Expression of SLC26A3, CFTR and NHE3 in the human male reproductive tract: role in male subfertility caused by congenital chloride diarrhoea

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Congenital chloride diarrhoea (CLD) is a rare inherited disease caused by mutations in the solute carrier family 26 member 3 (SLC26A3) gene. Disruption of intestinal Cl–/HCO3– exchange causes watery Cl– rich diarrhoea from birth, and recently male subfertility was observed as a novel manifestation. Expression of SLC26A3, together with interacting proteins cystic fibrosis transmembrane conductance regulator (CFTR) and Na+/H+ exchanger 3 (NHE3), was studied using immunohistochemistry in the testis (n = 2) and efferent ducts (ED) (n = 1) of patients with CLD (V317del genotype) and in the testis and epididymis (n = 11), seminal vesicle (n = 9) and prostate (n = 4) of the controls. SLC26A3 was immunolocalized in the head of the elongating spermatids (stages III–V) and CFTR in the elongating spermatids (stages III and IV) and pachytene (stages III–V) and diplotene spermatocytes. In the non-ciliated cells of the ED, apical expression of all three proteins was observed, but only SLC26A3 and CFTR were detected on the luminal border of the apical mitochondria-rich cells (AMRC) of the ductus epididymis and in the epithelium of the seminal vesicle. Only CFTR was present in the epithelium of the prostatic duct. In the patient with CLD, the expression of both SLC26A3 and CFTR was absent in the ED, but testicular expression was identical to that of the controls. These results suggest a primary role for SLC26A3 in male reproduction. Tissue-specific co-expression with CFTR and NHE3 supports diverse functions of SLC26A3 and may have an impact on pathophysiology of male subfertility both in CLD and in cystic fibrosis (CF), as well as spermatoceles.

Key words: CFTR/congenital chloride diarrhoea/male fertility/NHE3/SLC26A3

Introduction

Congenital chloride diarrhoea (CLD) is a rare autosomal recessive disease with a defect in the intestinal Cl–/HCO3– exchange (Norio et al., 1971; Holmberg et al., 1975). Cl– rich, acidic, watery diarrhoea begins in the uterus and leads to dehydration, hypochloremic metabolic alkalosis and failure to thrive soon after birth. With substitution therapy of sodium and potassium chloride, patients with CLD survive, and the long-term outcome is favourable (Holmberg, 1986; unpublished data). However, subfertility may be common among adult males (Högland et al., in press).

The molecular genetic basis of CLD has been identified, and over 30 mutations of the solute carrier family 26 member 3 (SLC26A3 alias DRA) gene have been shown to cause CLD, without evidence of phenotype-genotype correlation (Högland et al., 1996; Mäkelä et al., 2002). Previous immunohistochemical studies have revealed the expression of SLC26A3 in the apical brush border of the human intestinal epithelium, in the sweat gland and in the male seminal vesicle (Haila et al., 2000; Jacob et al., 2002). In the ileum and colon, SLC26A3 acts as an apical exchanger for Cl– and HCO3–, secondarily promoting the absorption of Na+, most likely through the Na+/H+ exchanger 3 (NHE3) (Holmberg et al., 1975; Melvin et al., 1999; Moseley et al., 1999; Lamprecht et al., 2002). In the duodenum and ductal systems, SLC26A3 is involved in electroneutral secretion of HCO3–, likely through a reciprocal molecular interaction with the cystic fibrosis transmembrane conductance regulator (CFTR) protein (Greeley et al., 2001; Ko et al., 2002, 2004; Rossmann et al., 2005).

Subfertility in males with CLD is characterized by oligoastheno-zooospermia with normal concentrations of sex hormones and normal testicular histology, as well as a tendency to form spermatoceles. A primary defect in Cl–/HCO3– transport in the male reproductive tract was proposed after the observation of a high concentration of Cl– and a low pH in the seminal plasma among these males (Högland et al., in press).

