Investigation of the function, cell surface expression and regulation of ABCA1 by using *in vitro* model systems

Doctoral Thesis

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INTRODUCTION

ABC (ATP-Binding Cassette) proteins form one of the largest protein families, the members of which are present in all living organism from bacteria to humans.

All ABC transporters contain two functionally and structurally distinct domains, the transmembrane domain (TMD) and the nucleotide-binding domain (NBD). ABC transporters are defined by the presence of three conservative sequences in the NBD also noted as ABC unit. This domain contains the catalytic site for ATP binding and hydrolysis. The other domain, the TMD is involved in recognition and translocation of the transported substrates. Amino acid substitutions in this domain lead to changes in substrate specificity of the transporter. A functionally active ABC protein typically consists of two NBDs and two TMDs. In most human ABC proteins, these four domains are present within one polypeptide chain (“full transporters”), however, in some other cases the transporters are formed by the association of two subunits (“half transporters”). In humans 48 ABC proteins have been identified. On the basis of sequential and structural similarities these transporters are classified into seven subfamilies ranging from ABCA through ABCG.

Depending on their action three different types of ABC proteins can be distinguished: i) active transporters, which perform uphill transport of substances across the membrane; ii) regulated ion channels; iii) and receptors, which transmit extracellular signals into the cell and regulate the function of other proteins. All three types of ABC transporters utilize the energy of ATP binding and hydrolysis to accomplish their function.

The ABCA1 is a defining member of the ABCA subfamily and structurally belongs to the full transporters. Regarding its tissue distribution,

ABCA1 is expressed ubiquitously with the highest expression levels in placenta, liver, lung, adrenal glands and testis. ABCA1 also exhibits high expression in tissue macrophages and macrophage-like cells. Subcellularly it localizes mostly to the plasma membrane, in polarized cells to the basolateral compartment.

The ABCA1 membrane protein plays a pivotal role in cellular lipid homeostasis. As an initial step of the reverse cholesterol transport pathway ABCA1 participates in the interactions of amphiphilic apolipoproteins (in humans primarily apoA-I) with cholesterol and phospholipids to generate nascent HDL particles, thus removing excess cellular cholesterol. During the ABCA1-dependent HDL generation not only phosphatidylcholine and cholesterol but other phospholipids are also effluxed from cells. Mutations in ABCA1 cause Tangier disease, a disorder characterized by HDL deficiency, hypercholesterolemia, cholesterol deposition in macrophages, and premature atherosclerosis. The dysfunction of ABCA1 leads not only to disturbed cholesterol homeostasis but also to impaired phagocytosis and incomplete platelet functions presumably as a consequence of defective exofacial exposure of phosphatidylserine. Nevertheless, the contribution of ABCA1 to the membrane lipid asymmetry in platelet activation and its role in the apoptotic phosphatidylserine exposure is still elusive.

Similar to other plasma membrane proteins, the cell surface expression and activity of ABCA1 are highly regulated at transcriptional and posttranscriptional levels by a wide variety of regulatory molecules. Transcriptional regulators include activators, such as cAMP or nuclear receptor activation, and inhibitors, e.g., inflammatory cytokines or TNF-α. Several posttranscriptional processes play important role in the modulation of ABCA1 protein stability and turnover. The protein degradation of ABCA1 is enhanced by calpain, a cystein protease; the protein activity is affected by various protein

kinases. The activity of ABCA1 can also be modulated by the membrane lipid environment.

Since the plasma membrane expression of ABCA1 is required for proper function; pharmaceuticals regulating the cell surface expression of ABCA1 have recently come into focus. The complex regulation of ABCA1 cell surface expression offers several potential targets for drug development strategies. Transcriptional modulation of ABCA1 through nuclear receptors affects a broad range of target molecules thus concomitantly induces numerous side-effects. Influencing the posttranscriptional processes, such as internalization, degradation and recycling of ABCA1 provides a potential alternative for drug development. Nevertheless, there is no available test system which is suitable for monitoring the plasma membrane level of ABCA1, independently from direct transcriptional regulation.

AIMS

The major goal of my study was to investigate the function, cell surface expression and regulation of ABCA1 protein by using *in vitro* model systems. In accordance with this purpose we intended to accomplish the following specific aims:

1. To study the role of ABCA1 in the Ca^{2+}-induced phosphatidylserine exposure at the cell surface.

For the functional measurements we planned to establish cell lines stably expressing ABCA1 by using retroviral expression system applicable for various cell types.

