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

Analysis of microRNA expression in metabolic and vascular diseases associated with enhanced platelet and endothelial cell activation

by Zsolt Fejes

Supervisor: Béla Nagy Jr., MD, PhD

UNIVERSITY OF DEBRECEN

KÁLMÁN LAKI DOCTORAL SCHOOL

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Members of the Examination Comittee:	Prof. Dr. Bálint Nagy, MD, PhD, DSc Prof. Dr. Kraszimir Kolev, MD, PhD, DSc

The Examination takes place at the Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, Debrecen; at 11:00 AM; 5th of December 2018

Head of the **Defense Committee**:	Prof. Dr. Zoltán Papp, MD, PhD, DSc
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The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, Debrecen; at 13:30 PM; 5th of December 2018

Introduction and former publications in this field

MicroRNAs (miRNA) are the members of non-coding RNA family, which have an important role in the posttranscriptional fine-tune regulation of genes involved in different cellular functions. They act via binding to either fully or partially complementary sequences within the 3' UTR of messanger mRNAs (mRNA), and inhibit the expression of target genes via repressing protein translation or promoting mRNA degradation. Among physiological conditions, miRNAs participate in the regulation of several metabolic pathways, cell differentiation, proliferation and apoptosis, while their abnormal expression has been linked to the development of inflammatory, malignant, metabolic and cardiovascular diseases.

In this thesis, we applied models for two common diseases which currently affect more and more individuals worldwide. Thrombotic and vascular complications associated with both type 2 diabetes mellitus (DM2) and ischemic coronary diseases have an important epidemiologic consequence as being represented among the leading causes of death.

Platelets and endothelial cells play a key role in hemostasis, since their normal function contributes to the maintenance of vascular integrity. Among abnormal metabolic and inflammatory conditions platelets and endothelial cells demonstrate enhanced activation status which may result in other vascular complications. It is well-known that platelet activation level increases in DM2, hence, platelets may become activated by a smaller stimulus and even at a higher degree. In coronary artery diseases, a large number of evidence is available about the clinical advantages of drug-eluting coronary stents (DES), e.g. with everolimus, since localy sustained drug level may decrease the risk of early complications, such as in-stent restenosis (ISR) in contrast to the classic bare-metal stents (BMS). In the background of these alterations, the role of activated endothelial cells is highly proposed.

The topic of this thesis is the characterization of platelet and endothelial cell derived miRNAs with the investigation of their pathomechanism in two diseases associated with enhanced platelet and endothelial cell activation. The medical reaserch on miRNAs has got in focus in the last couple of years, and thus, they may gradually become novel laboratory and prognostic biomarkers as well as new therapeutic targets.

The role of miRNAs in the regulation of gene expression

MiRNAs go through a multi-step maturation process before incorporate into the RISC complex (RNA-induced silencing complex). Their mature form is processed by the Dicer enzyme, which cleaves the „loop structure" of pre-miRNAs, and two mature miRNA duplexes are produced having cc. 18-25 nucleotides length. The leading strand is integrated into RISC complex with Ago2 protein, while the other strand becomes degraded.

MiRNAs prevents the expression of genes primarly in posttranscritional manner via two main mechanisms. First, they act via the inhibition of translation, and secondly, via the destabilization of mRNA with deadenilation and „decapping" mechanisms causing the final degradation of mRNAs. Based on their partial or complete complementary, miRNAs bind to the certain part of mRNAs. Each miRNA can regulate the expression of several hundreds of genes, and in turn, one gene can be regulated by more than one miRNA. About 60% of coding genes are under the regulation of miRNAs and thus, they may play an important role in the regulation of a number of physiological and pathological events.

Development of enhanced platelet activation

Platelets are produced by megakaryocytes (MK) in the bone marrow as well as in the lung tissue as resident cells based on a very recent animal study. They do not possess nuclei, and their normal life time is about 9-11 days. Among physiological circumstances, in case of vessel wall injury, liberated molecules (e.g. collagen, von Willebrand factor, etc.) can activate platelets through different receptors. Platelets aggregate with each other or with other cell types to adhere to the injured vessel wall as a part of primary hemostasis. Upon severe infection or inflammation, or in metabolic diseases, platelets become easily activated at a higher degree leading to potential thrombotic/vascular complications. Such stimuli can be hyperglycemia in DM2, Gram-positive or Gram-negative bacteria derived mediators (e.g. peptidoglycan or lipopolysaccharide) in sepsis, or high cholesterol level in lipid disorders, and the obesity itself. The generation of increased platelet activation status can be characterized with high surface and soluble P-selectin (CD62P) expression.

The RNA content of platelets

Although platelet do not have nuclei, they contain MK-derived mRNAs, miRNAs, several cell organelles (ribosomes, endoplasmatic reticulum, etc.) and enzymes (e.g. Dicer). Thus, platelets may be able to synthasize protein *de novo* (e.g. IL-1β) in response to specific stimuli. As there is no transcription in platelets, it seems that pre-mRNA „splicing" mechanism and the function of miRNAs have a much larger significance than in other nucleated cells. Currently, more than 500 platelet miRNAs have been described which modulate not only the expression of proteins in relation to platelet activation, but also those, which are involved in the prevention of platelet activation. Thus, this is how miRNAs may control the cellular activation processes and subsequent thrombus formation. Accordingly, altered miRNA levels may highly influence the level of platelet reactivity. In resting platelets, the RISC complex containing miRNAs can prevent the synthesis of activation-dependent proteins via the inhibition of mRNA function, while Dicer sustains the maturation of miRNAs. In case of platelet activation (e.g. by thrombin) mRNAs dissociates from the RISC and, hence, protein production may occur (e.g. Plasminogen activator inhibitor-1) in platelets. In DM2, due to increased intracellular Ca^{2+} concentration in platelet activation, Ca^{2+}-dependent calpains become activated that cleaves Dicer, and decreases its enzyme activity, so the expression of a number of mature miRNAs is lowered.

MiRNAs and their targer mRNAs in association with platelet activation

The direct relationship between some platelet miRNAs and their target mRNAs has been already approved. First, Landry and his coworkers discovered that miR-223 regulates the expression of P2Y12 ADP-receptor, which is higly involved in the regulation of platelet reactivity. Apart from this function, miR-223 regulates the expression of platelet β1-integrin, kidlin-3 and FXIII A subunit. Furthermore, miR-223 depleted mice showed increased platelet activation that underlines the central role of this miRNA in platelet function. Reactive platelets had lowered miR-96 level with elevated VAMP8 (Vesicle-associated membrane protein 8) mRNA and protein levels contributing to enhanced platelet secretion.

Altered platelet miRNA expression in DM2

Pancreatic Langerhans β-cells become gradually exhausted in DM2 and insulin resistance is generated casing hyperglycaemia that raise the degree of platelet activation

status. Beside endothelial cell dsyfunction, reactive platelets are also responsible for the development of cardiovascular complications in association with diabetes. Decreased platelet miR-223 and miR-146 levels were formerly reported raising the risk of stroke in DM2. In addition, lower miR-223, miR-126, miR-24, miR-197, miR-191 and miR-21 levels were found in plasma samples both in DM2 and severe atherosclerosis. Abnormal miRNA expression may also decrease the efficacy of anti-platelet therapies (e.g. miR-223 affects the effect of clopidogrel via P2Y12-receptors), and they may serve as pontential biomarkers in the early detection of DM2 and their complications as well (e.g. miR-103b).

The clinical implications of coronary bare-metal and drug-eluting stent implantation

For the treatment of coronary artery diseases, coronary stents are used in a wider range of implications thanks to the recent rapid development of interventional cardiology. In stead of classic bare-metal stents (BMS), drug-eluting stents (DES) are more frequently applied in those diseases with higher risk for thrombotic alterations, such as in DM2. According to recent clinical data, DES with everolimus or with other mTOR inhibitors are safer in acut myocardial infarct (AMI), atrial fibrillation, and stable angina compared to BMS as the risk of ISR is markedly lowered. However, data about the direct effects of DES and BMS on different cell activation have been contradictory in former clinical studies.

The role of everolimus in the regulation of cellular activation in endothelial cells

Everolimus is an mTOR (mammalian target of rapamycin) inhibitor that is used as an immunosuppressive agent primarly in patients who underwent an organ transplantation to prevent organ rejection. Its antiproliferative effect has been characterized in smooth muscle cells via the blockage of G1 phase of cell cycle. Moreover, everolimus decreased IL-8 production in neutrophils and their TNF-α induced adhesion was prevented to endothelial cells *in vitro*. The analog of everolimus could lower the level of VCAM-1 induced by TNF-α in HUVEC cells via the inhibition of mTORC2 (mTOR complex 2) activity. Of note, the effect of everolimus against endothelial cell activation and its potential transcriptional and posttranscriptional mechanisms have not been investigated as yet.

