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Examination of transglutaminases in proliferative vitreoretinopathy and neutrophil extracellular trap formation

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Examination takes place at the library of Research Center for Molecular Medicine, Faculty of Debrecen, University of Debrecen, May 31, 2018, 11 am

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The PhD Defense takes place at the Lecture Hall of Department of Dermatology, Faculty of Medicine, University of Debrecen, May 31, 2018, at 1 pm

1. Introduction

1.1 Transglutaminases

The Ca^{2+}-dependent transglutaminases (TGases) are multifunctional enzymes having role in physiological and pathological processes. TGases can catalyze the posttranslational modification of proteins via the formation of ε-(γ-glutamyl)-lysine isopeptide cross-links between lysine and glutamine residues.

TGases have been identified in microorganisms, plants, invertebrates and vertebrates. TGase Factor XIIIa can stabilize fibrin clots and participate in wound healing, the keratinocyte TGase (TG1) is involved in the terminal differentiation of keratinocytes and the ubiquitous tissue TGase (TG2) has various functions. The epidermal hair follicle TGase (TG3) participates in the terminal differentiation of the keratinocytes and formation of cornified envelope, the prostatic secretory TGase (TG4) plays a role in fertility. TGase 5 plays a role in keratinocyte differentiation and the formation of cell envelope, TGase 6 is expressed in the central nervous system and the recently discovered TGase 7 has role in metal ion binding and the conjugation of polyamines to proteins. Finally, there is a TGase-like protein, the erythrocyte protein band 4.2 that has no enzymatic activity and is an inactive form of TGase due to the presence of alanine instead of cysteine (Cys) in the active site.

1.2 Substrates and interaction partners of transglutaminases

TGases can exert their effect via interaction with substrates and interaction partners. For the detection of the functional role of TGases in different types of cells and tissues identification of their substrates and interactors is important. TRANSDAB is a database (http://genomics.dote.hu/wiki) generated to collect information about TGase substrate proteins and interaction partners. It contains more than 500 articles about 340 substrates and almost 80 interactors for TGases such as FXIIIa, TG1, TG2, TG3, TG4, TG5 and microbial TG.

1.3 The function of transglutaminases

All TGases can catalyze the formation of a thioester bond between the active site Cys and the γ-carboxamide group of glutamyl substrate and ammonia is released as product. Thereafter the acyl transfer to the ε-amino group of a peptide-bound lysine residue of amine donor substrate results in the formation of the isopeptide ε-(γ-glutamyl)-lysine bond. In transamidation reactions, monoamines and polyamines can participate as well. Besides transamidation, deamidation and isopeptide bond hydrolysis can be observed in case of different TGases.

TG1 is a Ca^{2+}-dependent enzyme involved in terminal differentiation of stratified squamous epithelia and in the formation of cornified cell envelope. The cross-linking provides strength and stability to the epidermis and participates in the formation of the protective barrier between the body and environment. The TRANSDAB database contains 14 substrates for TG1.

TG2 acts predominantly as a classic Ca^{2+}-dependent enzyme, but Ca^{2+}-independent enzymatic and non-enzymatic activities have been identified as well. TG2 as a Ca^{2+}-dependent enzyme found in several cell types and tissues has a variety of biochemical functions. It can catalyze Ca^{2+}-dependent post-translational modifications of proteins involving glutamine

deamidation, intraprotein and interprotein cross-link formation and incorporation of primary amines into proteins. TG2 has isopeptide bond cleavage activity, it can function as a protein kinase, can work as a G protein, can bind and hydrolyze GTP and has protein disulfide isomerase activity as well. The enzymatic and non-enzymatic activities of TG2 play a role in many critical cellular processes such as cell adhesion and growth, migration, differentiation, programmed cell death and extracellular matrix (ECM) assembly. It can also be present on the cell surface and in the ECM, where it cross-links and modulates several substrates making possible the ECM assembly and remodeling, the cell-matrix interactions providing tissue stability. TG2 plays an important role in cell attachment and spreading, wound healing, promotion and inhibition of angiogenesis.

1.4 Transglutaminases in diseases

TGases can participate in numerous human diseases. The activity of TG1 is dramatically reduced in lamellar ichthyosis which affects both the epidermis and hair. In patient with this skin disorder several mutations of the TG1 gene have been identified which are associated with deficient cross-linking of the cell envelope. In *TG1* knockout mice the skin barrier function was drastically impaired and these animals died within few hours.

TG2 can contribute to autoimmune-, inflammatory-, chronic degenerative-, malignant- and metabolic diseases. The absence of TG2 may cause delayed wound closure that can be valuable in reducing scar formation. The TG2 knockout (TG2KO) animals are viable and phenotypically normal, but they also carry several abnormalities in apoptosis, inflammatory and autoimmune reactions.

The proliferative vitreoretinopathy (PVR) is an abnormal wound-healing response in eye which can occur as a serious complication after surgery for retinal detachment. The exact pathogenesis of PVR is still not completely clarified. TG2 is known to be located in human PVR membranes where takes part in tissue stabilization and wound healing processes, but its exact role has not been understood yet.

