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ORIGINAL PAPER

Evaluation of cystine transport in cultured human kidney cells and establishment of cystinuria type I phenotype by antisense technology

Gunnar Wendt-Nordahl · Sreedhar Sagi · Christian Bolenz · Peter Alken · Maurice Stephan Michel · Thomas Knoll

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Abstract Cystinuria is a rare hereditary disease resulting in recurrent stone formation and the need for repeated invasive interventions. So far, two responsible genes have been identified which encode the two transporters, rBAT and $b^{0,+}$AT forming a heterodimer to transport cystine in proximal tubular cells (PTC) and whose defect results in increased excretion of cystine. A human cell line mimicing the phenotype of cystinuria in vitro is yet to be developed. Human kidney (HK)-2 is a PTC line derived from normal HK. After determining the presence of rBAT gene by RT-PCR and Western blot analysis, radioactively labeled cystine (S^{35}) was used to evaluate the functional presence of the amino acid transport in HK-2 cells when cultured in vitro. To achieve a cystinuria type I phenotype in HK-2 cells, the rBAT gene was silenced using antisense oligonucleotides complimentary to human rBAT mRNA. The reduced transport activity of cystine was then determined by radiolabeled cystine uptake measurements. RT-PCR and Western blot confirmed the expression of the rBAT gene in HK-2 cells. Considerable transport of the radio labeled cystine was observed in HK-2 cells and was linearly dependent on the incubation time with the amino acid. The cystine transport in rBAT knockdown cells after incubation with antisense oligonucleotides was significantly lower compared to control (0.76 vs. 0.98%; $P = 0.0008$), proving a transient knock-down of the rBAT gene. This study demonstrates the presence of the $b^{0,+}$ amino acid transport system in human proximal tubular HK-2 cells when cultured in vitro. Inhibition of this transport system is possible by using antisense technology. A permanent inhibition of the cystine transport, based on our model, would be useful for the development and evaluation gene therapeutic approaches.

Keywords Cystinuria · Inborn genetic diseases · Amino acid transport · Proximal renal tubules

Introduction

Cystinuria, an autosomal recessive disorder, is the cause of 1–2% of stones observed in adults and up to 10% of those occurring in children [1]. Cystinuric patients frequently form urinary tract calculi and subsequently require multiple urologic interventions. Both stone formation and the necessary interventions constitute a risk for the renal function in the patients [2]. The durability of long-term treatment success in patients with cystinuria is poor and only a small percentage of patients achieve and maintain the therapeutic goal of a low urinary cystine concentration. Medical treatment of cystinuria is only symptomatic and cannot completely prevent stone formation in most patients.

The uptake of cystine is mediated by the heterodimeric amino acid transporter system $b^{0,+}$, which is defective in cystinuric patients. Human $b^{0,+}$ transport activity is characterized by the sodium independent transport of dibasic amino acids, including cystine. The heavy chain rBAT associates with the light chain $b^{0,+}$AT to form the amino acid transport system $b^{0,+}$. The impaired reabsorption of

Gunnar Wendt-Nordahl, Sreedhar Sagi contributed equally to this work.

G. Wendt-Nordahl · S. Sagi · C. Bolenz · P. Alken · M. S. Michel · T. Knoll (✉)
Department of Urology,
University Hospital Mannheim,
Theodor-Kutzer-Ufer 1-3, 68135 Mannheim, Germany
e-mail: thomas.knoll@uro.ma.uni-heidelberg.de

poorly soluble cystine through the epithelial cells of the renal tubules results in an elevated concentration of urinary cystine leading to the formation of cystine calculi in the urinary tract. Mutations in the two genes, SLC3A1 (rBAT) and SLC7A9 ($b^{0,+}$AT) have been shown to lead to the cystinuria phenotype [3]. Cystinuria type I, the most common variant of the disease, results from a defect in the rBAT subunit of the amino acid transporter [4, 5].

In vitro experiments utilizing cell lines play a major role in understanding the molecular basis of the disease. Cell lines from kidneys of various animals have been used to characterize the cystine transporter [6, 7]. Also, xenopus oocytes have been used to a large extent to study cystine transport activity or molecular mechanisms of the heterodimeric amino transporters [5, 8–10]. However, a human cell line which mimics the phenotype of cystinuria in vitro is yet to be developed. Aim of this study was to verify the presence of a cystine transport mechanism in human proximal tubule cells (PTCs) when cultured in vitro and to generate a cystinuria phenotype in these cells. These experiments could provide primary data on cystine transport activity in human PTCs in case of permanent rBAT suppression. Such a cell line could serve as a valuable model to be used for the development of gene therapeutic approaches of cystinuria.