In the male reproductive tract, human SLC26A3 has been immunodetected in the epithelium of the seminal vesicle (Haila et al., 2000), and mouse homologous Slc26a3 mRNA has been found to be expressed in the non-ciliated cells of the efferent ducts (ED) of the testis, together with Cfr and Nhe3 (Lee et al., 2001). Both CFTR and NHE3 have been shown to be able to directly interact and, in the case of CFTR, also modulate the function of SLC26A3 (Greeley et al., 2001; Ko et al., 2002, 2004; Lamprecht et al., 2002; Rossmann et al., 2005). In addition, both CFTR and NHE3 are important regarding male fertility, as shown in the human disease cystic fibrosis (CF) and in knockout animal models (Anguiano et al., 1992; Zhou et al., 2001). This study was performed to assess the role of SLC26A3 in male fertility through its potential to interact with CFTR and NHE3 by analysing...
in detail the expression of these proteins in the human male reproductive tract.

Materials and methods

Patients and tissues

Testis biopsy specimens (n = 2), one of which contained several ED of the testis, were obtained during surgical extirpations of the large spermatoceles of two males with CLD (ages, 29 and 30 years). Both patients had a genetically confirmed diagnosis of CLD with the homozygous V317del genotype and had been shown to have abnormal sperm but normal testicular histology (patient numbers 4 and 5; Höglund et al., in press).

Control samples were archival specimens from adult men (range of age, 22–65 years) obtained from the Department of Pathology, HUSLAB, Helsinki University Hospital (Helsinki, Finland). The original reports of the pathologists were reviewed, and histologically healthy samples with normal spermatogenesis were used. The specimens included the testis and epididymis (n = 11) were from the patients orchitectomized for the following diagnoses: seminoma, cysta dermoides, cystadenoma, carcinoma embryonale and teratoma. The seminal vesicles (n = 9) and prostates (n = 4) were from the patients with radical prostatectomies because of focal prostatic carcinoma.

The study protocol was approved by the Institutional Review Board of the Hospital for Children and Adolescents, University of Helsinki.

Antibodies

Antiserum for SLC26A3, raised in rabbits against the synthetic peptide FNPSQEKGDKIDFT corresponding to nucleotides 2375–2416 of the published cDNA sequence (Gen bank number: L02785), was purchased from Research Genetics (Huntsville, AL, USA) and has been described previously (Haila et al., 2000). The antisera was diluted 1:350, and preimmune serum was used as a negative control in parallel sections.

A mouse monoclonal antibody against a synthetic peptide from the N-terminus of the human CFTR protein was purchased (CFTR Ab-2, MM13/4; NeoMarkers, Fremont, CA, USA) and was used at 1–2 μg/ml, and the IgG fraction of normal mouse serum (NMlgG; Labvision, Fremont, CA, USA) was used as a negative control in parallel sections.

A rabbit polyclonal, affinity-purified antibody for the C-terminal 22aa peptide of the rat NHE3 protein was purchased (NHE-31A; Alpha Diagnostics International, San Antonio, TX, USA) and used at 1 μg/ml, and the IgG fraction of normal rabbit serum (NRlgG; DAKO, Hamburg, Germany) was used as a negative control in parallel sections.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue blocks were cut into thin sections (~5 μm) using a microtome. The sections were mounted on glass slides and deparaffinized. Antigens were retrieved by boiling in a 10 mM citrate buffer (pH 7.0) for 10 min (NHE3), 15 min (SLC26A3) or 20 min (CFTR). Immunohistochemical staining was performed using EnVision kits (EnVision kit Rabbit K4010 for SLC26A3 and NHE3, and EnVision kit Mouse K4006 for CFTR; DakoCytomation, Carpinteria, CA, USA), according to the manufacturer’s instructions. The sections were incubated with the primary antibodies for 40 min (NHE3 and CFTR) and 60–80 min (SLC26A3) at room temperature or at 4°C overnight (SLC26A3). Diaminobenzidine solution (EnVision kit) was used for staining for 6–8 min, and Mayer’s hematoxylin (Merck, Darmstadt, Germany) was used for counterstaining. Finally, the slides were dried, and cover slips were placed on top.

The stages of spermatogenesis were analysed (Holstein et al., 2003), and the specimens of the ED and ductus epididymis were divided histologically into initial and terminal zones, according to the anatomical positions on the slides, and based on the cell morphology and location (Hermo and Robaire, 2002; Hess, 2002). In the seminal vesicles and prostates, higher glandular and lower unreactive ductal epithelium were histologically identified (Gartner and Hiatt, 1997).