Aims of the thesis

Our aim was to characterize platelet and endothelial cell derived miRNAs and to investigate their pathomechanism in two diseases associated with enhanced platelet and endothelial cell activation.

Analysis of platelet and MK derived miRNAs in DM2

We hypothesized that altered levels of platelet and MK miRNAs induced by hyperglycaemia contribute to elevated platelet activation status in DM2.

- We aimed to study the role of those miRNAs which are expressed at high quantity in platelets and may be related to platelet activation. The expression of miR-223, miR-26b and miR-140, and their target mRNAs (P2RY12 and SELP) was analyzed in isolated platelet and plasma samples of DM2 patients in comparison to obese and healthy controls.
- The diabetic conditions of the bone marrow were investigated with two different types of MK cell lines (MEG-01 and K562-MK) to observe the direct effect of hyperglycemia on miRNA and mRNA content of these cells.
- We wanted to prove the direct association of miR-26b and miR-140 with SELP mRNA via using specific miRNA mimics and inhibitors in MK cells.
- Altered Dicer function in DM2 was also analyzed in an indirect way by using calpain inhibitor (calpeptin) in MEG-01 MK cell samples *in vitro*.

Comparison of the effect of BMS and DES on cellular activation in stable angina patients

We hypothesized that coronary BMS implantation resulted in a higher level of platelet and endothelial cell activation than DES, thus, BMS patients might have clinical complications (e.g. ISR) at a higher risk.

- We wanted to compare the concentrations of activation-dependent soluble biomarkers in the presence of the two main types of coronary stent before implantation and after two different time points (after 24 hours and 1 month).
- Recruited patients were monitored for 6 months, and based on stent type and the development of ISR, biomarker values were futher evaluated in different subgroups.

Investigation of the inhibitory effect of everolimus on endothelial cell activation

We hypothesized that everolimus - as an mTOR inhibitor - eluted from DES was able to decrease the level of endothelial cell activation via modulating the expression of activation-dependent proteins.

- Our aim was to set a cellular model for the investigation of vascular inflammatory conditions using coronary artery (HCAEC) and venous (HUVEC) endothelial cell cultures for studying SELE and VCAM1 gene expression at transcriptional level *in vitro*.
- We analyzed the levels of mature and precursor forms of miR-181b related to the posttanscritional regulation of these genes in TNF-α-treated endothelial cell samples in the presence or absence of everolimus.
- The anti-inflammatory effect of everolimus was also monitored via the activation of NF-κB pathway and the altered expression of enhancer RNAs of SELE and VCAM1 genes.
- In the plasma samples of BMS- or DES-treated stable angina subjects, circulating miRNAs were quantified in the presence or absence of ISR caused by stent implantation.

Materials and methods

Patients and controls

DM2 subjects, obese and healthy controls

We performed a cross-sectional, case-control study involving 28 DM2 patients in comparison with 23 age- and gender matched healthy controls and 19 non-DM obese individuals. All participants gave written informed consents. The study was approved by the Ethics Committee of the University of Debrecen (permit number: 4102/2014) in accordance with the Declaration of Helsinki. Diabetic and obese subjects were enrolled from the Department of Internal Medicine Outpatients Clinic at the University of Debrecen. Exclusion criteria for enrollment included angina, intermittent claudication, severe inflammation, transient ischaemic attack, malignancy, pregnancy, autoimmune diseases, impaired liver or renal function and infectious diseases. Healthy controls were recruited among healthy volunteers or staff members from the Departments of Internal Medicine and Laboratory Medicine who underwent a detailed medical history, physical examination and routine laboratory tests and were free of any cardiovascular, cancer, metabolic or inflammatory disease.

Stable angina patients received BMS or DES coronary implantation

Forty-nine stable angina patients who underwent an elective coronary stenting during a period of 18 months: 28 were treated with BMS (Integrity®) and 21 received everolimus-eluting stents (Xience V® or Xience Prime®). Patients were selected for BMS or DES implantation at the decision of the operator (Dr. Tibor Szük, Institute of Cardiology, Faculty of Medicine, University of Debrecen). Exclusion criteria were AMI, chronic hematological diseases, malignancy, a history of previous stenting, and chronic inflammatory and autoimmune conditions. In addition, individuals who had any periprocedural complications such as procedure-induced acute occlusion or dissection were also excluded. This study was approved in advance by the Regional Ethics Committee of the University of Debrecen (permit number: 3510-2011) in accordance with the Declaration of Helsinki. All participants gave their written informed consent.

Blood sampling and sample preparation

Venous blood samples (10 mL) for platelet and plasma miRNA analysis were obtained by atraumatic venepuncture into Vacutainer® tubes containing 0.105 M

sodium citrate (Becton Dickinson) when DM2 patients attended for a follow-up appointment, or when obese and normal controls appeared for sampling at the Outpatient Clinic. White blood cell (WBC) count and platelet count were determined by Advia 2120 Hematology System (Bayer Diagnostics). Serum fasting glucose, total cholesterol, LDL- and HDL-cholesterol, triglycerides, C-reactive protein (CRP) as well as urine albumin and creatinine levels were measured by electrochemiluminescent immunoassay using a Cobas 6000 analyser (Roche Diagnostics). Serum fasting insulin was determined by a Liaison XL chemiluminescence analyser (DiaSorin). HbA1c was measured by HPLC (BioRad Laboratories).

We organized the collection of venous blood samples obtained from each BMS and DES patient by atraumatic venepuncture into Vacutainer® tubes containing 0.105 M sodium citrate (Becton Dickinson) at three different time points for the measurement of soluble biomarkers: (i) prior to stenting, (ii) at 24 hours after the procedure, and (iii) after a 1-month period of dual antiplatelet medication. Endothelial cell activation caused by the procedure was partly evaluated via von Willebrand factor antigen (vWF-Ag, BCS XP, Siemens) by immunoturbidimetry (BCS XP, Siemens). Serum C-reactive protein (CRP) and high sensitive cardiac troponin T (cTnT) were determined by electrochemiluminescent immunoassay (Cobas e411 instrument, Roche), D-dimer and fibrin monomer (FM) were measured by immunoturbidimetry (BCS XP), and fibrinogen level was investigated by Clauss method. These parameters were routinely analyzed by the Hemostasis and Integrated Clinical Chemistry Subunits of the Department of Laboratory Medicine.

Leukocyte-depleted platelet sample preparation

A volume of 2 mL PRP (platelet-rich plasma) was purified by anti-CD45-conjugated magnetic microbeads (Dynabeads®, Invitrogen) to obtain leukocyte-depleted platelet samples (LDP) according to the manufacturer's instructions. After the incubation of PRP with beads for 30 min at room temperature (RT), these samples were inserted into a magnetic separator (Becton Dickinson) for 2 min, and LDP was then transferred into a tube for additional centrifugation (1500 g, 15 min, RT). Platelet pellet was lysed with 1 mL TRI reagent (Molecular Research Center) and stored at -20 °C before RNA isolation.

Plasma sample preparation for the analysis of circulating miRNAs

In the DM2 study, PRP samples were further centrifuged at 1500 g for 15 min to obtain platelet-poor plasma (PPP). Frozen plasma samples of BMS and DES individuals were thawed and were then centrifuged (10 000 g, 1 min, RT). Then 750 µl TRI reagent was added into 250 µl PPP, and stored at -20 °C before RNA isolation.

Cell culturing and treatment

Culturing MEG-01 MK cells in hyperglycemic conditions

Human megakaryoblastic leukaemia cell line MEG-01 cells (ECACC) were cultured in RPMI-1640 medium (Sigma-Aldrich) with 10 % FBS (Sigma-Aldrich), 100 U/mL Penicillin and 100 µg/mL Streptomycin (Sigma-Aldrich) at 37 °C, 5 % CO_2. MKs were treated with high concentration of D-glucose (33 mM, Sigma-Aldrich) for 8 hours up to four weeks. Negative (osmotic) control sample was treated with D-mannitol (Sigma-Aldrich) at the same concentration for the same time period. After treatment, cells were washed once with sterile PBS, then lysed in 750 µl TRI reagent and stored at -20 °C before RNA isolation.

