 concentrations were determined using the Quantikine ELISA Human VCAM-1/CD106 Immunoassay ELISA kit (R&D Systems, Minneapolis, MN, USA), while soluble ICAM-1 concentrations were measured using the sICAM1 ELISA Kit (Sigma-Aldrich, St. Louis, MO, USA). Plasma vWF Ag levels were determined using an immunoturbidimetric method (BCS XP, Siemens, Munich, Germany).

The relative expression of extracellular miRNAs (miR-223, miR-181b, miR-126) was determined from peripheral plasma samples. Briefly, after being stored at -80°C, thawed plasma samples were centrifuged at 10,000 g for 1 min at (RT) and 400 μL of cell-free supernatants were spiked-in with 5 pmol mirVana cel-miR-39 mimic (Ambion, Austin, TX, USA). The expression of the microRNAs in the plasma was compared to this reference gene. Circulating miRNAs were then isolated with miRNeasy Kit (Qiagen, Hilden, Germany). The isolated total RNA was then reverse transcribed into cDNA using miRNAspecific stem-loop RT (reverse transcriptase) primer (500 nM, Integrated DNA Technologies, Leuven, Belgium) and TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Vilnius, Lithuania) according to the manufacturer's instructions. Quantification of miRNAs was performed using RT-qPCR (real-time quantitative polymerase chain reaction), in which universal reverse primer (100 μM, Integrated DNA Technologies), miRNA-specific forward primer (100 μM, Integrated DNA Technologies), Universal Probe Library probe #21 (10 μM, Roche Diagnostics, Mannheim, Germany), Taq DNA polymerase (5 U/μL, Thermo Scientific, Vilnius, Lithuania) and deoxynucleotide (dNTP) Mix (2.5 mM, Thermo Scientific) were used. Each measurement was performed in triplicates using QuantStudio 12K Flex qPCR instrument (Applied Biosystems by Thermo Fisher Scientific, Waltham, MA, USA). To normalize the results of miRNA level, the cel-miR-39 reference gene was measured in all the samples with the same RT-qPCR method.

Oligonucleotides and RT-qPCR assays were designed by the software (version 3.4) developed by Czimmerer et al.

Thrombin generation was measured in patient's PPP samples. Separations of PPPs were done by a two-step centrifugation of citrated blood samples. First, blood samples were centrifuged at 1500g for 15 min at (RT) then samples were further centrifuged at 10,000g for 10 min at RT. Thrombin generation was triggered by the reagent containing 1 pM tissue factor and 4 μM phospholipid. For the measurement of generated thrombin Fluoroskan Ascent FL fluorimeter with Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands) were used. The fluorescent signal was registered by the device and the kinetics of the reaction was characterized by a thrombin generation curve. The kinetics of thrombin generation was characterized by lag time, time to peak (ttPeak), while the quantity of generated thrombin was described by thrombin peak and endogenous thrombin potential (ETP). The measurements were performed in accordance with the manufacturer's instructions.

4. Statistical analyses

Statistical analysis was performed using SPSS (IBM SPSS Statistics for Windows, Version 25.0., Armonk, NY, USA). Normality was determined using Kolmogorov–Smirnov test. Continuous variables with normal distribution were displayed as mean ± standard deviation (SD), continuous variables with non-normal distribution were expressed as median and interquartile range (IQR). For comparisons, we used either Wilcoxon or Mann–Whitney U test. Categorical data on clinical states during their follow-up were evaluated using Chi2 test. Correlation between the type of intervention and the variables was explored using linear regression analysis. P values < 0.05 were considered statistically significant. In case of those patients ($n = 4$) in whom the third laboratory measurement was missed, last observation carried forward (LOCF) method was applied and the last known result was used to replace the missing data.