1.5 NET formation upon different factors

Human neutrophils are an important part of the immune system necessary to sense and control the different microbial infections. They are activated upon inflammatory stimuli leading to formation of neutrophil extracellular trap (NET) which is a unique form of cell death. During this process, neutrophils can eject the mixture of nucleoplasm and cytoplasm components resulting a web-like structure which can trap, neutralize and eliminate the invading pathogens.

1.6 Two-dimension gel electrophoresis and mass spectrometry in proteomics

Proteomics can help to understand the mechanism of cellular-, regulatory- and pathogenic processes, to screen the proteins of a cell, tissue or biological fluid and to identify and characterize the proteins. Two strategies, termed ‘bottom-up’ and ‘top-down’ proteomics, can be used to characterize proteins. During bottom-up proteomics, proteins are characterized by the analysis of peptides released from proteolytic digestion of complex samples. When the peptide mixture is fragmented and subjected to HPLC-MS/MS-based protein identification and quantification analysis, it is called shotgun proteomics. It is widely used for protein

identification, proteome profiling, protein quantification, protein modification detection and protein-protein interaction examination. Another strategy, the top-down proteomics was used for the determination of posttranslational modifications and examination of protein isoforms.

Two-dimension gel electrophoresis (2-DE) is a standard, high resolution method which is utilized for the examination of proteomic changes of various types of samples; retinal, muscle and colorectal tissues, Candida cells and meat were studied in normal and pathological conditions and the drug effect on protein expression was examined. The major advantages of 2-DE in proteomics is the robustness; this is a powerful and widely used method making possible the comparison of protein profiles generated as a response to various conditions. It allows the study of the expression patterns of proteins offering potential insights into protein regulation, function and interactions.

Mass spectrometry (MS) is a crucial technique for almost all proteomics experiments. It allows the identification and quantitative analysis of protein as well as the examination of the protein-protein interactions and posttranslational modifications. MS as a powerful analytical technique can separate gas phase ions according to their mass-to-charge (m/z) ratio and then records the relative abundance of the ions. As a result a mass spectrum of the molecule is generated. The mass spectrometer has three major parts: an ion source, a mass analyzer and a detector. The two most commonly used ionization techniques in proteomics are matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). MALDI and ESI are called 'soft' ionization techniques because only a minimal amount of analyte is fragmented during ionization. Both techniques are suitable for the ionization of small and large molecules as well as for the analysis of proteins and peptides. MALDI-MS is preformed to analyze relatively simple peptide mixtures, while ESI-MS system is preferred for the analysis of complex samples.

2. Aims of the study

1. Investigation of the dispase-induced wild-type (WT) and TG2 knock-out (TG2KO) mice models of PVR in order to get information how the protein profiles are changed in PVR using two dimensional electrophoresis and mass spectrometry.

- identification of proteins with significantly changed amount upon dispase treatment in WT and TG2KO mice
- examination of protein profile changes in the lack of TG2
- study of the network of proteins changed during PVR induction in WT and TG2KO mice

2. Proteomic analysis of the NET components in order to get insights on the types of cross-links present among NET proteins

- examination of cross-links of NET proteins through chlorinated polyamines
- examination of cross-links of NET proteins through ε-(γ-glutamyl)-lysine and bis-γ-glutamyl polyamine bonds catalyzed by TGases
- examination of the NET proteins and network of cross-links upon 5-(biotinamido)pentylamine and spermine treatments

3. Methods

3.1 Animal model of PVR

Each animal experiment was performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and protocols were approved by the Animal Care Committee of the University of Debrecen. Female 4-6 months old WT (n = 6) and TG2KO mice (n = 6) were anesthetized with pentobarbital and received one drop of 1 % procaine hydrochloride for local anesthesia and one drop of tropicamide for iris dilation. A proteolytic enzyme dispase was injected intravitreally into the right eyes resulting in PVR induction in mice. Into the eyes of the control animals sterile physiological saline solution was injected. Following injections Stratus Optical Coherence tomography (OCT) were taken to confirm PVR induction. Both of control and dispase-treated mice were sacrified at 14th day following injections when signs of PVR formation such as presence of epiretinal membrane and/or retinal detachment were noticed by OCT examination.

3.2 The preparation of vitreous bodies and protein samples purification

The eyes of the sacrificed mice were removed and after removal of the lens and of the cornea together with a scleral galler using scalpel blade, the vitreous bodies of mice were prepared. After the solubilization of vitreous bodies with lysis buffer, the lysates were sonicated in ice cold water bath for 5 min and centrifuged at 16.9 g for 10 minutes at 4°C. The supernatants were transferred into LoBind Eppendorf tubes and purified by Ready-Prep 2-D CleanUp Kit (Bio-Rad) according to the manufacturer's protocol. After precipitation and centrifugation, the pellets were dried and resuspended in 450 µl rehydration buffer and used immediately for isoelectric focusing (IEF).

