Studying the membrane proteins in red blood cells, identifying and characterizing the related genetic variants

Ph.D. Thesis

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Introduction:

Compartmentalization is one of the key features of eukaryotic cell complexity. Each compartment is surrounded by lipids, where the transfer of substances is possible only in a controlled manner, through membrane proteins. The 20-30% of the genes of most organisms encodes membrane proteins, and the human genome contains almost 10,000 membrane protein genes. These proteins are of great importance in drug discovery: most of the drugs modulate the activity of these proteins or act as a substrate of membrane transporters. So these proteins are the active participants in ADME-Tox (absorption, distribution, metabolism, excretion and toxicity of drugs) processes. Current estimates suggest that in the future more than half of the drugs can target membrane proteins.

Red blood cells are easily accessible and since there are a huge number of these cells in even a drop of blood, they can be used to quantitate membrane protein levels. However, determination of membrane protein levels is a challenge because the lipid environment makes it difficult to isolate them. Moreover several variants of these proteins can be found on red blood cells. The results of various proteomic experiments (mass spectrometry, 2D and 1D gel proteomics, 2D-nano-HPLC results) are inconsistent, often important blood group antigens have not been found, but proteins have been identified that are characteristic of other blood cells. But taken together these results, red blood cell membranes may harbor more than 300 different membrane proteins.

Our laboratory set up an antibody labeling technique to quantify the membrane proteins in red blood cells. Only one drop of blood is needed for our measurements. During these measurements, we found differences in the red blood cell expression for two of the proteins, PMCA4b and ABCG2, in some volunteers. One of the proteins was the main calcium ion exporter of red blood cells, PMCA4b. The other one is the ABCG2 xeno- and endobiotic transporter protein, which also plays an important role in the elimination of uric acid through gut, so there is a close correlation between ABCG2 dysfunction and the development of gout.

Objectives:

During my Ph.D. work I set up the following aims

1. I wanted to carry out genetic testing of volunteer donors with a lower level of PMCA4b protein, to identify the genetic variant(s) in the background of reduced expression.

Based on the results, I also wanted to understand the exact molecular mechanism of the identified variants on expression.

2. I wanted to carry out the genetic testing of volunteer donors and gout patients with a lower ABCG2 protein level to reveal the genetic variant(s) in the background of reduced expression.

Based on the results, I also wanted to understand the exact molecular mechanism of the identified variants on expression in this case.

Methods:

1. Determination of protein levels in red blood cells using our new method

Donors

Our internal control group consists of 155 (PMCA4b) and 127 (ABCG2) healthy young people. From these people we were taken blood with finger-prick. Besides these samples EDTA-anticouagulated gout related (n=64) and hyperuricemic (n=37) blood samples were obtained from our clinical partners (National Institute of Rheumatology and Physiotherapy, Prof. Gyula Poór and Dr. Márton Pálinkás).

Ghost forming and labeling

Before labeling we made membranes (“ghosts”) from red blood cells, which are permeable for antibodies by formaldehyde fixation. These ghosts were first incubated on 96-well plates with primary antibodies (anti-PMCA4b - JA3, anti-ABCG2 - Bxp-34) and labeled with Alexa Fluor-488 conjugated secondary antibody. Ghosts, by their small size, can be not easily separated from debris in the FSC-SSC channels, therefore we labeled them with wheat germ agglutinine (WGA-Alexa Fluor-647) for this purpose.

Flow cytometry

We performed the measurements on Canto II cytometer (BD) equipped with plate loader (HTS). With the first gate, we separate the ghosts from the non-fixed cells in the FSC-SSC channel. Next, based on WGA-labeling we separate the ghost from the debris-zone. The antibody signal is determined by Alexa Fluor-488 fluorescence on ghosts. Relative expression was calculated by dividing the median of the fluorescence of the wells containing the primary antibody with the median values of the same samples with isotype antibody.

2. Genetic analysis and DNA constructs

Sanger-sequencing

The genomic DNA was obtained from blood. Regions containing the exons and exon-intron boundaries were amplified with PCR by specific primers, then sent them for Sanger-sequencing.

TaqMan based genotyping

Identified mutations and SNPs were determined by TaqMan based qPCR method in a larger cohort. The specificity of the probes were validated by sequencing.

DNA constructs

The M71V mutant version of ABCG2 was generated by site directed mutagenesis in the pcDNA-ABCG2 vector. The mutant cDNA were then subcloned to pEGFP-C1 and pIRES2 vectors.

For the *ATP2B4* enhancer measurements I inserted the upstream intronic region of the haplotype into pGL3-basic vector. I amplified the wild type, the homozygous and the heterozygous sequences from people carrying the proper genotypes.

3. Analysis of the M71V variant in mammalian cells

I studied the effect of M71V mutation in HEK293H and HeLa cells. The cell lines were transfected with lipofectamine reagent. The treatments were carried out after 24 hour post transfection for further 24 hours before measurements: 1 mM 4-PBA (from Dr.