Results

1. Baseline characteristics of patients

59 patients were enrolled into this study, and 50 of them (84.7%) underwent successful intervention. The average age of the 50 patients is 61.78 ± 8.72 years, 60% of them are men. The median follow-up time was 8.5 months. It stands out clearly from the demographic characteristics and general clinical data that the patients in our study were an elderly patient population with several comorbidities. 29 patients (58 %) were treated with the intraluminal wire escalation technique and 21 patients (42 %) underwent the subintimal dissection procedure. Based on the general characteristics of the two subgroups, as well as the initial laboratory parameters used in routine diagnostics, the two subgroups were considered homogeneous, and there was no statistically significant difference in either anamnestic data or routinely available laboratory parameters at baseline.

2. Comparison of WE and DR techniques

2.1 Endothelial cell activation markers

In the WE-treated group, no significant differences were found when examining the VCAM-1 concentrations either 48 hours after the intervention or in the blood samples taken 3-6 months later compared to the baseline values. In contrast, in the DR group, a significant increase in VCAM-1 was observed after 48 h of the intervention (484 [406–789] ng/mL vs 366 [299–548] ng/mL, $p = 0.005$) and at 3–6 months (523 [430–681] ng/mL vs 366 [299–548] ng/mL, $p = 0.006$) compared to pre-PCI levels.

Similar results were obtained when examining ICAM-1 concentrations: there was no alteration in the WE group, but in the DR group ICAM-1 concentrations were significantly elevated in samples taken after 3–6 months (844 [700–1246] ng/mL vs 642 [412–743] ng/mL, $p = 0.037$) compared to the baseline values.

Quantity of vWF Ag was also measured, which characterizes endothelial cell activity. Quantification of vWF Ag in the WE group did not show any differences, while a significant elevation was observed in the DR group in the 48-hour samples (143.5 [114–221.3] % vs 136.5 [99.3–184] %, p = 0.002). Importantly, ABO blood group did influence the trend in vWF Ag level as when the results of vWF Ag levels in patients with blood groups 0 and non-0 were analyzed separately, this led to the same results.

2.2. Results from thrombin generation test

No significant differences were found in the lagtime and ttPeak values in the WE treated group. In contrast, in the DR group, a nearly significant decrease (8.38 [6.7–10.1] min vs 8.88 [7.51–10.46] min, p = 0.05) was observed in the lagtime at 48 hours after after the intervention compared to the baseline values, however, 3-6 months after PCI, the same difference became significant (6.5 [5.84–8.35] min vs 8.88 [7.51–10.46] min, p = 0.004), also compared to the preoperative results. Likewise, DR-treated patients showed a significant difference when examining the ttPeak parameter: both 48 hours after PCI (11.42 [9.05–13.26] min vs 12.26 [11.05–1333] min, p = 0.023) and 3–6 months later (9 [8 .0–10.26] min vs 12.26 [11.05–13.33] min, p = 0.002) a significant decrease was occured compared to pre-PCI values.

Examining the quantitative parameters of the generated thrombin, we obtained data consistent with the results of the parameters characterizing the kinetics. In case of the thrombin peak, no significant difference was found between the sampling times in the patient group treated with the WE technique. Among patients whose CTO was successfully opened by the dissection-reentry procedure, a significantly higher amount of thrombin generation was observed 48 hours after the intervention (312 [221–372] nM vs 244 [184–277] nM, p = 0.004), and this difference became even more pronounced 3-6 months after PCI (393 [316–441] nM vs 244 [184–277] nM, p = 0.001), compared to baseline values. Examining the ETP, a significant difference (p = 0.048) was shown between the WE-treated patients 48 hours after the intervention compared to

the pre-PCI values, however, more significant differences were found in the DR group. There was a significant difference between the 48-hour and baseline values ($p = 0.003$), which difference continued to increase 3-6 months later ($p = 0.002$) also compared to the baseline results.

2.3. Regression analysis

Linear regression analysis confirmed that development of significant differences in VCAM-1 ($p = 0.005$), lag time ($p = 0.001$), ttPeak ($p < 0.001$) an ETP ($p = 0.015$) after 3–6 months was dependent on the type of the intervention, while significant differences in ETP values ($p = 0.049$) and levels of vWF Ag ($p = 0.021$) observed after 48 h were related to the total length of the stents.