3.3 Two-dimension gel electrophoresis

Vitreal samples originating from the following groups were subjected to 2-DE: (1) dispase-treated WT (WT PVR), (2) physiological saline-treated WT (WT Ctrl), (3) dispase-treated TG2KO (TG2KO PVR) and (4) physiological saline-treated TG2KO (TG2KO Ctrl). Three biological replicates were used in each case. 24 cm IPG strips with immobilized pH gradient (pH 3-10) were passively rehydrated with extracted vitreous proteins at 20°C overnight. IEF was performed by applying 300 V for 3 hours, gradually increased to 3500 V in 5 hours and then held at 3500 V for 18 hours. The maximum current applied was 50 µA per gel. After IEF the IPG strips were equilibrated for 15 min in equilibration buffer containing 0.6 % dithiothreitol (DTT) (Bio-Rad) and and then in equilibration buffer containing 1.2 % iodoacetamide (IAA) (Bio-Rad) for 15 min. During the second dimension the strips were laid on the top of 12% SDS-polyacrylamide gels and covered with agarose (Bio-Rad). Using a Protean Plus Dodeca Cell (Bio-Rad) the electrophoresis was carried out simultaneously for each gel at 100 mA per gel for 24 hours until the bromophenol blue dye reached the bottom of the gel. All 12 gels were run together, under the same conditions. The gels were pre-fixed then stained with in-house prepared RuBPS fluorescent dye overnight. After staining the gels were washed and post-fixed. Gel images were scanned using Pharos FX Plus Molecular Imager (Bio-Rad).

3.4 Image analysis using Delta2D software

Gel images were evaluated using Delta2D (Decodon) software version 4.4. The gel images were grouped as follows: (1) WT PVR, (2) WT Ctrl, (3) TG2KO PVR and (4) TG2KO Ctrl. In order to study pairwise differences between groups three projects were created: in the first project, the WT Ctrl and WT PVR gel images were studied, while in the second project the TG2 KO Ctrl and TG2KO PVR gel images were examined and in the third project the WT PVR and TG2KO PVR gel images were compared.

Protein spots from each group were matched using the exact mode matching protocol and the group warping strategy of Delta2D software. The union mode was applied to generate a fused image containing all spots present on all of the gels. The fold change of mean normalized spot volume was calculated between groups and the significant differences were assessed by the Delta2D software using Student's t-test. Those spots were considered significantly different, where the p was $p < 0.05$. Spots showing significant differences were cut out for MS analysis.

3.5 In-gel digestion

The gel pieces were destained in 50% acetonitrile in 25 mM ammonium bicarbonate pH=8.5 solution, followed by reduction using 20 mM DTT for 1 hour at 56°C. Next alkylation was performed with 55 mM IAA for 45 min at room temperature (RT) in the dark then an overnight digestion with 100 ng stabilized MS grade trypsin (ABSciex) at 37°C was accomplished. The reaction was stopped with concentrated formic acid (FA) (VWR) and the tryptic peptides were extracted from the gel pieces and dried in speed-vac concentrator (Thermo Fisher Scientific). Samples were redissolved in 10 µl 1 % FA and used for mass spectrometry-based protein identification.

3.6 Protein identification by mass spectrometry

For protein identification the peptides were separated on Easy nLCII (Bruker) nanoHPLC. The chromatographic separation was performed using a 90 min water/acetonitrile gradient at 300 nl/min flow rate. The peptide mixture was loaded onto a Zorbax 300SB-C18 desalting column (5 mm x 0.3 mm, 5 µm particle size, Agilent), followed by separation on a 150 mm x 75 µm Zorbax 300SB-C18 analytical column (300 Å pore size, 3.5 µm particle size, Agilent). The mobile phase A was 0.1 % FA in LC-MS grade water, while the mobile phase B was acetonitrile containing 0.1 % FA. During the separation the percentage of phase B was increased from 0 % to 100 % in 60 min, then held at 100 % for 10 min, decreased to 0 % in 2 min, and finally was held at 0 % for 18 min.

The peptides eluted from the analytical column were analyzed in a 4000 QTRAP (ABSciex) mass spectrometer operated by Analyst software 1.4.2 (ABSciex). The positive ion mode MS/MS spectra and the Information Dependent Acquisition (IDA) method were applied. The first mass scan was between 400 – 1700 amu, an enhanced resolution separation was carried out to establish the charge state of the two most intensive precursor ions. For protein identification collision-induced dissociation spectra were obtained in enhanced product-ion mode (mass range 100 – 1900 amu) at scan rate of 4000 amu/sec and the rolling collision energy was applied with the maximum of 85 eV. The cycle time was 5.4 sec, the spray voltage was

2800 V, ion source gas was 50 psi, the curtain gas was 20 psi and the source temperature was 70°C. Based on the recorded MS/MS spectra proteins were identified with the ProteinPilot 4.5 software (ABSciex) using the UniProtKB/Swiss-Prot database. The minimum criteria for protein identification were the presence of two peptides per protein with at least 95 % confidence. In those cases where the protein identification was not successful using ProteinPilot software, a MASCOT search was carried out using the NCBInr database. The type of cleavage enzyme was defined as trypsin. The missed cleavage in all cases was set to maximum 1. In case of MASCOT search the variable modifications were set as: oxidation of Met and carbamidomethylation of Cys, while in case of ProteinPilot 4.5 search the Biological modification table implemented into the software was applied.

3.7 Functional analysis of proteomics changes

The gene ontology (GO) analysis was used for the functional analysis of the proteins. The Biological Process, Molecular Function and Cellular Localization according to GO was examined using String version 10.5. The network of differentially expressed proteins along with GO enrichment data was generated.