Materials and methods

Cell culture

HK (human kidney)-2 is a proximal tubular cell line derived from normal HK (ATCC Number: CRL-2190). HK-2 cells retain functional characteristics of proximal tubular epithelium and can reproduce experimental results obtained with freshly isolated PTCs. The cells were propagated in Keratinocyte-serum free medium (GIBCO-BRL) with 5 ng/ml recombinant epidermal growth factor and 0.05 mg/ml bovine pituitary extract. The cells were incubated at 37°C with 5% CO_2.

Determining the presence of rBAT gene in HK-2 cells

RNA from HK-2 cells was isolated using TRIZOL. RNA was reverse transcribed (RT) using Oligo DT primers. The presence of rBAT gene was determined by PCR of the cDNA using the hrBAT primers (Forward: 5′-TCC CGC TAA AAT AAG AAT AAG-3′; Reverse: 5′-GAT TGG AAA CAA AGC ATC TAT CTC-3′). For Western blot analysis, whole cell lysate from HK-2 cells was isolated. Hundred micrograms of total protein was run in a SDS-PAGE and transferred to a PVDF membrane. Peptide antibodies (primary) were generated against the rBAT part of the heterodimer (peptide sequence for human rBAT: MAE-DKSKRDSIEMSMKGC).

Amino acid uptake measurements in HK-2 cells

Radioactively labeled amino acid cystine (S^{35}) (Amersham Biosciences Europe) was used to determine the functional presence of this amino acid transport in PTCs (HK-2) when cultured in vitro. The HK-2 cells were cultured in K-SFM in 6-well plates. At confluence (80%), the medium was aspirated and cells washed with PBS. Buffer was then replaced by PBS supplemented with amino acid (uptake buffer). Uptake buffer consisted of 1 μCi of labeled cystine in 1 ml of PBS. One milliliter of uptake buffer was added to each well. Cells were incubated in time intervals of 5, 15 and 30 min in uptake buffer. Uptake was stopped by replacing the amino acid uptake buffer with ice cold PBS and washing for four times. Cells were lysed with 1 N NaOH. The lysate was then added to vials with scintillation fluid (SF; Ready Safe, Beckman) and measured using a Beckman Liquid scintillation counter to determine the uptake of S^{35} labeled cystine by the cells. Zero time uptake was used as a control for non-specific binding.

rBAT gene silencing using antisense oligonucleotides

To achieve a cystinuria type I phenotype in HK-2 cells, the rBAT gene was silenced using antisense technology. A transient knock out of the gene was achieved by using oligonucleotides complimentary to the human rBAT mRNA. Sense: 5-TGC CCA AGG AGG TGC TGT TC-3 (starting at base 203 of coding region); antisense: 5-GAA CAG CAC CTC CTT GGG CAT-3 (starting at base 222 of the coding region). Six micromolar concentration of the antisense oligonucleotides were added to the cells and incubated for 48 h. The transport activity of the transporter was then determined by the radioactively labeled cystine uptake measurements as described above. Controls were treated with the same concentration of non-sense oligonucleotides or received no treatment.

Statistical analyses

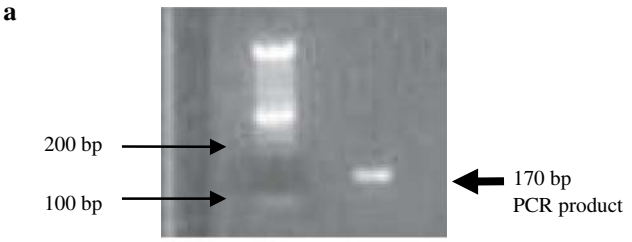
All numerical results are expressed as mean ± standard deviation (SD). $P < 0.05$ was considered statistically significant. A two sided t test was performed with the SAS statistical software.

Results

The constituent expression of the rBAT transporter protein in HK-2 cells when cultured in vitro was analyzed by

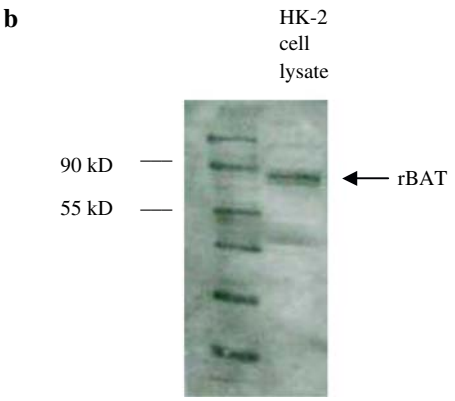
RT-PCR. The PCR product (170 bp) of the cDNA using hrBAT primers verified the presence of the rBAT gene as shown in Fig. 1a. Similarly, Western blot analysis of the rBAT protein from the whole cell lysate at ~85 kDa showed the functional expression of the rBAT gene as displayed in Fig. 1b. To examine the transport activity of cystine in human PTCs radiolabeled cystine was used. As shown in Fig. 2 considerable transport of the radiolabeled cystine was observed in HK-2 cells. Cellular uptake for cystine was time dependent and reached 0.05, 0.18 and 1.04% after incubation for 5, 15 and 30 min, respectively.