Human chronic myelogenous leukemia cell line K562 cells (kindly provided by Dr. Tamás Varga, University of Debrecen, Department of Biochemistry and Molecular Biology) were cultured in RPMI-1640 medium with 10 % FBS, 100 U/mL Penicillin and 100 µg/mL Streptomycin at 37 °C, 5 % CO_2. Cells were differentiated by using 20 ng/mL phorbol-12-myristate-13-acetate (PMA) for 5 days. The differentiation of these cells was followed via the surface CD41 and CD61 percent positivities and mean fluorescence intensity (MFI) values by flow cytometry. For immunophenotyping, MK cells were incubated with anti-CD41-PE or anti-CD61-PE and CD45-FITC antibodies (Becton Dickinson) for 15 min in the dark at RT. A total of 100.000 events were acquired on a FC-500 flow cytometer (Beckman Coulter). Results were expressed as the percentage of positive MK cells. Mature MKs were then exposed to high concentration of D-glucose (33 mM) for 8 hours up to five days to provide similar hyperglycemic conditions as described for MEG-01 cells above. Finally, treated cells were washed with sterile PBS, then lysed in 750 µl TRI reagent and stored at –20 °C before RNA isolation.

Culturing endothelial cells among *in vitro* inflammatory conditions

Human coronary artery endothelial cells (HCAEC, Cell Applications) were cultured in ready-to-use MesoEndo Cell Growth Medium (Cell Applications) at 37°C, 5% CO_2. In parallel, HUVECs were specifically isolated for this study and were removed from human umbilical veins by exposure to dispase and cultured in medium 199 (M199, Gibco) containing 15% fetal bovine serum (Gibco), antibiotic, antimycotic solution (1%, Sigma), heparin (5 U/mL, Teva) and endothelial growth supplement (7.5 ug/mL, Sigma). HUVEC cells and the special medium were kindly provided by Dr. Viktória Jeney and Dr. Enikő Balogh (University of Debrecen, Internal Medicine Institute).

HCAEC and HUVEC cells (3 x 10^5/well) were treated in 6-well plates with recombinant TNF-α (100 ng/mL, Gibco) for 1-24 hours to generate cellular inflammatory conditions as an *in vitro* model of stent-induced EC inflammation. In parallel, the effect of everolimus on EC activation was studied using everolimus (0.5 μM, dissolved in DMSO, Sigma) in the presence of TNF-α for the same time period above. After treatment, cells were washed once with sterile Hanks' Balanced Salt solution (Sigma), then lysed in 750 μL TRI reagent (Molecular Research Center) and stored at -20°C before RNA isolation.

Laboratory analyses

Flow cytometry analysis of platelet activation level via P-selectin expression

Investigation of platelet activation level via surface P-selectin expression was performed. Whole blood samples (40 μL) were fixed, then platelets were identified by a fluorescein-isothiocyanate (FITC)-conjugated monoclonal antibody to GPIX (CD42a-FITC). Platelet activation was detected by phycoerythrin (PE)-labelled anti-P-selectin (CD62-PE). As a control for immunolabelling with anti-CD62 antibody, platelets were incubated with PE-coupled non-immune mouse IgG1 antibody (Becton Dickinson). A total of 10.000 dual-colour labelled platelet events were acquired on a FC-500 flow cytometer (Beckman Coulter). Results were expressed as the percentage of double positive platelets.

Total RNA extraction

Total RNA including miRNA was isolated from LDP and plasma by TRI reagent according to the manufacturer's recommendations. The purity and the concentration of

separated RNA samples were verified by a NanoDrop spectrophotometer (Thermo Scientific). Total RNA samples were stored at –70 °C.

MiRNA specific stem-loop RT-qPCR analysis

The expression of miRNAs in platelets, in plasma samples and in MK cells was analysed by Universal ProbeLibrary (UPL)-probe based stem-loop RT-qPCR assay. This technique included two steps: 1) miRNAs (10 ng total RNA) were transcribed into cDNA via miRNA specific reverse transcription using miRNA-specific stem loop-RT primer (500 nM, Sigma-Aldrich) and TaqMan® MicroRNA® Reverse Transcription Kit (Applied Biosystems), and 2) miRNA quantification was performed by RT-qPCR using designed universal reverse primer (100 μM, Sigma-Aldrich), miRNA-specific forward primer (100 μM, Sigma-Aldrich) and UPL probe #21 (10 μM, Roche Diagnostics) with Taq polimerase (5 U/μl, Thermo Scientific) and dNTPs (2.5 mM, Thermo Scientific). All measurements were done in triplicates on a QuantStudio 12 K Flex qPCR instrument (Applied Biosystems). The reference gene RNU43 was used for normalisation.

RT-qPCR analysis of mRNAs, precursor miRNAs and enhancer RNAs

cDNA synthesis was performed with High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's recommendation with minor modifications. Initial amount of RNA in LDP was 500 ng per reaction, while 1000 ng per reaction was used in the MK experiments. Quantitative PCRs for pre- and pri-miRNAs, mRNAs and eRNAs were performed using LC-480 instrument (Roche Diagnostics) with Light Cycler 480 SYBR Green I Master mix (Roche Diagnostics) and gene specific primers (10 μM, Integrated DNA Technologies). For normalization, we used the reference gene RPLP0 (36B4).

Identification of enhancer RNAs

Processed ChIP-seq data were downloaded from the NCBI GEO depository (GEO accession number: GSE53998). Integrative Genomics Viewer (IGV2.3, Broad Institute) was used for data browsing and creating representative snapshots. We reanalyzed the unstimulated and TNF-α-treated HUVEC cells-derived publicly available NF-κB transcription factor subunit p65, RNA Polymerase II (RNAPII), active histone mark H3K27Ac, and active transcription start site mark H3K4m3-specific ChIP-seq data sets. We wanted to identify TNF-α-activated transcription factor-bound enhancers in the

neighboring genomic regions of VCAM-1 and E-selectin genes. Bioinformatic analyses and the design of eRNA-specific primers were performed by Dr. Zsolt Czimmerer and Attila Horváth (University of Debrecen, Department of Biochemistry and Molecular Biology). HCAECs were then treated with TNF-α (100 ng/mL) with or without everolimus (0.5 μM) for 1 hour, and we then quantified the levels of two selected eRNAs (SELE_-11Kb and VCAM1_-10Kb) by RT-qPCR.

Quantification of circulating miRNAs in plasma samples

Total RNA was extracted from plasma samples obtained after 1 month of intervention from BMS and DES subjects to analyze the levels of circulating miRNAs with regards of the development of early ISR in 20% of BMS individuals. Patients were divided into 4 subgroups: i) the DES cohort, ii) the entire BMS cohort, iii) those BMS subjects without complications, and iv) BMS patients with ISR.

To analyze and to screen the profile of circulating miRNAs, we randomly selected 3 RNA samples from each cohort and TaqMan Open Array (Applied Biosystems) was performed to measure the level of 754 miRNAs in parallel. cDNA samples were generated by Megaplex primer pools A and B (Set v3.0, Applied Biosystems). Preamplification reactions were then performed using TaqMan PreAmp master mix (Applied Biosystems) and Megaplex PreAmp primers (Set v3.0, Applied Biosystems). Samples were placed on a TaqMan Open Array panel containing specific primers and fluorescent probes by the assay module of a QuantStudio 12 K Flex qPCR instrument (Applied Biosystems). Reactions were run based on the manufacturer's recommendations. The evaluation of the results was finally processed by Thermo Fisher Cloud System and Expression Suite Software v1.0.3 programs. MiRNAs showing the largest alteration in screening were further validated by UPL-probe based RT-qPCR in all *ex vivo* specimens. In case of Open Array analysis, we used a global normalization, and the most stable miR-24 was assessed to normalize the level of individual miRNAs. During the operation of the Open Array measurements, Dr. Szilárd Póliska (University of Debrecen, Department of Biochemistry and Molecular Biology) supervised the overall analysis.

As miR-181b may regulate the expression of SELE and VCAM1 mRNAs, we also investigated its expression in plasma samples, as this miRNA was not present in the original repertoire of the Open Array panel.