2.4. Clinical follow-up of recruited CTO patients

During the median 9 months of follow-up period, complication (e.g. myocardial infarction) in the WE group developed in only one (3.44 %) patient, while there were no complications in the DR cohort, and no death occurred in either group. Consequently, no significant difference was found in TVR and MACE between the two groups ($p = 0.473$). On the basis of data on clinical status obtained during the follow-up, we concluded that the two variables, i.e. the type of intervention and the presence of symptoms were independent from each other ($p = 0.865$).

3. Activation markers analysed according to the characteristics of interventions

3.1. Surface P-selectin expression of platelets

In case of patients with low (0 or 1) J-CTO score, no significant difference was found in platelet surface P-selectin expression between blood sampling times. Patients with higher J-CTO (2–3) score values showed a significantly elevated platelet P-selectin positivity 2 days after the intervention (4.07 [3.18–7.17] % vs. 3.17 [2.48–4.22] %, $p = 0.004$) compared to baseline

levels. A shorter duration of PCI (40–117 min) resulted in no change in surface P-selectin expression. When the procedure time was between 118–255 min, significantly elevated expression of P-selectin was measured 2 days after the CTO-PCI (3.64 [2.69–6.94] % vs. 3.16 [2.4–4.08] %, p = 0.013) compared to baseline values. No significant difference was found in surface P-selectin expression of platelets between the blood sampling times nor in the shorter, neither in the longer stented group.

3.2. Soluble P-selectin concentration

Patients with lower J-CTO scores showed no alteration in the concentrations of soluble P-selectin after CTO-PCI, however in the higher J-CTO score group, significantly elevated soluble P-selectin levels were measured 3–6 months after the intervention (54.2 [39.7–73.2] ng/mL vs. 45.8 [36–70.1] ng/mL, p = 0.028) compared to pre-intervention levels. No change was observed under relatively short procedures, while patients who have undergone long intervention (118–255 min) showed significantly elevated soluble P-selectin levels 3–6 months after the PCI (54.8 [42.8–76.4] ng/mL vs. 49.5 [35.4–73] ng/mL, p = 0.023) compared to pre-PCI levels. The two subgroups created based on the total stent length showed no alteration in soluble P-selectin concetration after CTO-PCI.

3.3. Analysis of the relative expression of circulating microRNAs

In the WE group, no significant difference was found in the relative expression of miR-223, while a decreasing tendency occurred in the DR cohort 3–6 months after the CTO-PCI compared to baseline samples (0.012 [0.011–0.026] vs. 0.026 [0.017–0.045], p = 0.053). Analyzing the results according to the three other categories, no significant alteration was found either in the 2 day or in the 3–6 month samples. The same can be claimed for miR-126, no significant change in the relative expression of miR-126 occurred between the post- and pre-PCI values in any categorization. In theWE group, significantly lower expression of circulating

miR-181b was detected after 3–6 months during the monitoring compared to the baseline values (0.023 [0.009–0.073] vs. 0.04 [0.014–0.104], p = 0.042). In contrast, patients treated with the DR technique also showed reduced miR-181b levels throughout the study intervals, but did not reach a significant difference between two time points. Shorter total stent length also resulted in a significantly lower expression of miR-181b at 3–6 months after the intervention (0.016 [0.007–0.049] vs. 0.037 [0.014–0.073], p = 0.031) compared to baseline values. In the group with longer stents, no significant alteration was observed. A nearly significant decrease occurred in the relative expression of miR-181b 3–6 months after the intervention (0.022 [0.011–0.053] vs. 0.034 [0.015–0.07], p = 0.068), when patients received shorter duration of treatment, compared to the baseline levels. The longer duration of the procedure showed no significant alteration in relative miR-181b expression between pre- and post-PCI values.