After having proved the presence of the cystine transport mechanism in HK-2 cells when cultured in vitro, we knocked down the rBAT gene using antisense oligonucleotides to establish a cystinuria type I phenotype. The cystine uptake in the presence of antisense oligonucleotides reaching 0.76% after 30 min was significantly reduced compared to 1.04% (control) and 0.98% reached after incubation with non-sense oligonucleotides ($P = 0.0008$), as displayed in Fig. 3. This proves the transient knock out of the rBAT gene in presence of antisense oligonucleotides.



Lane 1: 100 Kb ladder

Lane 2: PCR product (170 bp) of rBAT primers



Lane 1: Santa Cruz marker

Lane 2: HK-2 cell lysate rBAT protein (~85kD)

Fig. 1 **a** Identification of the rBAT gene expression in HK-2 cells by RT-PCR. RNA from HK-2 cells was reverse transcribed to cDNA. PCR was performed on the cDNA using hrBAT primers to identify the rBAT gene. Agarose gel electrophoresis of the RT-PCR product from HK-2 cells shows the PCR product at 170 bp (*lane 2*). **b** Western blot identification of the rBAT protein expression in HK-2 cells. Whole cell lysate was electrophoresed using SDS-PAGE and the proteins were transferred to the PVDF membrane. The rBAT protein (~85 kDa) was identified by Western blot using the peptide primary antibody

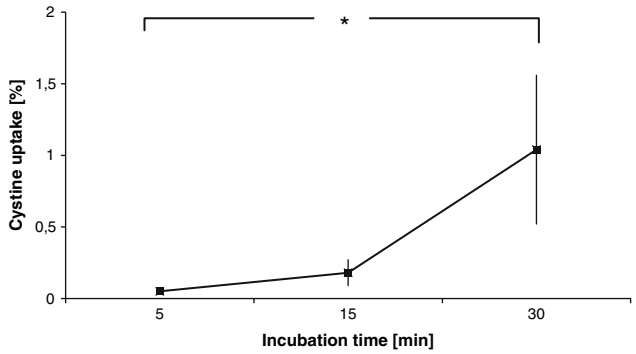


Fig. 2 Time dependent increase in uptake of radiolabeled S^{35} cystine into HK-2 cells (*$P = 0.005$). Uptake values are the % of counts per minute of the radiolabeled amino acid found in the cell lysate to the total amount of labeled amino acid given to the cells and are expressed as mean ± SD

Discussion

Cystinuria, an autosomal recessive disorder, is characterized by the frequent formation of urinary tract stones requiring multiple stone removing procedures. The disease has a significant impact on the patients' quality of life and constitutes a risk to the renal function [2]. Although preventive measures including forced diuresis, urine alkalization and medical cleavage of the disulfide bond have been shown to be effective in reducing stone formation, the preventive treatment of cystinuric patients in clinical routine is

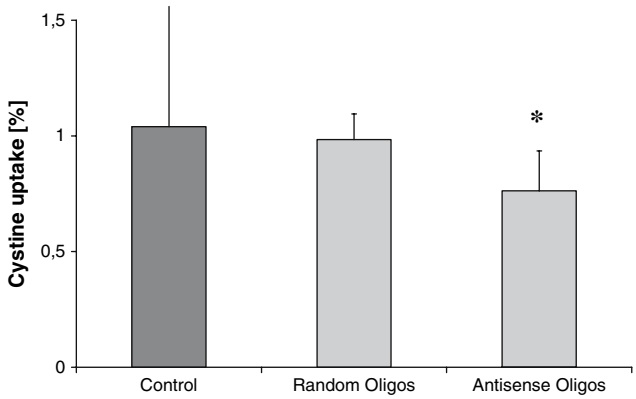


Fig. 3 Uptake of radiolabeled cystine into HK-2 cells after treatment with antisense oligonucleotides (oligos) was significantly reduced compared to control (no oligos) and treatment with random oligos (*$P = 0.0008$). Uptake of S^{35} cystine was measured after 30 min incubation with antisense oligonucleotides and control oligonucleotides. Uptake values are % of counts per minute of the radiolabeled amino acid found in the cell lysate to the total amount of labeled amino acid given to the cells and are expressed in mean ± SD

often unsatisfactory [1]. In contrast, a gene therapeutic approach might offer cure to the patients. To develop such an approach, full understanding of the underlying molecular processes is required. It has been shown, that cystinuria is caused by the defective amino acid transporter system $b^{0,+}$ in proximal tubular cells. In the heterodimeric transport system $b^{0,+}$ the heavy chain rBAT associates with the light chain $b^{0,+}$AT [5, 6].