Transfection of MEG-01 cells and endothelial cells with miRNA mimics or inhibitors

MKs pretreated with D-glucose (33 mM) for 24 hours were centrifuged and resuspended in Opti-MEM I Reduced Serum Medium (Gibco) with 3 % FBS, 100 U/mL Penicillin and 100 μg/mL Streptomycin. The overexpression of miRNAs was performed using mirVana® miR-26b and miR-140 mimics (20 pmol, Ambion) with Lipofectamine RNAiMAX® Transfection Reagent (Invitrogen) for 24 hours at 37ºC and 5 % CO_2. In parallel, a negative control sample was treated with mirVana® miRNA mimic negative control (NEG-01, 20 pmol, Ambion). In turn, anti-miR-223 and anti-miR-26b (40 pmol, Ambion) were transfected into MEG-01 cells for 24 hours with Lipofactamine reagent to suppress specifically and individually these miRNA levels. Simultaneously, a sample was treated with a negative control inhibitor (NEG-01, 40 pmol, Ambion). After transfection, total RNA was extracted and miRNAs and mRNAs were quantified as described above.

The transfection of HCAECs with specific miR-181b mimic was performed based on the manufacturer's instructions. Briefly, these cells were treated with TNF-α (100 ng/mL) for 1 hour in MesoEndo medium, and Opti-MEM I Reduced Serum Medium (Gibco) with 3% FBS, 100 U/ mL Penicillin and 100 μg/mL Streptomycin was added to the cells for transfection. The overexpression of miR-181b was done using mirVana miR-181b mimic (25 pmol, Ambion) with Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) for 24 hours at 37°C and 5% CO_2. In parallel, negative control samples were treated with mirVana miRNA mimic negative control (NEG-01, 25 pmol, Ambion). After transfection, total RNA was extracted and this miRNA with SELE and VCAM1 mRNAs were quantified as described above.

Analysis of Dicer function in hyperglycemic MEG-01 cells

We applied a MK cell model where 3×10^5/mL MEG-01 cells were treated by a calpain 1 and 2 inhibitor, calpeptin (10 μmol/L, Sigma Aldrich) for 24 hours. The effect of calpeptin on miRNAs was assessed in the following settings: calpeptin alone, calpeptin together with high glucose, and calpeptin before and after glucose treatment. Untreated and hyperglycemic MK cells served as control samples. Mature miR-223 and miR-26b levels were measured by RT-qPCR in all these samples as described above.

Measurement of soluble protein levels by ELISA kits

The concentrations of all circulating cellular activation-dependent biomarkers were determined by using commercially available enzyme-linked immunoassays (ELISA, R&D Systems) according to the manufacturer's instructions. Before analysis, samples were centrifuged (10 000 g, 1 min, RT) to gain cell-free plasma or supernatant samples.

P-selectin protein concentration was also determined in platelet lysates and plasma samples of DM2 subjects and healthy controls. For the intracellular P-selectin measurements, platelets (1.25×10^8 cells/sample) were lyzed by 1% TritonX-100 and a special buffer containing protease inhibitors, and protein level was then determined along with plasma samples.

In the samples of stented individuals, the activation of platelets was monitored via soluble P-selectin, soluble CD40L and platelet derived growth factor-BB (PDGF-BB) levels. The levels of soluble VCAM-1, soluble ICAM-1, soluble E-selectin were determined for the evaluation of endothelial cell activation. Vascular inflammatory events were also monitored via TNF-α concentrations measured in *ex vivo* plasma samples after 1 month of stenting.

Two endothelial cell activation markers soluble E-selectin and VCAM-1 levels were studied in the supernatants of TNF-α-stimulated HCAEC samples with or without everolimus treatment.

Detection of nuclear factor kappa B (NF-κB) pathway activation in endothelial cells

HCAEC cells were seeded onto sterile uncoated microscope slides at a density of 5×10^4 cells/slide and cultured for 2 days. HCAECs were stimulated with TNF-α (100 ng/mL) for 1 hour in the absence or presence of everolimus (0.5 μM) or DMSO, and were then fixed with ice-cold methanolacetone (50 v/v %) for 10 min. Non-specific antibody binding sites were blocked with FBS for 15 min. For primary labelling of NF-κB p65 subunit, rabbit anti-human p65 (100 μg/mL, Santa Cruz Biotechnology, AB_632037) was used followed by secondary staining with Alexa Fluor 488-conjugated goat-anti-rabbit IgG (Invitrogen). Cell nuclei were labeled with Hoechst 33342 (Invitrogen). Samples were observed by Zeiss Axio Scope.A1 fluorescent microscope (HBO 100 lamp) (Carl Zeiss Microimaging). Images were analyzed with ZEN 2012 v.1.1.0.0. software (Carl Zeiss Microscopy), and for the NF-κB staining the

ratio of nuclear and perinuclear fluorescence intensity was calculated. Dr. Judit Váradi and Dr. Ferenc Fenyvesi (University of Debrecen, Department of Pharmaceutical Technology) contributed to the staining of cells for NF-κB p65 and the interpretation of results.

Statistical analyses

The Kolmogorov-Smirnov test was used for the evaluation of the normality of the data. In case of normally distributed parameters unpaired t-test was used, while parameters with non-normally distribution were analysed by Mann-Whitney U analysis. Comparison of multiple groups was performed using ANOVA or Kruskal-Wallis with post hoc test. Differences in various parameters among study groups were tested using Chi square test. $P<0.05$ probability level was regarded as statistically significant. Pearson's correlation coefficient (r) was used to explore relationship between the levels of soluble adhesive receptors and circulating miRNAs. Analyses were performed using GraphPad Prism (version 4.0 and 6.01) and SPSS (version 19.0) softwares.

Results

Analysis of platelet and MK miRNAs in DM2

Demographical and laboratory parameters of patient cohorts

The two different control groups were comparable with DM2 patients based on their demographical characteristics. Variables associated with diabetes showed significant differences between diabetics and normal controls (e. g. body mass index [BMI], serum fasting glucose, fasting insulin and HDL-C), while non-DM obese individuals had an alteration in BMI (35.8 [31.6-37.3] kg/m^2 and HDL-C ($P<0.05$) versus healthy volunteers.

Increased platelet activation was observed in these diabetics based on significantly elevated level of surface P-selectin expression (7.2 ± 5.1 %, $P<0.001$), in contrast to both healthy (1.8 ± 0.9 %) and obese controls (3.5 ± 1.5 %, $P=0.003$). Under statin treatment, normal lipid levels or only mild hypertriglyceridaemia were detected in these patients, thus, we could exclude the modulatory effect of hypercholesterolaemia.

P-selectin was measured intracellularly and in its soluble form in plasma, since during platelet activation, surface-bound P-selectin receptors may be shedded. Compared to healthy controls, platelets from DM2 subjects contained significantly higher level of this protein (250 ± 33 vs. 304 ± 39 ng/mL, $P=0.045$). In addition, soluble P-selectin concentrations in plasma samples showed an elevation vs. controls (26.6 ± 7.4 vs. 35.4 ± 5.6 ng/mL, $P=0.050$).

Platelet miRNAs are altered in DM2

Our aim was to investigate the background of altered platelet function in DM2 via measuring the level of some of the most abundant platelet miRNAs that might be involved in platelet activation. Platelet miR-223 was already proved to regulate the expression of ADP-receptor P2Y12, which modulates platelet sensitivity to various stimuli. Based on databases, two other platelet miRNAs, which may target the SELP mRNA that encodes P-selectin, are the miR-26b and miR-140. We found that the level of mature miR-223 ($P=0.003$), miR-26b ($P=0.001$) and miR-126 ($P<0.001$) as well as that of miR-140 ($P=0.041$) measured in a selected population were downregulated in diabetes versus healthy controls, and the alterations were even more prominent versus BMI-matched obese subjects ($P<0.001$ in case of all miRNAs).

We then studied the potential causes of attenuated level of affected platelet miRNAs to see whether DM2 platelets were incapable of processing pre-miRNAs into mature miRNAs. When the levels of pre-miR-223 and pre-miR-26b were quantified in platelets by RT-qPCR, there was a lower expression of these pre-miRNAs: platelet pre-miR-223 showed significantly decreased levels (P=0.040) as well as pre-miR-26b (P=0.093) was lowered compared to normal controls.

Circulating miRNAs are reduced in *ex vivo* plasma specimens

Platelets are a major contributor to the circulating pool of miRNAs. We wondered if plasma miRNAs were also downregulated in diabetic patients. As seen in the platelet form, circulating miR-223 (P=0.003), miR-26b (P=0.001), miR-140 (P=0.041) and miR-126 (P<0.001) showed a significant decrease in contrast to healthy controls.