Discussion

Since the beginning of the PCI era, there have been many studies on how percutaneous intervention and accompanying balloon dilatation and stent implantation affect the vasculature, endothelial cells, platelets and the coagulation cascade. Most of these studies focused on the topic in relation with acute coronary syndrome and stable ischemic heart disease population, however quite a few studies deal with the group of patients with chronic total coronary occlusion, so the results from the quantitative comparison of biomarkers can only be evaluated in the light of studies conducted on populations with ACS and stable angina.

1. Comparison of subgroups treated with WE- and DR-technique

In this comparison, we first performed the quantitative determination and comparison of endothelial cell activation markers. While there was no difference between the blood sampling times in the WE-treated patients, the group of patients treated with the dissection-reentry technique showed significantly higher VCAM-1 and ICAM-1 concentrations 3-6 months after the intervention, compared to the baseline values. In case of VCAM-1, a significant concentration increase was already observed 2 days after PCI. In a study conducted in 2010, in which a population of patients suffering from stable angina and undergoing elective PCI was examined, a significantly higher concentration of VCAM-1 was measured 3 days after the intervention, which returned to the baseline level on the 7th day. Another study from 2016 compared patient groups undergoing BMS and DES implantation. Patients who received BMS showed a significantly increased VCAM-1 concentration 24 hours after PCI, while the increase was not significant in case of DES implantation. Patients in whom in-stent restenosis was confirmed during the follow-up showed significantly higher ICAM-1 plasma levels already 1 month after the intervention compared to patients without restenosis. Jin et al investigated the correlations between late ST and endothelial dysfunction biomarkers after sirolimus-eluting stent implantation. ST was registered in 41 people, and their results were compared with the

values of a control patient population of 82 people, matched according to age, gender and coronary lesion. Significantly higher concentrations of vWF, VCAM-1 and ICAM-1 were measured in patients undergoing ST compared to the control group. In our study, ST was confirmed only once in the WE-treated group, so we cannot draw far-reaching conclusions about the relationships between the development of complications and biomarker levels. Although the patients treated with the DR technique showed significantly higher levels of VCAM-1, ICAM-1 and vWF in our study than the patients in the WE group, no complications developed during the follow-up period. Overall, it can be declared that the patients whose CTO was opened using the dissection-reentry technique with more extensive vessel injury showed a greater degree of alteration in the biomarkers characterizing endothelial cell activation than those whose lesion could be opened with an intraluminal technique.

To the best of our knowledge, this study investigates for the first time thrombin generation potential after PCI in plasma of patients with CTO. Recently, patients with a residual thrombin generation after PCI and stent implantation showed a significantly higher risk for long-term cardiovascular death. Based on the obtained TG values, it is striking that the results point in one direction: significant differences in the TG parameters in the DR group, while the same parameters did not or did not change significantly among patients treated with the WE technique. Examination of the 48-hour plasma samples of the DR group revealed that compared to baseline values, TG started earlier and was associated with a higher maximum thrombin concentration, which was also reflected in higher ETP values. These changes persisted 3-6 months after PCI, moreover, the degree of the difference became statistically more significant. In comparison, patients treated with the wire escalation technique did not show the same differences. The results of the TG tests allow us to conclude that the DR technique can contribute to thrombus formation through increased endothelial cell activation, which can lead to the development of thrombotic complications. Despite these data above, there was no

difference in the early and late complication rate and stent thrombosis rate between the WE and DR treated groups. The dual antiplatelet therapy seems to be effective against the elevated thrombosis risk found in the DR treated group, which is reflected in the vigorous vWF activity and in the TG results at the level of laboratory measurements.