Results

SLC26A3

In the testis, stage-specific labelling of the SLC26A3 protein was found in the head of the elongating spermatids (Sb-Sc) of stages III–VI but not in the fully developed spermatozoa (Figure 1). Immunoreactivity was localized at the cells’ periphery, seen first as a polarized labelling on the other border of the elongating spermatids (stage III), then as a hood in the head of the elongating spermatids (stage IV) and finally as a band surrounding the centre of the head of the spermatids (stages V and VI). There were no differences in the staining patterns between the patients with CLD and the controls (Figure 1).

In the initial zone of the ED, the luminal side of the non-ciliated cells showed immunoreactivity for SLC26A3, whereas the ciliated cells remained negative (Figure 2). In the terminal zone of the ED, no specific immunostaining for SLC26A3 was observed (data not shown). The ED of the patient with CLD was of the histologically proximal type and showed no labelling for SLC26A3 (Figure 2).

In the epididymides of the control patients, the luminal side of the apical mitochondria-rich cells (AMRC) showed specific expression of SLC26A3, whereas other cells remained negative (Figure 2). Both the high glandular epithelium and the lower unreactive ductal epithelium of the seminal vesicles of the controls showed intense apical immunoreactivity for SLC26A3 (Figure 2), whereas the prostates remained repetitively negative for SLC26A3 (data not shown).

CFTR

Labelling of CFTR was observed in the pachytyene spermatocytes of stages III–V and in the diplotene spermatocytes (stage VI), in which the signal was located around the cell nucleus, probably on the cell membrane (Figure 1). In the elongating spermatids (Sb), a cranially polarized staining (stage III) and a hood-like staining (stage IV) were observed similarly to SLC26A3. Later spermatids and the fully polarized staining (stage III) and a hood-like staining (stage IV) were observed similarly to SLC26A3. Later spermatids and the fully
developed spermatozoa remained negative for CFTR (Figure 1). There were no differences in the expression profiles of CFTR in the seminiferous tubules of the patients with CLD and the control subjects (Figure 1).

Similar to SLC26A3, immunoreactivity for CFTR was observed in the apical brush border of the non-ciliated cells of the initial zone of the ED in the control patients, but the staining was absent in the terminal zone (data not shown) and in the patient with CLD (Figure 2).

In the epididymides of the control individuals, the signal for CFTR was observed on the luminal side of the AMRC, similar to SLC26A3 (Figure 2). In the seminal vesicles of the controls, no staining was observed in the high glandular epithelium (data not shown), but strong labelling for CFTR was present, together with SLC26A3, on the luminal side of the lower ductal epithelium (Figure 2). In the prostates of the controls, the apical border of the epithelium of the prostate ducts showed intense immunoreactivity for CFTR (Figure 2), whereas the higher glandular epithelium of the prostate remained negative (data not shown).

NHE3

Staining for NHE3 remained negative in the seminiferous tubules, epididymides, seminal vesicles and prostates (data not shown). Instead, NHE3 showed immunoreactivity in the apical border of the non-ciliated cells of the initial zone of the ED, both in the controls and in the patient with CLD (Figure 2).

Discussion

The molecular basis of male infertility is heterogeneous and poorly understood. Recently, we demonstrated that a single gene disorder, CLD, is associated with both male subfertility and a tendency to form spermatoceles, suggested to be caused by the disruption of SLC26A3 at multiple sites of the male reproductive tract (Höglund et al., 2002, 2004). Lack of the functioning of SLC26A3 with secondary inhibition of NHE3 in the ED was proposed to be responsible for poor reabsorption of luminal fluid, leading to the dilution of sperm, maturation defects and spermatoceles (Höglund et al., in press). Furthermore, joint activities of SLC26A3 and CFTR, as shown in the pancreatic duct (Greeley et al., 2001), were thought to regulate the HCO3− secretion and the pH in the male reproductive tract as well. In this study, immunohistochemistry of SLC26A3, CFTR and NHE3 was performed in several human tissues of the male reproductive tract to further assess their role in male fertility.

Previous studies have characterized CFTR and NHE3 as important proteins for normal male fertility. Males with CF are infertile mainly because of the congenital bilateral absence of the vas deferens (CBAVD) (Anguiano et al., 1992), but poor sperm quality with low pH and abnormal morphology has also been observed, even among carrier males of CFTR mutations (Denning et al., 1968; Kaplan et al., 1968; van der Ven et al., 1996). NHE3 is not associated with any known human disease, but the disruption of its function in knockout mice (NHEKO) has been shown to lead to luminal accumulation of fluid and massively dilated ED associated with infertility (Zhou et al., 2001; Hess, 2003).