Hyperglycemia represses miRNA levels in MKs

We also studied the effect of hyperglycaemia on miRNA expressions in MKs as abnormal glucose concentration might also depress miRNAs in these cells, which may consequently deliver less miRNAs into platelets. For this purpose, we used two different MK cell culture models (MEG-01 and K562-MK) where MK cells were cultured under hyperglycemic conditions by adding 33 mmol/L D-glucose for a period of 8 hours and up to four weeks. To exclude the osmotic effect of carbohydrates, D-mannitol at the same concentration was also used as a negative control sample for the same time period. There was a gradually decreasing level of miR-223, miR-26b and miR-140 and a statistically significant alteration (P<0.010 in miR-223 and miR-26b, P<0.05 in miR-140) was observed by 24 hours in both MK cells. When these metabolic circumstances were maintained in MKs for a longer period of four weeks, even weaker expressions were found (P<0.001). In contrast, D-mannitol treatment did not affect the miRNA levels in MKs at any time point.

The level of pre-miR-223 and pre-miR-26b was also analysed in MKs after 8 hours up to four weeks of treatment when the most significant difference was seen in mature miRNAs. In contrast to mature miRNAs, we did not find a lower level of pre-miRNAs in MK cells within 24 hours compared to baseline value, but significantly augmented level of these pre-miRNAs was detected by week 4 suggesting the overexpression of pre-forms in the lack of mature miRNAs due to altered Dicer activity and other potential compensatory mechanism.

Lower platelet miRNA expressions are associated with elevated target mRNA levels in platelets and MK cells by hyperglycemia

According to a former publication, miR-223 targets P2RY12 mRNA in platelets and MKs, while miR-26b and miR-140 target SELP mRNA predicted by TargetScanHuman program (Release 7.0, www.targetscan.org). We detected significantly ($P=0.036$) increased levels of P2RY12-specific mRNA in DM2 platelets in contrast to healthy controls. In parallel, SELP mRNA was significantly overexpressed ($P=0.005$) in the presence of depressed miR-26b and miR-140 expressions in diabetics versus normal individuals. In parallel, glucose treated MEG-01 MK cells showed a similar tendency in terms of both mRNAs ($P<0.05$) after 24 hours.

Platelet miR-26b and miR-140 regulate the SELP expression in platelets and MK cells

In order to prove the direct association between these platelet miRNAs and the SELP gene expression, two different approaches were taken: i) miR-26b or miR-140 was individually overexpressed in MEG-01 MKs pretreated by hyperglycaemia via using specific miRNA mimics for 24 hours. We first checked if overexpression of these miRNAs successfully occurred in MKs, and it was also excluded whether these miRNA mimics affected the expression of the other miRNAs. As a negative control, a vehicle miRNA mimic (NEG-01) was applied. The relative level of SELP mRNA was significantly attenuated in MKs ($P<0.05$) as a result of the overexpression of either miR-26b or miR-140 mimics compared to negative control sample, which was considered as 100 %. Furthermore, we performed experiments with MEG-01 MKs pretreated with anti-miR-26b and anti-miR-140 for 24 hours. There was significantly enhanced SELP mRNA level ($P<0.05$) in both cases vs. the control sample.

Abnormal Dicer function also contributes to impaired miRNA levels in DM2

We wanted to explore whether Dicer function was also modulated by hyperglycemia resulting in decreased miRNA expression in platelets with diabetes. For this purpose, the MEG-01 MK model was assessed when these cells were incubated with a specific calpain inhibitor for 24 hours. We showed that calpeptin alone caused a massive elevation in miRNA levels due to the inhibition of platelet calpains that could not cleave Dicer. Furthermore, when calpeptin was used together with high glucose or only after the glucose treatment, there was a substantial raise in mature miRNA levels. Finally, in the sample with calpeptin added prior to glucose, miR-223 ($P=0.009$) and

miR-26b (P=0.027) expression were significantly elevated compared to the control sample treated with high glucose only.

Comparison of the effect of BMS and DES on cellular activation in stable angina

Demographical and clinical parameters of stented patients

In this study, 28 subjects with BMS and 21 with DES were enrolled to investigate the levels of platelet and endothelial cell activation specific biomarkers. These cohorts were comparable in age and gender and there was no difference in comorbidities (DM2, hypertonia, hypercholesterolemia, etc.) and smoking habits. After implantation, patients were followed for 6 months, and no stent thrombosis occurred, while 6 BMS subjects had ISR.

We first analyzed the levels of the D-dimer and the FM assay before and after the procedure in order to detect whether stenting induced any hemostasis activation. The D-dimer and fibrinogen did not undergo any change. The median post-implantation FM concentrations increased significantly (P=0.012) in the first 24 hours in the DES group as compared with the baseline value despite UFH administration during the operation, and the level was still elevated at 1 month (P=0.021). The median FM levels remained in the reference range (<10 mg/L) throughout the 6-month follow-up. The myocardial ischemia generated by the intervention was followed via the serum cTnT. After stenting, several patients exhibited significantly elevated cTnT levels within 24 hours in both cohorts (P=0.0001 in BMS; P=0.042 in DES), which had returned to the baseline by 1 month.

Increased platelet activation is sustained after stenting

The platelet activation was monitored and after intervention, P-selectin positivity subsequently increased during the administration of anti-platelet medication, independently of the stent type (3.3 [2.3–3.8] % in BMS vs. 2.5 [2.3–3.2] % in DES, P=0.574). Gradually rising post-intervention soluble CD62 levels were observed in both cohorts and, similarly to the membrane-bound form, the soluble P-selectin concentrations were still higher at 1 month relative to the baseline values, especially in the BMS subjects, where this alteration was statistically significant (P=0.048). The plasma levels of soluble CD40L were above the reference range (<100 pg/mL) throughout the study period, in agreement with other platelet markers.

After a vascular injury induced by stenting, a large quantity of PDGF-BB can be released from the activated platelets that stimulates the proliferation of smooth muscle cells in the vessel wall. To find a possible explanation why the BMS subjects were more susceptible to restenosis than DES patients, we determined the plasma levels of PDGF-BB in the presence of a detectable hyperfunction of platelets. After 1 month, the patients with ISR exhibited a still higher level as compared with the BMS patients without complications and the entire DES cohort. Moreover, the PDGF-BB levels decreased significantly (P=0.004) in the DES individuals, resulting in a lower chance for ISR.

Comparison of endothelial damage generated by the two different types of coronary stent

No difference (P=0.154) in baseline vWF-Ag was observed between the two patient groups, while significantly higher plasma vWF-Ag concentration was found in sample 2 in the BMS group relative to the baseline value (190 [173–195] % vs. 152 [142–167] %; P=0.046). In the DES cohort, no such alteration was observed. The BMS patients exhibited a significantly elevated median soluble VCAM-1 level at 24 hours after the intervention versus the baseline (610 [501–806] ng/mL vs. 512 [449–703] ng/mL; P=0.046) in contrast with the DES individuals. Soluble ICAM-1 demonstrated only a modest elevation after 1 month of stenting and this alteration was at a similar degree in BMS and DES groups, however, in agreement with soluble E-selectin, its level was much higher in ISR.

Elevated level of endothelial cell activation markers in ISR

Among BMS patients, there were 6 cases (cc. 20 %) who had ISR during the follow-up period, however, there was no complication in the DES cohort, thus, we reanalyzed the results of endothelial cell activation markers after subgrouping. Soluble CD40L and ICAM-1 levels primarly indicate the activation of leukocytes. Although there was no difference between entire BMS and DES cohorts, ISR was associated with much higher soluble ICAM-1 (P=0.046) and CD40L (P=0.032) concentrations showing a larger degree of cellular activation.

In addition, endothelial cell activation specific E-selectin, VCAM-1 and vWF levels were also higher in ISR individuals compared to DES group. This alteration was

even larger in soluble E-selectin and vWF (P=0.032 and P=0.011), while in case of VCAM-1 only modest tendency was observed (P=0.160).

Investigation of the effect of everolimus on endothelial cell activation *in vitro*

Elevated E-selectin and VCAM-1 mRNA levels induced by TNF-α are downregulated by everolimus in ECs *in vitro*

We first investigated whether elevated expression of EC activation dependent adhesion molecules E-selectin and VCAM-1 could be observed in EC cultures under inflammatory conditions with or without everolimus (0.5 μM) *in vitro*. E-selectin and VCAM-1 mRNA and protein levels were analyzed in HCAECs and HUVECs after treatment with TNF-α in the presence or absence of everolimus. TNF-α stimulation resulted in a robust elevation in both mRNA levels compared to baseline sample. In contrast, everolimus in the presence of TNF-α significantly, however, not completely lowered these mRNA levels in HCAECs (P<0.001) and in HUVECs (P<0.001) as well. No alteration in these mRNA levels was found by everolimus alone or by vehicle (DMSO) with TNF-α vs. untreated baseline samples.