András Váradi), 1 μM colchicine (Sigma-Aldrich). The surface expression was determined with an antibody labeling the outer epitope of the protein (5D3 antibody). For the functional analysis of ABCG2 I used Hoechst 33342 dye. Before transport measurements I incubated the samples for 5 minutes in 37 °C with or without Ko-143 specific inhibitor. The measurement started with the adding of Hoechst dye. I analyzed the transport function in the first 80 seconds (this is the linear phase of the dye accumulation) with flow cytometry using continuous sampling. I only studied the GFP positive cells, while they were transfected.

4. Analysis of the *ATP2B4* enhancer

The enhancer measurements were carried out in HEK293H and K562 cell lines. The cells were transfected with lipofectamine, and I measured the luminescence after 48 hours. The relative expression from the region was determined with dual-luciferase assay (Promega) according to the manufacturer's protocol.

5. RBC membrane preparation and Western-blot

I isolated the RBC membranes with Wolf-Schatzman method. I loaded equal amounts of proteins to acrylamide gel (protein concentrations were determined with Lowry-Folin method). After electrophoresis I blotted the proteins into PVDF membrane and immunostained it. We used the following antibodies: anti-pan PMCA (5F10), anti-PMCA4 (JA9), anti-PMCA4b (JA3), anti-PMCA1 (NR1). Then I labeled the membrane with horse radish peroxidase (HRP)-conjugated anti-mouse secondary antibody. Then I incubated it in Pierce ECL Western Blot substrate and the results were developed in x-ray films. I determined the protein quantities with luminografy.

6. Calcium transport measurements in RBCs

I used Fluo-4 fluorophore based calcium influx measurements for determining the calcium efflux capacity of RBCs with flow cytometry. I preincubated the red blood cells with Fluo-4, and I iniciated the calcium influx with adding ionomycin. The calcium ion concentrations and the ionomycin concentration were adjusted, that the normal wild type red blood cells could reset the initially low intracellular calcium ion concentrations within 15 minutes.

Results:

1. The analysis of PMCA4b

1.1 The Western-blot analysis of red blood cell membranes

We examined with Western-blot two people with reduced protein levels and one with normal protein level according to our previous flow cytometry expression measurements. We used 4 antibodies for this purpose: PMCA4b, PMCA4, PMCA1, and pan PMCA labeling antibodies. The results showed that the PMCA4b was not replaced by PMCA4a variant, so there is no mutation in the gene which affects the splicing. The size of the PMCA4b protein was correct, just its expression was reduced. There was no compensation by PMCA1.

1.2 Exon-sequencing of the ATP2B4 gene and qPCR genotyping

I selected six people with different expressions of PMCA4b (two very low, two medium and two normal), and I performed Sanger-sequencing in the exons and exon-intron boundaries of their *ATP2B4* gene. The sequencing results revealed three halotypes (common SNPs inherited together, "alleles", labeled H1, H2, H3) and one SNP in the 5 prime region located near the promoter. None of them cause any change in the amino acid sequence, since they are located in the non-coding region of the gene or cause synonym change. I selected a "tagging-SNP" for the three haplotypes, which are constant SNPs in the region, and I performed qPCR based genotyping in the whole voluntary donor sample set. The results showed that the haplotype 1 is responsible for the reduced PMCA4b protein levels.

1.3 Calcium efflux measurements in RBCs

I performed the efflux measurements in RBCs from a homozygous haplotype carrier, a heterozygous carrier and in a wild type volunteer. Unlike the wild type, the RBC of heterozygous and homozygous volunteer showed impaired PMCA4b function, their red blood cells weren't able to extrude the calcium in the examined time interval. We succeeded to confirm that the reduced protein levels, caused by a haplotype, also involve the loss of pump function.

1.4 Dual-luciferase measurements

The haplotype caused significant changes in the expression only in case of erythroid K562 cell line. In case of HEK293 the haplotype had no effect in the expression. Probably this region is an erythroid specific enhancer, which only works in erythroid cell lines.

2. The analysis of ABCG2

2.1 The expression levels of ABCG2 in gout related and control samples

We performed ABCG2 expression studies in the RBCs of 64 gouty patients, 37 age and sex matched volunteers and 127 healthy controls. Comparison of the gouty cohort and the whole control group I concluded that the ABCG2 levels significantly reduced in gouty patients.

2.2 TaqMan based qPCR genotyping of Q141K variant

The Q141K variant is very common in Hungary and we know from previous experiments that it reduces the protein expression. So I performed TaqMan based qPCR analysis of Q141K variant in our cohorts. The results showed that the SNP significantly reduces the protein levels both in homo- and heterozygous form and there were more carrier among the gouty patients. The mean ABCG2 expression of heterozygotes was 82%±17%, and for homozygous carrier it was 56%±7% compared to wild types.

2.3 Sanger-sequencing

In some blood donors, the decreased ABCG2 expression in red blood cell was not explained by the presence of Q141K (all showed an almost 50% ABCG2 expression). In these cases, Sanger-sequencing was performed on the entire encoding and exon-intron boundary regions of the gene. I found three individuals who carried the R236X mutation in heterozygous form, causing the early termination and degradation of the protein. I found a volunteer carrying the R383C variant. In addition, I found two (one gout related and one control) individuals with a new non-characterized mutation causing M71V amino acid change (n.211A> G, rs148475733).