The comparison of the safety of the two different CTO opening techniques was also the subject of the study. As complication was registered in only 1 case, in the WE group (presumably due to the low number of cases and short follow-up time), we could not assess the safety of the two techniques in this study. However, studies analyzing the same issue are available in the international literature. Godino et al. conducted a study involving patients who underwent successful CTO-PCI, in which WE and the guided subintimal tracking and reentry (guided-STAR) technique were compared. There was no significant difference in the periprocedural MI, the ST during the follow-up, or the cardiovascular mortality between the two groups. On the other hand, the incidence of restenosis in patients treated with the guided-STAR technique was found to be higher, and the independent predictor of restenosis was the length of the stent. Azzalini et al compared WE, modern DR and older DR procedures. Patients treated with WE and modern DR techniques showed similar TVR and MACE rates, however, in those treated with old DR techniques, the TVR rates were higher, consequently the MACE rates were also higher. In Rinfret's study, the frequency of MACE did not differ significantly when comparing the two procedures, and it was confirmed by multivariate analysis that the DR technique did not affect the long-term outcome. Data in the literature are somewhat contradictory, however, after an earlier and more extensive DR intervention, restenosis may develop more frequently.

2. Activation markers analysed according to the characteristics of interventions

In this part of the research, the surface P-selectin expression of platelets, the concentration of soluble P-selectin in plasma, and the relative expression of microRNA-223, -181b and -126 were investigated. In the present study, flow cytometric measurements showed a significant

degree of platelet activation 2 days after CTO-PCI, if it was complicated and prolonged. After the spread of percutaneous interventions, several studies were published on the effects of PCI on platelet activation. In these studies, surface P-selectin expression of platelets of patients who underwent diagnostic coronary angiography and stent implantation was usually compared. Inoue's study found a significant increase in the proportion of P-selectin positive platelets after PCI compared to the pre-intervention state. In Nagy et al.'s study, a significantly higher rate of CD62-positive platelets was identified in stented patients 15 minutes after PCI than in case of those who underwent only angiography. The data from the international literature point in the same direction as the results of our present research: shortly after the more stressful, complex, intervention with extensive vascular injury, a significantly higher level of platelet activation was detected compared to baseline levels.

The concentration of soluble P-selectin was also measured by the research groups named above. Nagy et al. found no difference between the concentrations of soluble P-selectin in stented patients and those who underwent coronary angiography, while Inoue et al. measured significantly higher soluble P-selectin concentrations in the coronary sinus immediately after stent implantation compared to baseline values. The results of soluble P-selectin obtained from our study are consistent with data from similar studies: 3-6 months after prolonged, complex interventions, significantly higher P-selectin concentrations were detected compared to pre-PCI values. Patients with a higher J-CTO score received a long, complicated, stressful intervention, the vascular damaging effect of such interventions is also more pronounced. This was also manifested in the surface P-selectin expression of platelets. A more stressful intervention causes the activation of a larger amount of platelets. P-selectin bounded to platelets is first transformed into a soluble form by the elimination of platelets within a relatively short time, and then disappears from the circulation, reducing surface P-selectin expression and initially increasing the concentration of the soluble form. However, the high soluble P-selectin concentration

measured 3-6 months later is much more due to the endothelial cells. This explains the significantly higher soluble P-selectin concentration measured 3-6 months after the intervention and the surface P-selectin expression decreasing to the initial level, since platelets activated in connection with PCI are no longer in the circulation months later.

Although many studies have investigated the biology of microRNAs and their potential role in cardiovascular diseases, there has not yet been a study involving the analysis of microRNAs in a population suffering from chronic total coronary occlusion. In the present research, the relative expression of miR-223, miR-181b and miR-126 was determined using the reference gene *cel-miR-39*. In the case of miR-223, we only found a noteworthy result in the comparison of the WE vs. DR patient groups: In the group of DR-treated patients, an almost significant relative decrease in expression ($p = 0.053$) was detected 3-6 months after the intervention, compared to the pre-PCI data. An animal experimental research conducted by Dai et al. identified the role of miR-223 in inhibiting angiogenesis. This role of miR-223 may explain the almost significant decrease in relative miR-223 expression observed in the DR-treated group, as the remodelling and angiogenesis-stimulating processes are more active after interventions with extensive vascular injury. When examining miR-126, no significant differences were found between patient groups and blood sampling times, while contradictory data were obtained for miR-181b. We found a decrease in the relative miR-181b expression in patients who underwent a simpler, shorter, less stressful procedure. The levels of the microRNAs analyzed in the present study usually decrease in processes involving an inflammatory reaction and the activation of endothelial cells and platelets. This seemed to be confirmed only in case of miR-223. Since one mRNA is regulated by the fine-tuning of dozens of microRNAs, and the examined microRNAs are also involved in the modification of several targets, therefore, the results contrary to expectations lead to the conclusion that miR-181b and miR-126 are also involved in the regulation of other processes in addition to those identified so far, and that the