3.8 Isolation of neutrophils from human venous blood

50 ml of human venous blood from healthy volunteers was collected into vacutainer tubes containing EDTA and centrifuged for 15 min, at 500 g on RT in order to remove plasma containing thrombocytes. Ethical approval was obtained from the Ethics Committee of the University of Debrecen, Debrecen, Hungary (DEOEC RKEB/IKEB Prot. No. 2745-2008). Cells were transferred into 50 ml tube and 12 ml 3 % dextran (Pharmacocosmos, Dextran T500) in saline solution (sterilized using 0.22 μm filter, TPP Spritzen) was added to the cells. Then the tube was filled up to 50 ml with saline solution and incubated for 30 min on RT. After floating foam containing red blood cells was removed, supernatant containing white blood cells was collected and diluted 4 times with 20 mM PBS-EDTA in 15 ml tube. Cells were centrifuged for 7 min, at 300 g at RT. After supernatant was discarded, pellet was suspended in 3 ml 20 mM PBS-EDTA and cells were layered on Histopaque gradient and then centrifuged for 30 min, at 300 g at RT. Supernatant was removed and neutrophils were collected into 50 ml tube and washed 2 times with 50 ml 20 mM PBS-EDTA. After centrifugation for 7 min, at 300 g at RT, the rest of the thrombocytes was removed and cells were suspended in 30 ml RPMI 1640 medium. Cells were centrifuged again for 7 min, at 300 g at RT, supernatant was removed and cells were suspended in 1 ml RPMI 1640 medium. The cells were counted, NET was induced and used for protein isolation.

3.9 Purification of NET proteins

$2.0x10^6$ neutrophils were seeded in 6-well tissue culture plates in 4 ml RPMI 1640 medium. Neutrophils were pre-incubated with RPMI 1640 medium or 5-(biotinamido)-pentylamine ($BPNH_2$) or spermine (SPM), then activated with phorbol-12-myristate-13-acetate (PMA) for 4 h at 37°C in a 5% CO_2 atmosphere. After removing the medium, all wells were carefully washed by pipetting 1 ml of fresh and pre-warmed RPMI 1640 medium containing protease inhibitor cocktail and incubated for 10 min at 37°C. After the medium was removed,

the formed NETs were digested for 20 min in 1 ml fresh of RPMI medium with 10 Unit/ml DNase-1 (Worthington), and the samples were centrifuged at 16.9 g at 4°C. To precipitate the proteins 0.5 ml of supernatant was mixed with 1.5 ml of ice-cold acetone and incubated overnight at -20°C. Samples were centrifuged at 10.4 g for 30 min at 4°C. Then pellets were solubilized in 200 µl Laemmli buffer and frozen at -70°C for further analysis.

3.10 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Total concentration of NET proteins was determined with Bradford assay. The bovine serum albumin was used as a standard in the protein assay. 20 µg NET protein was separated on 12 % polyacrylamide gel in a Mini Protean Tetra Cell (Bio-Rad). Electrophoresis was carried out for 1.5 hours at 30 mA constant current and ProSieve™ Protein Ladder (Lonza) as a protein molecular weight marker was used. Protein staining was performed by PageBlue™ Protein Staining Solution (ThermoScientific), gels were scanned using Pharos FX Plus Molecular Imager (Bio-Rad) and the gel image analysis was done by QuantityOne Software (Bio-Rad). The band intensities and the estimated molecular weight for each band was calculated.

3.11 MS/MS based investigation of cross-linked NET proteins using StavroX software

StavroX 3.2.10 software was applied to identify possible cross-links among the NET proteins. The MS/MS data in Mascot generic format (.mgf) including all recorded spectra were used for the analyses. Amino acid sequences of the previously identified NET proteins were imported from UniProtKB in FASTA format. Enzymatic cleavage sites of trypsin were defined as C-terminal to lysine and arginine and blocked by proline. Variable modifications were set as oxidation of Met and carbamidomethylation of Cys. SPM (spermine), SPD (spermidine) and PUT (putrescine) were applied as probable cross-linkers, searching for the presence of the following cross-links: 1) Lys-polyamine-Lys cross-links with 200 Da, 143 Da and 86 Da mass shift, respectively, 2) Lys-polyamine-Met cross-links with 200 Da, 143 Da and 86 Da mass shift, respectively, 3) Gln-polyamine-Gln cross-links with 166 Da, 109 Da and 54 Da mass shift, respectively, 4) Gln-Lys cross-links with 17 Da mass shift. The calculated mass shifts were implemented into the software and the MS/MS spectra were searched for b- and y- ions corresponding to all possible cross-links. The results were manually analyzed and those hits were accepted where the score value of the identified cross-linked proteins was positive and contained at least four b- or y-ions in series. The sequence of the cross-linked peptides and the position of the cross-link was imported to Excel files and utilized for the visualization of the network of cross-linked NET proteins.