Inflammation-raised E-selectin and VCAM-1 concentrations were significantly decreased by everolimus (P=0.001, P<0.001, respectively) in agreement with their altered mRNA levels in ECs above. Hence, these *in vitro* results provide some explanation about the lower level of EC activation with less E-selectin/VCAM-1 in DES individuals.

Everolimus lowers inflammatory events in endothelial cells

To investigate if everolimus downregulates adhesive molecule expression via modulating a global inflammatory response in ECs, HCAECs were treated with TNF-α that caused a robust elevation in IL-1β and IL-6 mRNA levels already by 1 hour and further increased by 4 hours. In contrast, everolimus moderately, but significantly lowered IL-1β (P=0.002) and IL-6 mRNAs (P=0.039) already after 1 hour, which were more obvious after 4 hours of treatment (P=0.002, P=0.004, respectively). Based on these results, TNF-α-induced EC inflammation could be interrupted by everolimus.

Effect of everolimus on NF-κB nuclear translocation in HCAECs

Translocation of the p65 NF-κB subunit into the cell nuclei reliably evaluates the degree of an inflammatory reaction at cellular level. To further analyze the direct effect

of everolimus in inflammation-stimulated ECs, early NF-κB p65 nuclear translocation was studied in HCAEC cultures after stimulation with cell culture medium, or TNF-α (100 ng/mL) in the absence or presence of everolimus (0.5 μM) or with its solvent for 1 hour. The nucleus/cytosol intensity of p65 staining was studied in these cells. We found that compared to TNF-α stimulated cells, everolimus treatment in the presence of TNF-α significantly decreased the p65 staining in the cell nucleus ($P<0.001$). In contrast, TNF-α with DMSO sample did not show alteration in p65 staining. Accordingly, everolimus was shown to directly influence EC activation via interrupting NF-κB pathway.

Transcriptional regulation of E-selectin and VCAM-1 genes and the expression of enhancer RNAs by everolimus

We here re-analyzed the unstimulated and TNF-α-treated HUVEC-derived publicly available NF-κB transcription factor subunit p65, RNA Polymerase II (RNAPII), active histone mark H3K27Ac, and active transcription start site mark H3K4m3-specific ChIP-seq data sets. As we expected, TNF-α-induced RNAPII binding was observed at both SELE and VCAM1 gene bodies. In addition, two enhancers were identified in the neighboring genomic regions of both genes associating with TNF-α-induced p65 and RNAPII binding. We measured the eRNA expression at one selected TNF-α-activated p65 transcription factor bound enhancers in the neighboring genomic regions of both genes including SELE_-11Kb and VCAM1_-10Kb. TNF-α induced eRNA expression at both enhancers ($P<0.05$) compared to the baseline sample. However, the TNF-α-augmented eRNA expression was significantly reduced by everolimus treatment ($P=0.027$, $P=0.017$, respectively). Overall, we suppose that everolimus inhibits EC activation via altering the TNF-α-induced transcription of EC activation-related genes, such as SELE and VCAM1.

Posttranscriptional regulation of EC inflammation

Although E-selectin and VCAM-1 expressions were found to be highly regulated at transcriptional level in this experimental system, we sought to study the role of post-transcriptional regulator of these receptors upon EC inflammation. Since miR-181b modulated VCAM-1 and E-selectin expression in HUVECs among *in vitro* conditions, we here analyzed the levels of this miRNA in TNF-α-stimulated ECs with or without everolimus as their potential key effector. Both HCAECs and HUVECs were treated by

recombinant TNF-α for 1-4 hours to analyze miR-181b expression along with inflammation-specific miRNAs. As expected, miR-155 and miR-146a as well as the biomarker of EC dysfunction miR-185 were elevated by TNF-α ($P<0.001$) compared to untreated sample in both EC cultures. However, everolimus caused significantly decreased miR-155 and miR-146a levels, with lower miR-185 expression.

Importantly, the level of miR-181b was downregulated by the inflammatory stimulus ($P<0.001$) and the treatment with everolimus restored their expression in both EC cultures ($P=0.042$, $P=0.049$).

We subsequently studied whether altered levels of these mature miRNAs above were due to their abnormal transcriptional regulation. Therefore, the levels of pre- and pri-miR-155, and both precursors of miR-181b were quantified by RT-qPCR in HCAECs stimulated with TNF-α with or without everolimus. We found that the levels of these miRNA precursors were altered in the same manner as seen in mature miRNAs. These findings suggest that miR-155 and miR-181b expressions were modulated at transcription level by TNF-α stimulation and everolimus in ECs.

MiR-181b regulates the SELE and VCAM1 expression in endothelial cells

We wanted to confirm the relationship between miR-181b and SELE as well as VCAM1 in HCAECs stimulated with TNF-α by using transfection of specific miR-181b mimic. The overexpression of miR-181b was produced by its specific mimic. As a consequence, SELE ($P=0.006$) and VCAM1 ($P<0.001$) mRNA levels were significantly decreased in the coronary endothelial cells versus control samples transfected with the NEG-01 control mimic. Based on these results, we confirmed that miR-181b targets E-selectin and VCAM-1 in HCAECs.

Extracellular miRNAs levels in endothelial cell activation upon the development of ISR

By using OpenArray analysis, we detected 66 miRNAs plasma samples. In ISR 17 miRNAs were downregulated with fold change of ≥ 1.5 (e.g. miR-126, miR-223, miR-424), while 23 miRNAs were upregulated at similar extent (e.g. miR-155, miR-185) compared to those BMS patients without complicatios and all DES subjects.

In *ex vivo* plasma samples in the presence of higher TNF-α concentrations, circulating miR-155 ($P<0.01$) and miR-185 ($P<0.001$) levels were also higher than normal suggesting the presence of vascular inflammation in ISR. The expression of E-

selectin and VCAM-1 regulator miR-181b was significantly lower in ISR in contrast to BMS (P=0.035) and DES (P=0.034) cohorts. MiR-424 being another endothelial cell activation specific biomarker that regulates the expression of vWF. Results obtained from the OpenArray analysis could be validated in the entire patient groups in terms of miR-424 showing lowered level (P<0.01) vs. those subjects without any complications. In ISR patients, miR-126 (P=0.036) and miR-34a (P<0.001) were decreased in comparison to the DES group, however, their role in vascular inflammatory events has been described, hence, we used them as „control miRNAs" during these analyses.

In aggrement with our hypothesis, there was a negative correlation between lowered miR-181b and upregulated E-selectin (r=-0.375, P=0.049) and VCAM-1 plasma levels (r=-0.441, P=0.019), as well as between miR-424 and vWF levels (r=-0.647, P=0.009). Accordingly, we suppose that decreased miRNAs may contribute to the generation of enhanced endothelial cell activation upon ISR.

Discussion

Because of the relatively frequent incidence of the development of DM2 associated inflammatory and thrombotic complications as well as coronary stent implantation related clinical alterations, it is important to thoroughly investigate the abnormal cellular events in the background of these conditions. In the last couple of years there is a growing body of knowledge about intracellular processes not only at protein level, but also at RNA level, which are involved in the regulation of pathological progresses. To study the details of these alterations, we analyzed platelet and MK derived miRNAs in connection with enhanced platelet activation in DM2. In parallel, we characterized the molecular regulatory mechanisms in the background of ISR associated with increased endothelial cell activation generated after coronary stenting and the inhibitory effect of everolimus on these cellular events.

Platelets carry a large number miRNAs and mRNAs with relatively high expression. MiRNAs fine-tune posttranscriptionally the expression of several genes via targeting their mRNAs. For many decades, there was a doubt if platelets without nuclei and having the avarage life time of 8-12 days might contain functional RNA molecules, and thus, they might be able to produce proteins in response to certain challenges. Some recent publications reported the role of certain platelet miRNAs and their target mRNAs in diseases in which platelets become activated, e.g. in DM2 or sepsis. Vascular and thrombotic complications have a large epidemiological impact in DM2 especially in developed countries. The frequent and often long-term hyperglycemic clinical conditions may result in the activation of different cell types, for example MK cells in the bone marrow and circulating platelets in the blood flow. Among abnormal metabolic milieu, platelet acivation level increases, and more reactive platelets appear in the circulation which can be activated more easily by a minor stimulus and even at higher extent. Increased platelet activation status can be observed by elevated surface and soluble P-selectin levels not exceptionally in DM2, but already in obesity.