endothelial cell and platelet activation is also affected by other microRNAs, the investigation of which was not part of our study.

Summarizing the data, it is clear that in case of prolonged, complicated and stressful CTO-PCI, we have to reckon with increased platelet activation in the short term and increased endothelial cell activation in the long term, which may be the consequences of extensive vascular injury. Enhanced platelet activation shortly after the procedure may predispose to a higher incidence of ST, while increased endothelial activation can result in restenosis in the treated segment in the long term. Despite the observed increased cell activation, we did not observe a higher complication rate in connection with prolonged complicated interventions, such as the DR technique. Based on the results regarding platelet activation, the administration of novel types of antiplatelet agents after a complex, challenging intervention in the early postoperative period should be considered. Complications appearing in the late follow-up period may be caused by the enhanced endothelial cell activity, thus our study can highlight the importance of research, which targets the attenuation of the activation of endothelial cells.

Summary

In present study we investigated endothelial cell and platelet activation in connection with percutaneous coronary intervention in patients suffering from chronic total coronary occlusion. For this purpose, the surface-bound P-selectin of platelets, the plasma concentration of soluble adhesion molecules, the relative expression of selected microRNAs, and the thrombin generation potential were determined from blood samples obtained at three different time points.

In the first part of our research, laboratory parameters of the WE- and DR-treated group were compared. Based on the tests, we confirmed that patients treated with the DR procedure show increased endothelial cell activity in long term, which is indicated by the increased plasma concentration of adhesion proteins and the increased thrombin generation potential as well.

In the second round, cell activation changes related to the characteristics of the intervention were analyzed. For this purpose, the members of the patient population were divided into two parts based on four criteria. We found, that patients with high J-CTO score who underwent prolonged, complicated intervention show a significantly increased platelet activation in short term, while in long term, endothelial cell activation mostly dominates, which is supported by the significantly elevated concentration of soluble P-selectin, that persists even months after the procedure. Data on the relative expression of selected microRNAs are somewhat contradictory, so they did not contribute to drawing conclusions from the research.

Our laboratory results indicate, that long-lasting, complex interventions with extensive vascular injury are associated with increased platelet activation and thus a higher ST risk in the early follow-up period, while the late postoperative period is characterized by remarkable endothelial cell activation, which may presumably also be responsible for late complications.

New scientific achivements

1. In connection with the DR treatment of patients suffering from chronic total coronary occlusion, a significantly higher degree of endothelial cell activation can be measured months after the intervention than in case of using the wire escalation technique.

2. In the DR-treated patient group, all the four parameters of the thrombin generation test show an increased activity of the coagulation cascade both in short and long term after the intervention, in contrast to the patient group treated with the wire escalation technique.

3. Based on clinical data, the type of intervention (WE vs. DR) and the symptoms occurring during the follow-up period are independent of each other.

4. In case of high J-CTO score (2 or 3) and long intervention (118-255 min), a significantly higher platelet activation can be measured 2 days after the intervention than with a low (0 or 1) J-CTO score and short (40-117 min) type of intervention.

5. In case of high J-CTO score (2 or 3) and long intervention (118-255 min), a significantly higher level of endothelial cell activation can be measured months after the intervention than with a low (0 or 1) J-CTO score and short (40-117 min) type of intervention.

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