2. To explore the mechanism of ABCA1 in the phosphatidylserine exposure by using mutant ABCA1 variants.

In addition to the wild-type protein we intended to generate ABCA1 variants carrying mutations in the catalytic sites or a mutation indentified in a patient with Scott syndrome, a rare bleeding disorder. To make specific and sensitive detection of the protein feasible we planned to introduce an extracellular hemagglutinin (HA) epitope into the wild type and mutants ABCA1 variants.

3. To develop a quantitative *in vitro* test system suitable for monitoring the cell surface expression of ABCA1.

We aimed to establish different mammalian cell lines stably expressing HA-tagged ABCA1 variants and to characterize them in terms of expression level, subcellular localization and function of ABCA1. In order to demonstrate the applicability and reliability of our model system we planned to study the effect of different substances which are known to influence ABCA1 trafficking and degradation.

4. To investigate the effect of various pharmaceuticals on the cell surface expression of ABCA1. The compounds to be studied include cholesterol level-lowering and HDL level-increasing drugs as well as chemicals known to influence the expression or function of ABCA1.

METHODS

For the generation of retroviral vectors, the human ABCA1 cDNA was cloned into a bicistronic retroviral vector, which contains the neomycin resistance cassette gene. To generate the HA-tagged ABCA1, a cassette containing the coding sequence of the HA epitope was introduced into the ABCA1 cDNA. Vectors for the inactive MK (K939M), KM (K1952M) and MM (K939M/K1952M) Walker A mutants and the Scott mutant RQ (R1925Q) ABCA1 variant were generated by PCR mutagenesis technique. In order to establish HA-ABCA1 variants-expressing B lymphocytes, HeLa, HEK293 and MDCKII cell lines we transformed packaging cell lines for the particular retrovirus production and transduced the target cells by collected virus particles. Cell lines stably expressing the transgene were generated by specific selection method suited for each cell type. To obtain cell lines with comparable ABCA1 expression levels, the transduced cell lines were cloned and clones were pooled or alternatively the cells were sorted by fluorescence-activated cell sorter.

The ABCA1 mRNA level of the established cell lines was determined by conventional and real-time quantitative PCR. The protein expression levels were monitored by Western analysis on whole cell lysates or membrane preparations. The subcellular localization of HA-tagged ABCA1 variants was studied by immunofluorescence staining followed by confocal microscopy.

To investigate the functional properties of ABCA1 protein variants various *in vitro* methods were applied. The cell surface phosphatidylserine exposure associated with ABCA1 function was studied by specific binding of fluorescently-labeled annexin V and detected by flow cytometry. The effect of several ABC transporter inhibitors was also examined. The functional properties of HA-tagged ABCA1 variants were investigated by apoA-I-dependent cholesterol efflux and apoA-I binding measurements. For the former

the cells were labeled with ^{3}H-cholesterol and the effluxed cholesterol was determined from the supernatants by liquid-scintillation counting. For the apoA-I binding measurements apoA-I was conjugated with cyanine 5 fluorochrome and the cell surface binding was detected by flow cytometry.

The cellular localization of ABCA1 was visualized by indirect immunofluorescence labeling with anti-HA antibody detected by flow cytometry.

RESULTS

The role of ABCA1 in the Ca^{2+}-induced cell surface exposure of phosphatidylserine was studied on immortalized B cells from a patient with Scott syndrome. These cells, provided by Prof. C. Higgins, express a mutant form of ABCA1 (R1925Q), and exhibit impaired Ca^{2+}-induced phosphatidyl-serine translocation. We transduced these cells with the ABCA1 by using retroviral expression system and multistep transduction protocol. Our functional studies revealed that expression of wild type ABCA1 restores the defective phosphatidylserine translocation observed in the parental Scott B cells. We confirmed the role of ABCA1 by the inhibitory effect of glyburide, an ABCA1 inhibitor, on the phosphatidylserine exposure in both B cells from healthy subjects and ABCA1-transduced Scott B lymphocytes. Our results suggest that ABCA1 actively participates in the Ca^{2+}-induced phosphatidylserine exposure.