Most former publications reported altered level of circulating miRNAs measured in plasma or serum samples in DM2 and obese individuals, and only a few data are known about the change in miRNAs of human MK cells and platelets in these diseases. Others previously claimed that reactive platelets had lower miR-96 expression and increased VAMP8 mRNA and protein levels in relation to elevated platelet secretion.

Platelet miR-223 was descibed as a regulator of mRNA of P2Y12 ADP-receptors in platelets and MK cells. These data suggest the function of platelet miRNAs in regulation of particular platelet protein or receptor expression in both normal and abnormal conditions, however, many of these miRNAs with their target mRNAs have not been indentified.

Based on former publications and public databases or prediction programs (pl. www.mirbase.org) we here analyzed those miRNAs in DM2 which may control platelet protein expression upon platelet activation. To the best of our knowledge, no previous data are available on the regulatory miRNAs of P2Y12 and P-selectin receptors in DM2. For this purpose, we quantified mature and precursor miR-223, miR-26b, miR-140 and miR-126 in platelet and plasma samples with their target (P2RY12, SELP) mRNAs. The modulatory effect of hyperglycemia in MK cell cultures was also investigated *in vitro*, and the association of miR-26b and miR-140 with SELP mRNA was detected.

Although there were no acute thrombotic complications in either patients, based on high surface P-selectin positivity, enhanced platelet activation was observed in diabetic subjects in comparison to controls, as we had earlier published not only in DM2 but in obesity as well. In both platelets and plasma samples, intracellular/soluble forms showed higher concentrations in DM2 vs. controls suggesting that P-selectin was not only secreted at higher level, but was produced with larger quantity by metabolic stimulus. These events may result in the generation of larger number of heterotipic aggregates and related inflammatory and atherothrombotic complications.

Our aim was to analyze miRNAs in connection with abnormal platelet function in diabetes. MiR-223 levels were decreased in diabetic platelets and elevated P2RY12 mRNA expression than control samples. These altered miR-223 and P2RY12 levels may contribute to high platelet reactivity.

MiR-26b was previously described in the regulation of IL-6 expression, but no data were known about its relationship with SELP mRNA. Platelet miR-26b and miR-140 levels were significantly decreased in DM2 in contrast to healthy and obese individuals. In contrast, SELP mRNA were elevated in diabetes mellitus. The direct relationship between miR-26b, miR-140 and SELP mRNA by using specific miRNA mimics and inhibitors after transfection of MEG-01 MK cells. Mimics caused highly

elevated miR-26b and miR-140 expression with lowered SELP mRNA level. Anti-miR-26b and anti-miR-140 decreased miRNA level and also caused elevated SELP mRNAs. Of note, other miRNAs may be also involved in the regulation of P-selectin in platelets.

The lower miRNA content of platelets may suggest that less miRNAs are packed into platelets from MK cells. To study this aspect, MK cell cultures (MEG-01, K562-MK) were maintained in high glucose milieu to measure miRNA expression. There was a decreasing tendency in miRNA levels in both MK models after 24 hours, and even larger alteration was seen after 1-4 weeks. These results are in agreement with former data about altered MK cell function in hyperglycemia producing platelets with elevated expression of surface glycoprotein receptors.

We wanted to explore whether Dicer function was also modulated by hyperglycemia resulting in decreased miRNA expression in platelets with diabetes. For this purpose, the MEG-01 MK model was assessed when these cells were incubated with a specific calpain inhibitor for 24 hours. We showed that calpeptin alone caused a massive elevation in miRNA levels due to the inhibition of platelet calpains that could not cleave Dicer. Furthermore, when calpeptin was used together with high glucose or only after the glucose treatment, there was a substantial raise in mature miRNA levels. Finally, in the sample with calpeptin added prior to glucose, miR-223 and miR-26b expression was significantly elevated compared to the control sample treated with high glucose only.

In this thesis, we compared the effect of BMS and DES coronary stents in terms of cellular activation in stable angina patients, and among *in vitro* conditions transcritional and posttranscriptional regulatory mechanisms of endothelial cell activation induced by everolimus of DES were observed.

Recently, several clinical studies demonstrated the beneficial impact of DES vs. BMS. After implantation, DES eluates mTOR inhibitors having anti-inflammatory and anti-proliferative proporties (e.g. everolimus, sirolimus, and their analogues, etc.) in two phases for about 2-3 months. These drugs result in slower endothelization and lower smooth muscle cell proliferation leading to a smaller risk of ISR. However, the effects of DES and BMS on cellular activation is still not fully exlored.

We first analyzed the levels of the D-dimer and the FM assay before and after the procedure in order to detect whether stenting induced any hemostasis activation. The D-

dimer and fibrinogen did not undergo any change. The median FM concentration increased significantly in the first 24 hours in the DES group as compared with the baseline value despite UFH administration during the operation, and the level was still elevated at 1 month. The median FM levels remained in the reference range (<10 mg/L) throughout the 6-month follow-up. The myocardial ischemia generated by the intervention was followed via the serum cTnT. After stenting, several patients exhibited significantly elevated cTnT levels within 24 hours in both cohorts, which had returned to the baseline by 1 month.

The platelet activation was monitored and after intervention, P-selectin positivity subsequently increased during the administration of anti-platelet medication, independently of the stent type. Gradually rising soluble CD62 levels were observed in both cohorts and, similarly to the membrane-bound form, the soluble P-selectin concentrations were still higher at 1 month relative to the baseline values especially in the BMS subjects, where this alteration was statistically significant. The plasma levels of soluble CD40L were above the reference range (<100 pg/mL) throughout the study period, in agreement with other platelet markers.

To find a possible explanation why the BMS subjects were more susceptible to restenosis than DES patients, we determined the plasma levels of PDGF-BB in the presence of a detectable hyperfunction of platelets. After 1 month, the patients with ISR exhibited a still higher level as compared with the BMS patients without complications and the entire DES cohort. Moreover, the PDGF-BB level decreased significantly in the DES individuals, resulting in a lower chance for ISR.

No difference in baseline vWF-Ag was observed between the two patient groups, while significantly higher plasma vWF-Ag concentration was found in sample 2 in the BMS group relative to the baseline value. In the DES cohort, no such alteration was observed. The BMS patients exhibited a significantly elevated median soluble VCAM-1 level at 24 hours after the intervention versus the baseline in contrast with the DES individuals. Soluble ICAM-1 demonstrated only a modest elevation after 1 month of stenting and this alteration was at a similar degree in BMS and DES groups, however, in agreement with soluble E-selectin, its level was much higher in ISR.

Among BMS patients there were 6 cases (cc. 20 %) who had ISR during the follow-up period, however, there was no complication in the DES cohort, thus, we

reanalyzed the results of endothelial cell activation markers after subgrouping. Soluble CD40L and ICAM-1 levels primarly indicate the activation of leukocytes. Although there was no difference between entire BMS and DES cohorts, ISR was associated with much higher soluble ICAM-1 and CD40L concentrations showing a larger degree of cellular activation. In addition, endothelial cell activation specific E-selectin, VCAM-1 and vWF levels were also higher in ISR individuals compared to DES group. This alteration was even larger in soluble E-selectin and vWF, while in case of VCAM-1 only modest tendency was observed.

Finally, we investigated the effect of everolimus of DES on endothelial cell activation *in vitro*. We first investigated whether elevated expression of EC activation dependent adhesion molecules E-selectin and VCAM-1 could be observed in EC cultures under inflammatory conditions with or without everolimus (0.5 μM). E-selectin and VCAM-1 mRNA and protein levels were analyzed in HCAECs and HUVECs after treatment with TNF-α in the presence or absence of everolimus. TNF-α stimulation resulted in a robust elevation in both mRNA levels compared to baseline sample. In contrast, everolimus in the presence of TNF-α significantly, however not completely lowered these mRNA levels in HCAECs and in HUVECs as well.

Inflammation-raised E-selectin and VCAM-1 concentrations were significantly decreased by everolimus in agreement with their altered mRNA levels in ECs above. Hence, these *in vitro* results provide some explanation about the lower level of EC activation with less E-selectin/VCAM-1 in DES individuals.