Our study demonstrates the existence of the $b^{0,+}$ amino acid transport system in human proximal tubular HK-2 cells when cultured in vitro. It was evident by RT-PCR and Western blot analysis that the rBAT portion of the rBAT–$b^{0,+}$AT heterodimer is existent in HK-2 cells. By using radiolabeled cystine the time-dependant uptake of the amino acid was demonstrated also on a functional level. The assay using labeled cystine (S^{35}) has already been shown to be a reliable technique to study cystine transport in other studies [10–12].

As the rBAT–$b^{0,+}$AT heterodimer is the main apical reabsorption system for cystine in the kidney [13], this human tubular cell line can be used for further studies to characterize cystine transport in vitro in order to improve comprehension of the pathomechanisms of the disorder.

Until this study, most of the previous studies were carried out in Xenopus oocytes, where wild type or mutated cRNA of the rBAT and/or $b^{0,+}$AT was injected into the cells and cystine uptake was determined [5, 8–10]. However, the functional characterization of the rBAT–$b^{0,+}$AT heterodimer proved to be difficult as the Xenopus oocytes exhibit a very strong endogenous transport activity, which is almost indistinguishable from its mammalian counterpart [14]. Furthermore, as the $b^{0,+}$ transporter is not naturally expressed in oocytes, the functional properties may not be identical to those displayed in native tissue. Therefore, the characteristics of the amino acid transport mediated by rBAT can be better studied in mammalian cell models that express rBAT constitutively.

Experiments have been performed on kidney cell lines obtained from various animals. In MDCK cells human rBAT cDNA was expressed using vectors and cystine transport was measured [7]. Chairoungdua and colleagues studied the functional expression in COS-7 cells as they believed that Xenopus oocytes were not suitable for the functional expression of $b^{0,+}$ due to the abundant expression of endogenous transporters [15]. They found a significant transport activity of cystine mediated by expression of rBAT in COS-7 cells. Although the described models delivered important information on cystine transport activity, the characterization in a human cell line would be ideal.

Such a cell line mimicking the phenotype of cystinuria in vitro has not been developed until this study. The HK-2 cells used in this study are derived from HK and duplicate the environment of HK in vitro. The $b^{0,+}$ amino acid transport system is constitutively expressed in these cells thereby providing a natural transport of cystine. Thus, we demonstrated the naturally occurring cystine transport in HK-2 cells when cultured in vitro.

As a first step in developing gene therapeutic approaches for the disease, a model mimicking cystinuria in vitro has to be established. Antisense oligonucleotides complimentary to human rBAT mRNA have been used to inhibit the rBAT mediated uptake of cystine in Xenopus oocytes [16, 17] and mammalian cell lines [18]. A cystinuric phenotype in a human cell line has not been described until this study. Our work demonstrates that a cystinuria like condition can be achieved in HK-2 cells by using antisense oligonucleotides. In our study we used antisense technology to silence the SLC3A1 (rBAT) gene to suppress the $b^{0,+}$ transport system leading to a state comparable to human cystinuria. Based on our results further studies can be performed to characterize the inhibition of the $b^{0,+}$ amino acid transport system in HK-2 cells by antisense oligonucleotides in more detail.

To evaluate gene therapeutic approaches of cystinuria, a human cell line originating from PTCs displaying a permanent knock down of the $b^{0,+}$ transport system would be ideal. Our results using antisense technology to achieve a transient suppression of rBAT may give the primary data in establishing such a model. By using lenti-viral gene delivery into HK-2 cells a permanent knock down of rBAT might be achieved to obtain a stable cystinuria type I phenotype. Such a model may prove valuable in developing gene therapeutic approaches of the disease.

Conclusion

In conclusion, we demonstrated the functional presence of the $b^{0,+}$ amino acid transport system in a human PTC line. Furthermore, we were able to achieve a cystinuria like phenotype in these cells by using antisense technology. Our results may serve as a basis for future experiments establishing a permanent knock down of rBAT to obtain a stable cystinuria type I phenotype in HK-2 cells.

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