4. Results

4.1. Proteins characteristic for PVR induction by dispase treatment in wild type (WT) mice

Dispase treated WT C57/BL6 mice were used to study proteins involved in pathogenesis of PVR. 2-DE was performed on vitreous samples derived from three Ctrl and three dispase-treated WT mice vitreous. Gels were stained using homemade RuBPS and the gel images were scanned and analyzed by Delta2D software. The WT Ctrl group was created from the three gel

images originating from physiological saline-treated samples, while the WT PVR group was constituted from the three gel images originating from dispase-treated samples. A fused image was generated by superimposing each gel image and 698 spots could be detected.

In case of each gel the intensity of all spots was determined and the fold change between the groups was calculated. The intensity of 30 out of 698 spots indicated significant ($p < 0.05$) changes upon dispase treatment and these 30 spots were excised from the gel, digested by trypsin and subjected to HPLC-MS/MS-based protein identification.

In case of 19 spots, different forms of crystallins were identified. 8 out of 19 spots contained alpha-crystallin A and the amount of different forms of crystallins (except alpha-crystallin B) was reduced. The expression level of intracellular proteins decreased in the dispase-treated WT samples compared to the physiological saline treated ones.

In case of spots 11-14, 16-18, 22 and 24 more than one protein with similar peptide counts reflecting almost equal quantities of all proteins was detected. The combined effect of these proteins could be observed in such cases, therefore there is no information about the changes of each individual protein.

4.2 Proteins characteristic for PVR induction by dispase treatment in TG2KO mice

Mice models are used to examine the function of well-defined proteins as far as the KO animals for the studied protein can easily be created. In order to present the feasibility of the mice PVR model in elucidating the role of TG2 in PVR, dispase and physiological saline solution, respectively was injected into the eyes of TG2KO animals. The strategy used for the investigation of WT Ctrl and WT PVR samples was applied in case of TG2KO samples as well. The vitreous samples were analyzed by 2D electrophoresis followed by protein staining and image analysis. On one of the gel images from the physiological saline-treated TG2KO mice eyes less than 100 spots could be detected, so this gel image was excluded from further analyses. The images from the TG2KO Ctrl group created from two gel images originating from physiological saline-treated TG2KO mice eyes and from the TG2KO PVR group created from three gel images originating from dispase-treated TG2KO mice eyes were superimposed to generate the fused image

866 spots were detected, out of which 97 showed significant ($p < 0.05$) changes in their intensities upon dispase treatment. The spot intensity in case of 33 spots elevated, while in case of 64 spots reduced. Some spots within the basic pH and lower MW region of the gels could be detected only on TG2KO PVR gels; in spite of otherwise good quality of protein separation, very few spots were visible in the gel region corresponding to basic, low MW proteins in case of the physiological saline treated samples.

The spots showing significantly different intensities were cut out and 37 of them were identified with HLPC-MS/MS-based analysis. Most of the spots showing altered intensity changes upon dispase treatment contained alpha-crystallin, but in this case, their level was elevated upon dispase treatment. The amount of G-protein, heterogeneous nuclear ribonucleoprotein and tubulin was reduced upon dispase treatment, being in accordance with previous results obtained by the analysis of WT mice. The amount of glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, of cytoskletal protein beta-actin-like protein 2, of recoverin, peroxiredoxin 2, serotransferrin and alpha-2 macroglobulin

was reduced as well. The level of beta-crystallin A1, ferritin light and heavy chains and grifin increased in TG2KO PVR samples.

When the changes upon dispase injection in WT and TG2KO mice, respectively, were compared 4 of the studied proteins were changed both in WT and TG2KO mice. The level of tubulin beta 5 reduced upon dispase treatment independently of the presence of TG2, while the decrease in the level of alpha-crystallin A, beta-crystallin A1 and grifin detected in WT mice changed to an increase in the TG2KO mice as a response to dispase injection. There was one spot in case of alpha-crystallin A where reduction in the protein amount could be observed in dispase-treated TG2KO mice.

4.3 Proteins affected by the lack of TG2 in mice vitreous during PVR formation

The proteins whose expression depends on TG2 during PVR formation were investigated by comparing gel images of WT PVR to TG2KO PVR. The only difference between the two groups was the presence/absence of TG2, and this resulted in significantly different expression in case of 41 spots.

15 spots were previously identified by HLPC-MS/MS of which 12 spots contained alpha- or beta-crystallin and in the other spots ferritin heavy chain, griffin and tubulin was present.

4.4 Functional analysis of proteins differentially expressed upon dispase treatment

The network of proteins showing significant changes upon dispase treatment was generated by String 10.4. In case of WT mice the network contained 17 proteins and 14 possible protein-protein interactions analyzed at medium stringency. The enriched biological and molecular functions were eye development and protein binding indicating that most of the proteins implicated in PVR development may play a role in the correct development and structural stabilization of the eye.

The network of proteins with significantly different amount in TG2KO mice contained 18 proteins and 24 protein-protein interactions. The enriched biological and molecular functions indicate active metabolic and homeostatic mechanisms involving oxidoreductase-, L-lactate dehydrogenase-, thioredoxin peroxidase activities and ferric iron binding.

The localization of proteins in TG2KO PVR was mostly in extracellular region and vesicles, while in case of WT mice it was mainly in myelin sheath.