To investigate if everolimus downregulates adhesive molecule expression via modulating a global inflammatory response in ECs, HCAECs were treated with TNF-α that caused a robust elevation in IL-1β and IL-6 mRNA levels already by 1 hour and further increased by 4 hours. In contrast, everolimus moderately but significantly lowered IL-1β and IL-6 mRNAs already after 1 hour, which were more obvious after 4 hours of treatment. Based on these results, TNF-α-induced EC inflammation could be interrupted by everolimus.

Translocation of the p65 NF-κB subunit into the cell nuclei reliably evaluates the degree of an inflammatory reaction at cellular level. To further analyze the direct effect of everolimus in inflammation-stimulated ECs, early NF-κB p65 nuclear translocation was studied in HCAEC cultures after stimulation with cell culture medium, or TNF-α

(100 ng/mL) in the absence or presence of everolimus (0.5 μM) or with its solvent for 1 hour. The nucleus/cytosol intensity of p65 staining was studied in these cells. We found that compared to TNF-α stimulated cells, everolimus treatment in the presence of TNF-α significantly decreased the p65 staining in the cell nucleus. In contrast, TNF-α with DMSO sample did not show alteration in p65 staining. Accordingly, everolimus was shown to directly influence EC activation via interrupting NF-κB pathway.

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Although E-selectin and VCAM-1 expression were found to be highly regulated at transcriptional level in this experimental system, we sought to study the role of post-transcriptional regulator of these receptors upon EC inflammation. Since miR-181b modulated VCAM-1 and E-selectin expression in HUVECs among *in vitro* conditions, we here analyzed the levels of this miRNA in TNF-α-stimulated ECs with or without everolimus as their potential key effector. Both HCAECs and HUVECs were treated by recombinant TNF-α for 1-4 hours to analyze miR-181b expression along with inflammation-specific miRNAs. As expected, miR-155 and miR-146a as well as the biomarker of EC dysfunction miR-185 were elevated by TNF-α compared to untreated sample in both EC cultures. However, everolimus caused significantly decreased miR-155 and miR-146a levels, with lower miR-185 expression. Importantly, the level of miR-181b was downregulated by the inflammatory stimulus and the treatment with everolimus restored their expression in both EC cultures.

We subsequently studied whether altered levels of these mature miRNAs above were due to their abnormal transcriptional regulation. Therefore, the levels of pre- and pri-miR-155, and both precursors of miR-181b were quantified by RT-qPCR in HCAECs stimulated with TNF-α with or without everolimus. We found that the levels of these miRNA precursors were altered in the same manner as seen in mature miRNAs. These findings suggest that miR-155 and miR-181b expression were modulated at transcription level by TNF-α stimulation and everolimus in ECs.

We confirmed the relationship between miR-181b and SELE and VCAM1 in HCAECs stimulated with TNF-α by using transfection of specific miR-181b mimic. The overexpression of miR-181b was produced by its specific mimic. As a consequence, SELE and VCAM1 mRNA levels were significantly decreased in the coronary endothelial cells versus control samples transfected with the NEG-01 control mimic. Based on these results, we confirmed that miR-181b targets E-selectin and VCAM-1 in HCAECs.

By using OpenArray analysis, we detected 66 miRNAs plasma samples. In ISR 17 miRNAs were downregulated with fold change of ≥ 1.5 (e.g. miR-126, miR-223, miR-424), while 23 miRNAs were upregulated at similar extent (e.g. miR-155, miR-185) compared to those BMS patients without complicatios and all DES subjects. In *ex vivo* plasma samples in the presence of higher TNF-α concentrations, circulating miR-155 and miR-185 levels were also higher than normal suggesting the presence of vascular inflammation in ISR. The expression of E-selectin and VCAM-1 regulator miR-181b was significantly lower in ISR in contrast to BMS and DES cohorts. MiR-424 being another endothelial cell activation biomarker that regulates the expression of vWF. Results obtained from the OpenArray analysis could be validated in the entire patient groups in terms of miR-424 showing lowered level vs. those subjects without any complications.

In aggrement with our hypothesis, there was a negative correlation between lowered miR-181b and upregulated E-selectin and VCAM-1 plasma levels, as well as between miR-424 and vWF levels. Accordingly, we suppose that decreased miRNAs may contribute to the generation of enhanced endothelial cell activation upon ISR.

Summary

MicroRNAs have been described as novel posttranscriptional fine regulators of gene expression in the pathomechanism of various diseases. Platelets and endothelial cells can become activated under abnormal metabolic or inflammatory conditions increasing the risk of thrombotic and vascular complications. In this thesis, our aim was to investigate platelet and endothelial cell derived miRNAs in metabolic and vascular disorders associated with enhanced cell activation.

Due to decreased levels of Dicer and pre-miRNAs, we detected lower platelet and circulating miR-223, miR-26b and miR-140 expression in type 2 diabetes mellitus (DM2) in contrast to obese and normal controls. In response to hyperglycemia, the miRNA levels were decreased in both MEG-01 and matured K562-MK cells among *in vitro* conditions. Lowered miR-26b and miR-140 levels were related to elevated P-selectin (SELP) mRNA and protein levels, while decreased miR-223 was associated with higher P2RY12 mRNA expression in platelets and MK cells. The direct link between miR-26b, miR-140 and SELP mRNA was proved by using miRNA mimics and inhibitors in MEG-01 cells.

Coronary bare-metal stent (BMS) and drug-eluting stent (DES) induced platelet and endothelial cell activation at different extent. By the application of BMS causing higher soluble P-selectin, VCAM-1 and vWF plasma levels, there were 6 cases who suffered from early is-stent restenosis (ISR) out of 28 patients vs. the DES cohort. In addition, ISR was associated with much higher soluble E-selectin and VCAM-1 concentrations indicating a larger degree of endothelial cell activation.

Everolimus could depress TNF-α induced SELE and VCAM1 mRNA and protein levels via the inhibition of p65 nucleus translocation of the NF-κB pathway and the expression of enhancer RNAs. MiR-181b plays an important role in the posttranscriptional regulation of E-selectin and VCAM-1 gene expression. In *ex vivo* plasma samples, decreased miR-181b and miR-424 showed a strong correlation with soluble E-selectin, VCAM-1 and vWF levels elevated in ISR.

In summary, there may be a number of impaired molecular events in the development of clinical complications of different diseases, which need to be explored to promote treatment and prevention more sufficiently.

New scientific results

1. Lower level of platelet and circulating miR-223, miR-26b and miR-140 can be detected in DM2, and these alterations are caused by decreased Dicer function and lower level of pre-miRNAs.

2. The miRNA content of MEG-01 and K562 MK cells is also decreased significantly by hyperglicemia among *in vitro* conditions.

3. Decreased miR-26b and miR-140 expression caused elevated P-selectin (SELP) mRNA and protein levels, while lowered miR-223 was associated with higher P2RY12 mRNA in platelets and MK cells.

4. The direct relationship between miR-26b, miR-140 and SELP mRNA was approved by miRNA mimics and inhibitors in MEG-01 cells.

5. Coronary bare-metal stents (BMS) and everolimus eluting stents (DES) altered the level of platelet and endothelial cell activation at different extent in stable angina.

6. By the application of BMS, more patients had early in-stent restenosis (ISR), which was associated with higher soluble E-selectin, VCAM-1 and vWF plasma concentrations demostrating a higher level of endothelial cell activation.

7. Everolimus decreased TNF-α induced SELE and VCAM1 mRNA and protein levels via the inhibition of NF-κB pathway p65 nucleus translocation and the expression of enhancer RNAs. Consequently, endothelial cell activation was suppressed by this drug in HCAEC cells.

8. MiR-181b plays an important role in the posttranscriptional regulation of E-selectin and VCAM-1 genes. Based on the altered levels of pre- and pri-miR-181b, the expression of mature miR-181b was also influenced by TNF-α and everolimus via transcriptional manner in HCAEC cells.

9. In *ex vivo* samples of coronary stented patients, decreased miR-181b and miR-424 showed a strong correlation with elevated soluble E-selectin, VCAM-1 and von Willebrand factor concentrations, respectively, in ISR.

List of publications

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List of publications related to the dissertation

1. **Fejes, Z.**, Czimmerer, Z., Szűk, T., Póliska, S., Horváth, A., Balogh, E., Jeney, V., Váradi, J., Fenyvesi, F., Balla, G., Édes, I., Balla, J., Kappelmayer, J., Nagy, B. Jr.: Endothelial cell activation is attenuated by everolimus via transcriptional and post-transcriptional regulatory mechanisms after drug-eluting coronary stenting.
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