2.4 TaqMan based genotyping for the mutations in a larger cohort

Additional DNA samples from 278 healthy blood donors were tested for the presence of mutations with qPCR-based genotyping probes. The minor allele frequencies were the following: MAF(R236X)=0.0037; MAF(M71V)=0.0049; MAF(Q141K)= 0.0963. R383C did not occur in these samples. M71V occurs at similar frequencies compared to other rare variants, about 1% of the Hungarian (caucasian) population carries it in heterozygous form.

2.5 Membrán expression studies in mammalian cell lines

In both HEK293 and HeLa cells, the expression level of M71V protein is about 60-70% compared to the wild type, similar to the Q141K variant. So in these transient expression studies I also approved the reduced protein levels caused by the mutation.

2.6 Functional studies in mammalian cells

The protein carrying the M71V variant transported the Hoechst 33342 dye. The pump function is intact, only the protein level decreases in case of this variant.

2.7 Modulation of the expression of ABCG2 variants by chemical chaperons

Colchicine treatment, which has been also used as drug in gout, significantly increased the protein levels of both wild type and mutant versions of the ABCG2 protein. The 4-PBA affected significantly only the wild type protein.

Conclusions:

1. The red blood cell measurements are suitable for determining previously unknown variants

During red blood cell expression measurements only a drop of blood needed to determine 10-20 membrane protein levels simultaneously within 3 hours. During my work I used this method to identify SNPs in the Hungarian population which may affect the membrane protein levels of a calcium exporter protein, PMCA4b and the endo- and xenobiotic transporter ABCG2.

- In the case of PMCA4b, I found a haplotype in an erythroid specific enhancer that has a protective effect on malaria in according to GWA studies.

- In the gene of ABCG2 I found more defective variants. The R236X causes the early termination and degradation of the protein. The R383C causes the loss of an important salt bridge, resulting protein inactivation and destabilization. Besides these, I found a new, previously unrecognized variant, the M71V, which is relatively common in the Hungarian population.

2. The red blood cell measurements may be useful from a prognostic or diagnostic point of view

- In case of PMCA4b, the identified haplotype showed closed correlation with the red blood cell expressions, so this method can be used to detect the haplotype.

- There are several variants in ABCG2 that affect the level or activity of the protein. Based on GWA studies, other SNPs in regulatory regions of ABCG2, such as intronic or promoter variants, may also play a role in gout, so they may somehow influence the activity or expression of the ABCG2 protein. Volunteers with lower ABCG2 levels were also found in red blood cell expression measurements, but the exon sequencing did not reveal the background variant or other regulatory modifications in the gene. Therefore, red blood cell-based membrane level determination may be particularly beneficial in the clinical examination of this protein.

The red blood cell measurements that we have set up, - besides the new variant identification - may be suitable for direct quantitative determination of membrane proteins for prognostic or diagnostic purposes. In the literature, I did not find any other similar method that would allow the quick, easy and parallel determination of protein levels from a drop of blood. Most molecular diagnostic laboratories equipped with flow cytometers, so the method can be used to assist clinicians in the future.

3. Limitations of red blood cell expression studies

- There are more than 300 membrane proteins in red blood cells according to the latest mass spectrometry data. However, there may be proteins that are not expressed on these cells, so in these cases the method is not suitable for protein expression determination.

- We need good antibodies for membrane protein labeling. During our work we tried a total of 40 antibodies, and only 35 of them could even detect our proteins. Only 11 of them gave the signal suitable for the quantitative measurement.

4. The significance of the haplotype affecting PMCA4b

I identified a haplotype in the PMCA4b coding *ATP2B4* gene, which:

- caused 75% reduction in protein expression in case of heterozygotes and 50% in case of homozygotes. Other PMCA isoforms were not affected.

- has about 0.1 minor allele frequency in Hungary, but in malarial endemic countries it is much more frequent.

- affects calcium extrusion of red blood cells, because it is the main calcium exporter protein of these cells.

- localizes in an erythroid specific enhancer, which directs protein expression in erythroid cell lines. In other cell types probably have no effect on expression.

5. Significance of SNPs and mutations identified in *ABCG2*

Examining gouty patients and healthy controls, I found that:

- the levels of ABCG2 in gouty patients are reduced.

- one of the common variant of ABCG2, Q141K, is more frequent in patients than in controls. This variant causes reduced expression in red blood cells as well.

In addition, I found more people with about 50% expression compared to wild types. In these people, I identified three rare mutations:

- the R236X causes the early termination and degradation of the protein.

- the R383C amino acid substitution destroys one of the critical salt bridges of the protein, therefore the protein does not work and unstable.

- the M71V has not been identified so far. By simple cell base methods I proved, that the M71V protein is active, but unstable and degrades. I succeeded in correcting this with colchicine.

Publications:

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