In conclusion, PVR mice model could be successfully utilized to analyze the protein changes upon dispase induced PVR formation. The results are in accordance with data reported in the literature. The highest changes in protein amounts upon dispase treatment were observed in case of crystallins. We demonstrated for the first time the increased level of alpha-crystallin B in vitreous upon dispase treatment. Therefore it seems logical that crystallins can be released into the vitreous after retinal injury, which often precedes the PVR formation. The alteration of crystallin network was observed upon dispase treatment in TG2KO mice. Despite the similar pathological outcome at the time point of the analysis a different crystallin profile change was observed in the WT PVR compared to TG2KO PVR. These data show that more than one type of change in the profile of crystallins might be associated with PVR in mice. Considering that

dispase is a protease causing proteolysis and TG2 is a crosslinking enzyme which generates isopeptide bonds, we can speculate on the possible outcomes of proteolysis in the absence of TG2. It is possible that the lack of protease-resistant cross-link formation leads to an increased crystallin cleavage by dispase.

4.5 Proteomic analysis of the components in neutrophil extracellular trap formation (NET) upon different treatments

Protein profile of the isolated NET was investigated by proteomic approach. 20 μg of isolated NET proteins of Ctrl, $BPNH_2$ and SPM-treated samples originating from three donors were separated on 12 % SDS-polyacrylamide gels.

Gel image analysis of Ctrl, $BPNH_2$ and SPM treated NET samples was performed. We observed a highly reproducible pattern of protein bands after separation on SDS-polyacrylamide gel. Alterations in band intensities upon $BPNH_2$ and SPM treatment could be observed, however the differences were not statistically significant.

Bands were excised and subjected to HPLC-MS/MS based protein identification. We could identify 16 NET proteins, 14 out of them have already been published. Leukocyte elastase inhibitor and protein S100-A6 have not been identified as NET components yet. Some proteins were identified in bands corresponding to their theoretical MW, but many of them migrated to different positions which might indicate extensive protein processing.

4.6 Investigation of polyamines mediated protein cross-links by chlorinated polyamines and transglutaminase reaction

In order to detect the presence of potential cross-links formed between the identified proteins, the MS data were analyzed using StavroX. The presence of four possible cross-links were examined. Chloramines of SPM, SPD and PUT can be formed upon the myeloperoxidase (MPO) activity. The chloramines can react with protein-bound lysines or methionines resulting in Lys-polyamine-Lys or Lys-polyamine-Met cross-links. SPM, SPD and PUT can also form cross-links between protein-bound glutamines in a reaction catalyzed by the TGase. Beside this, TGases are able to catalyze the formation of isopeptide bonds between protein-bound Lys and Gln, therefore the presence of γ-Gln-ε-Lys cross-links was also monitored.

We could detect bis-ε-lysyl polyamine and ε-lysyl-S-methionyl polyamine cross-links formed by chlorinated polyamine incorporation. In addition, we could find bis-γ-glutamyl polyamine as well as ε-(γ-glutamyl)-lysine cross-links typically catalyzed by transglutaminases. The number of incorporated chlorinated SPM and SPD did not change significantly while the PUT incorporation increased in $BPNH_2$ treated samples. A similar tendency was observed in the number of transglutaminase catalyzed bis-γ-glutamyl polyamine and ε-(γ-glutamyl)-lysine cross-links.

These data indicate the presence of both chloramine-mediated and TGase-mediated cross-links. The network of cross-linked proteins was drawn manually using the results of StavroX analysis and alterations as a result of the administration of $BPNH_2$ and SPM were observed.

In conclusion, we have demonstrated that covalent crosslinking of the NET proteins as an integral part of functional NET formation can stabilize the NET structure. In case of all

samples the pattern of NET proteins was highly reproducible but the protein composition of the bands was distinct. Protein S100-A6 and leukocyte elastase inhibitor as novel NET proteins were identified by MS analysis. Many proteins were identified in bands not corresponding to their MW suggesting an extensive post-translational processing of proteins during NET formation involving probably both cross-link formation and proteolysis. The fact that the SPM and $BNPH_2$ treatments did not altered significantly the pattern of NET proteins but rather resulted in minor changes in protein composition indicates the robustness of the NET-forming biochemical systems. Our results show that endogenous covalent polyamine conjugation and enzymatic cross-linking of NET proteins contribute to the overall stability of NET and are essential for its biological functions. Based on MS/MS data of protein cross-links formed in the NET samples we have demonstrated that chlorinated polyamines covalently incorporated into NET proteins by TGase and polyamines chlorinated at both of their primary amino groups can form cross-links between NET proteins contributing to NET stability. We also confirmed the presence of ε-(γ-glutamyl)-lysine cross-links. These data indicate that the presence of polyamines, MPO and TGase together is required for the proper NET stabilization.

5. Summary

Transglutaminases (TGases) are widely investigated Ca2+-dependent enzymes with diverse functions. They play a role in numerous essential physiological and pathological processes via catalyzing the isopeptide bond formation between lysine and glutamine residues leading to the formation of ε-(γ-glutamyl)-lysine bonds.