To examine the mechanism of ABCA1 in the phosphatidylserine translocation we transduced B cells with inactive mutants of ABCA1 (MK, KM and MM) as well as with a variant carrying a missens mutation (RQ) indentified in the particular Scott's patient. To assure sensitive and specific immunodetection of the protein we introduced an HA-epitope into the ABCA1 variants. Our measurements with these cells revealed functional association between ABCA1 and Ca^{2+}-induced phosphatidylserine exposure, since only the wild-type HA-ABCA1 restored the defective function; neither the non-functional NBD mutants nor the RQ mutant was able to repair the defective phosphatidylserine exposure. The effect of these ABCA1 mutants were also studied in healthy B cells. Interestingly neither the NBD mutants nor the RQ mutant had a dominant negative effect on the Ca^{2+}-induced phosphatidylserine exposure. To further explore the mechanisms phosphatidylserine translocation next we studied the effect of glyburide and the plasma membrane cholesterol levels on this process. The Ca^{2+}-induced phosphatidylserine exposure was not

inhibited by glyburide or cholesterol overload either in healthy B cells or in the ABCA1-transduced Scott B cells.

To investigate the regulation of trafficking and cell surface expression of ABCA1 we generated an *in vitro* cellular model system for monitoring the plasma membrane level of ABCA1. We established cell lines stably expressing the functional HA-tagged ABCA1 or the NBD mutant variants by using retroviral transduction. The introduced extracellular HA-epitope allowed us to detect cell surface expression of ABCA1 by flow cytometry and confocal microscopy.

To study the functional properties of our HA-tagged ABCA1 variants we measured the apoA-I-dependent cholesterol efflux and apoA-I binding. These measurements clearly demonstrated the full functionality of the HA-tagged wild-type ABCA1 protein and the inactiveness of the NBD mutants. We also investigated the effect of drugs which influence the plasma membrane expression of ABCA1 on the apoA-I cell surface binding in cells expressing the functional ABCA1 protein or the inactive mutant (MM) variant. Our results revealed that blocking of ABCA1 degradation by a calpain inhibitor results in increased apoA-I cell surface binding. In contrast, reduced apoA-I binding was observed in response to cyclosporin A, which increases ABCA1 on the cell surface and blocks its function. These drugs had no effect on the apoA-I binding in the MM ABCA1-expressing cells, further confirming the defectiveness of the MM variant.

Next we demonstrated the applicability of our model system for monitoring ABCA1 cell surface expression by applying various substances with known effect on ABCA1 trafficking and degradation, i.e., ALLN, a calpain inhibitor and Brefeldin A. By having a verified test system, we studied the effect of various drugs on the cell surface expression of ABCA1 variants. Several cholesterol level-lowering pharmaceuticals, e.g., atorvastatin and niacin, as well as some calcium-channel blockers, e.g., nifedipine and

verapamil, have been tested but no alteration in the plasma membrane expression of ABCA1 was observed in response to these drugs in either the functional or the inactive ABCA1-expressing cells. In contrast, cyclosporin A increased the plasma membrane appearance of ABCA1, whereas glyburide, the ABCA1 inhibitor had no effect in either cell types.

Our studies revealed an unexpected effect of ezetimibe, a blocker of the intestinal cholesterol absorption. This drug significantly reduced the cell surface expression of the functional ABCA1. Interestingly, this effect of ezetimibe was found to be associated with ABCA1 function, since no reduction in the cell surface expression of the inactive ABCA1 variant was observed.

CONCLUSIONS

In the presented work we performed a multiple analysis for the functional characterization of human ABCA1 protein. We studied the role of ABCA1 in the phosphatidylserine cell surface exposure. Our results revealed that expression of wild type ABCA1 restores the defective phosphatidylserine translocation, a phenotype observed in a patient with Scott syndrome. The recovered function was inhibited by an ABCA1 inhibitor. This is the first demonstration of the contribution of ABCA1 to the cell surface exposure of phosphatidylserine. Our results confirm the association between ABCA1 dysfunction and a bleeding disorder.

We examined the effect of inactive ABCA1 variants and a mutant variant carrying a missense mutation indentified in the particular Scott patient. We concluded that the defective phosphatidylserine exposure is complemented solely by the functional ABCA1 expression; the inactive mutant did not restore this defective function. Expression of mutant variants did not alter the phosphatidylserine exposure in healthy B cells by a dominant negative effect.

We developed a model system which is suitable for sensitive and reliable monitoring of the plasma membrane level of ABCA1. By using these model cells the effects of several pharmaceuticals with known cholesterol-lowering or HDL level-raising effects were tested. This approach revealed an unknown effect of ezetimibe, this drug blocks not only the intestinal cholesterol absorption but inhibits the cell surface appearance of ABCA1 protein. Our findings demonstrate the applicability of a novel and reliable model system for studying cellular routing of ABCA1, opening up new opportunities to identify unknown interaction of drugs with this key transporter of cholesterol metabolism.

PUBLICATIONS

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