In the first study our aim was to identify proteins associated with proliferative vitreoretinopathy (PVR) in the vitreous bodies of mice using two dimensional electrophoresis and mass spectrometry-based protein identification. We could demonstrate proteins related to PVR in the vitreous bodies of mice both in the presence and lack of TG2. Highest changes in the amount of different forms of crystallins upon dispase treatment were detected. In addition a different crystallin profile change was observed in the wild-type PVR group compared to TG2 knock-out PVR group. The utilization of PVR mice model along with two-dimension gel electrophoresis to analyze the protein changes upon dispase induced PVR formation and the effect of the lack of TG2 was successful. The results are in accordance with data reported in the literature, but further functional studies are needed to elucidate the molecular basis of this phenomenon.

In the second study our aim was to investigate the protein cross-links and how the protein crosslink-profile changes during neutrophil extracellular trap (NET) formation upon BPNH2 and SPM treatments. NET proteins were analyzed by electrophoresis and HPLC-coupled tandem mass spectrometry on a 4000 QTRAP mass spectrometer. Based on MS/MS data, the site and the type of cross-links formed were identified using StavroX protein cross-link examination software. We could demonstrate the changes in the protein cross-linking patterns upon the applied treatments and according to our data for the cross-linked protein network formation two processes are responsible: both the TGase and the myeloperoxidase (MPO)-catalyzed polyamine chloramine mediated crosslinks are important. Our data showed that the presence of MPO and TGases together is required for proper NET stabilization.

6. Publications

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Subject: PhD Publikációs Lista

Candidate: Bernadett Márkus
Neptun ID: GGSPAW
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

List of publications related to the dissertation

1. **Márkus, B.**, Pató, Z., Sarang, Z., Albert, R., Tőzsér, J., Petrovski, G., Csősz, É.: The proteomic profile of a mouse model of proliferative vitreoretinopathy.
   FEBS Open Bio. 7 (8), 1166-1177, 2017.
   DOI: http://dx.doi.org/10.1002/2211-5463.12252
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2. Csomós, K., Kristóf, E., **Márkus, B.**, Csomós, I., Kovács, G., Rotem, O., Hodrea, J., Bagoly, Z., Muszbek, L., Csősz, É., Fésüs, L.: Protein cross-linking by chlorinated polyamines and transglutamylation stabilizes neutrophil extracellular traps.
   Cell Death Dis. 7 (8), e2332, 2016.
   DOI: http://dx.doi.org/10.1038/cddis.2016.200
   IF: 5.965

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List of other publications

3. **Márkus, B.**, Szabó, K., Pfliegler, V. P., Petrényi, K., Boros, E., Pócsi, I., Tőzsér, J., Csősz, É., Dombrádi, V.: Proteomic analysis of protein phosphatase Z1 from Candida albicans. *PLoS One. 12* (8), e0183176, 2017.
DOI: http://dx.doi.org/10.1371/journal.pone.0183176
IF: 2.806 (2016)

4. Csősz, É., Lábiscsák, P., Kalló, G., **Márkus, B.**, Emri, M., Szabó, A., Tar, I., Tőzsér, J., Kiss, C., Márton, I.: Proteomics investigation of OSCC-specific salivary biomarkers in a Hungarian population highlights the importance of identification of population-tailored biomarkers. *PLoS One. 12* (5), e0177282, 2017.
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DOI: http://dx.doi.org/10.1016/j.jprot.2016.08.009
IF: 3.914 (2016)

Total IF of journals (all publications): 17,634
Total IF of journals (publications related to the dissertation): 8,108

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

26 October, 2017

7. Conference presentations and posters

Oral presentation:

Bernadett Márkus, Endre Kristóf, István Csomós, György Kovács, László Fésüs, Éva Csősz, Investigation of the neutrophil extracellular trap patterns elicited by different stimuli, 10th Molecular, Cell and Immune Biology Winter Symposium, Debrecen, 2017.

Márkus Bernadett, Lábiscsák Péter, Kalló Gergő, Emri Miklós, Csősz Éva és Márton Ildikó: Szájüregi laphámrák (OSCC) - specifikus biomarkerek azonosítása tömegspektrometriás és immunológiai módszerek segítségével a magyar populációban gyűjtött nyálmintákból, MTA DAB Genomika Proteomika Munkabizottság, Omikai Fórum, 2016.

Jakob Bernadett, Csősz Éva, Tőzsér József, Emri Miklós, Szabó Adrienn, Redl Pál, Tar Ildikó, Kövér Zsanett, Olasz Lajos, Molnár Gyöngyi, Piffkó József, Barabás Péter, Barabás József, Márton Ildikó: A magyar populációban alkalmazható szájüregi laphámrák-specifikus nyál biomarkerek azonosítása tömegspektrometriás és immunológiai módszerek segítségével, A Magyar Fogorvosok Egyesületének Árkövy Vándorgyűlése, Szeged, 2016.

Jakob Bernadett, Dr. Éva Csősz, Prof. Dr. Tőzsér József, Dr. Emri Miklós, Dr. Szabó Adrienn, Dr. Redl Pál, Dr. Tar Ildikó, Dr. Kövér Zsanett, Prof. Dr. Olasz Lajos, Dr. Molnár Gyöngyi, Prof. Dr. Piffkó József, Dr. Barabás Péter, Prof. Dr. Barabás József, Prof. Dr. Márton Ildikó: Fehérje alapú szájüregi laphámrák (OSCC) biomarkerek azonosítása az Észak-Alföldi régióban gyűjtött nyálmintákból, Debreceni Fogászati Napok, Debrecen, 2016.

Bernadett Jakob, Selected Reaction Monitoring (SRM)-based targeted proteomics method development for brain protein analysis, 9th Molecular Cell and Immune Biology Winter Symposium, Debrecen, 2016.

Bernadett Jakob, Analysis of the protein crosslink profile during Neutrophil Extracellular Trap formation, 8th Molecular Cell and Immune Biology Winter Symposium, Debrecen, 2015.

Posters:

Berandett Márkus, Endre Kristóf, István Csomós, László Fésüs, Éva Csősz, Analysis of the protein crosslink-profile changes and the neutrophil extracellular trap patterns elicited by different stimuli, Transglutaminases in Medicine, Debrecen University Symposium, Debrecen, 2017.

Tímea Székely, Bernadett Márkus, Éva Csősz, László Fesüs, Update to TRANSDAB – a collection of transglutaminase substrates and interaction partners, Transglutaminases in Medicine, Debrecen University Symposium, Debrecen, 2017.

Bernadett Márkus, Krisztina Szabó, József Tőzsér, Éva Csősz, Viktor Dombrádi: Proteomic analysis of protein phosphatase Z1 functions in Candida albicans, Hungarian Molecular Life Sciences, Eger, 2017.

Tímea Székely, Bernadett Márkus, Éva Csősz, László Fesüs, TRANSDAB – Reloaded, 10th Molecular, Cell and Immune Biology Winter Symposium, Debrecen, 2017.*

Eszter Deák, Gergő Kalló, Bernadett Jakob, Eszter Szalai, Éva Csősz, Adrienne Csutak: Alterations of tear proteins in patients with type 1 diabetes mellitus, 10th Central and Eastern European Proteomic Conference, Budapest, Magyarország, 2016.

Bernadett Jakob, Gergő Kalló, Péter Lábiscsák, Miklós Emri, Fera Mária, Ildikó Tar, Adrienn Szabó, Pál Redl, Éva Csősz, Ildikó Márton: Proteomics investigation of OSCC-specific salivary biomarkers in Hungarian population, 10th Central and Eastern European Proteomic Conference, Budapest, Magyarország, 2016.

Bernadett Jakob, Éva Csősz, Miklós Emri, Gergő Kalló, Ildikó Márton: Investigation of OSCC-specific salivary biomarkers in Hungarian population using targeted proteomics and immunological methods, 21st Congress of the European Association of Dental Public Health, Budapest, Magyarország, 2016.

Éva Csősz, Péter Lábiscsák, Bernadett Jakob, Gergő Kalló, Miklós Emri, Ildikó Tar, Mária Fera, József Tőzsér, Csongor Kiss and Ildikó Márton: Proteomics examination of OSCC-specific salivary biomarkers in a Hungarian population, Magyar Biokémiai Egyesület Vándorgyűlése Szeged, Magyarország, 2016.

Bernadett Jakob, Endre Kristóf, Krisztián Csomós, László Fésüs and Éva Csősz: Analysis of changes in Neutrophil extracellular trap (NET) proteins profile using proteomic methods, 3rd FEBS3+ Meetings, Portoroz, Szlovénia, 2015.

Bernadett Jakob, Endre Kristóf, Krisztián Csomós, László Fésüs and Éva Csősz: Analysis of the protein crosslink-profile changes during Neutrophil Extracellular Trap formation, 33rd Informal Meeting on Mass Spectrometry, Szczyrk, Lengyelország, 2015.

Bernadett Jakob, Katalin Petrényi, József Tőzsér, Éva Csősz, Viktor Dombrádi: Revealing the functions of protein phosphatase Z1 in Candida albicans with proteomic methods, II. Life Sciences, Eger, 2015.

Zsuzsanna Pató, Bernadett Jakob, Réka Albert, Zsolt Sarang, Goran Petrovski, József Tőzsér, Éva Csősz: Analysis of proteins involved in proliferative vitreoretinopathy using proteomics methods, 8th Molecular Cell and Immune Biology Winter Symposium, Debrecen, 2015.

Jakob Bernadett, Pató Zsuzsanna, Petrovski Goran, Csősz Éva: A proliferatív vitreoretinopátiás (PVR) elváltozásra jellemző fehérje mintázatok tanulmányozása egér üvegtesti mintákban, Vaszkuláris és Kardiális Kutatóhálózat tudományos ülés, Debrecen, 2014.

Bernadett Jakob, Endre Kristóf, Krisztián Csomós, László Fésüs and Éva Csősz: The effect of different treatments on the characteristic of crosslinked proteins in Neutrophil Extracellular Trap, Annual Meeting of the Hungarian Biochemical Society, Debrecen, 2014.

Bernadett Jakob, Endre Kristóf, Krisztián Csomós, László Fésüs and Éva Csősz: Analysis of crosslinked proteins in Neutrophil Extracellular Trap,, 32nd Informal Meeting on Mass Spectrometry, Balatonszárszó, 2014.

8. Keywords

Transglutaminases, proliferative vitreoretinopathy, neutrophil extracellular trap, 2D gel electrophoresis, mass spectrometry, polyamines, cross-link

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