This study shows for the first time that SLC26A3 is immunolocalized in the elongating spermatids, non-ciliated cells of ED and AMRC of the ducts epididymis and confirms the previous report of expression of SLC26A3 in the epithelium of the seminal vesicle (Haila et al., 2000). Both SLC26A3 and CFTR were detected in the elongating spermatids, AMRC of the epididymis and ductal epithelium of the seminal vesicle, whereas SLC26A3, CFTR and NHE3 were all found in the non-ciliated cells of the epithelium of the ED. This implicates the potential of these proteins to interact and regulate the functions of each other. These regulatory properties are at least partially mediated through the interactive domain STAS (sulphate transporter anti-sigma factor antagonist) of SLC26A3 and through a C-terminal PDZ (PSD-95, Dlg1, ZO-1) interaction motif of SLC26A3 with the potential to target, cluster and regulate the function of several other proteins. Interaction between the R-domain of CFTR and the STAS-like domain of SLC26A3 stimulates the activity of both transporters, resulting in increased secretion of HCO3− and fluid from the epithelium (Ko et al., 2002, 2004), whereas interaction of the PDZ-interacting motif ETkF (single-letter amino acid code) in the C-terminus of the SLC26A3 protein is likely to mediate the joint action of SLC26A3 and NHE3 in the intestine through the adapter protein E3KARP (NHE3 protein kinase A regulatory protein), promoting the absorption of NaCl (Melvin et al., 1999; Lamprecht et al., 2002).

Based on the immunolocalization of SLC26A3, CFTR and NHE3 on the luminal side of the non-ciliated cells of the human ED and the known functions of the ED (Chulow et al., 1998; Hess, 2003), it is likely that SLC26A3 acts, in conjunction with CFTR, as a ductal HCO3− secretor and as an absorber of NaCl and fluid in conjunction with NHE3. Both these functions have previously been proposed in vitro for the pancreas (Greeley et al., 2001; Ko et al., 2002, 2004) and intestine (Melvin et al., 1999; Lamprecht et al., 2002), respectively, and in vivo for the intestinal phenotype for the loss of both Na+ and
SLC26A3 studies of the pathogenesis of male infertility. Furthermore, it would ties of the SLC26A3 protein will provide interesting prospects to further and motility. The normal testicular histology of the patients with CLD perhaps together with CFTR, may similarly affect sperm maturation SLC26A3 in the AMRC of the epididymis and in the seminal vesicle, which could result in the clinical phenotype with hypo-osmotic fluid tendency to form spermatoceles, and acidic Cl– rich seminal plasma, and are proposed to be secondary to CBAVD (Denning et al., 1968). There has been limited data available on the expression of cfr in the human testis (Tizzano et al., 1994; Larriba et al., 1998). In rats, a role for CFTR in sperm volume reduction has been proposed for its expression in the round and elongating spermatis (Gong et al., 2001). Whether SLC26A3 or CFTR has a primary role in the human spermatogenesis remains to be clarified.

In a single patient with CLD, we repetitively found evidence for lack of expression of both SLC26A3 and CFTR in the ED but concomitant presence of NHE3. Unfortunately, no other samples from patients with CLD were available for evaluation of the effects of the mutated SLC26A3 protein on the expression patterns of CFTR and NHE3, e.g. in the ductus epididymis and seminal vesicle. However, data based on this single patient suggested the role of CFTR in the pathogenesis of CLD and possibly, a converse role of SLC26A3 to that of CF. Both our patients had the Finnish founder mutation V317del, which is equivalent to the mutant V310del murine slc26a3. Expression and functional studies have shown V310del to remain in the endoplasmic reticulum (ER) with concomitant loss of all Cl⁻/HCO₃⁻ exchange capacity (Ko et al., 2002), whereas some epithelial and stronger intracytoplasmic (ER region) signal for the intestinal SLC26A3 mRNA and protein have been observed in human patients with the V317del mutation (Haila et al., 2000). Interestingly, the testicular tissues obtained from two patients with CLD showed normal expression of both SLC26A3 and CFTR.

We conclude that male subfertility in CLD is caused by the disruption of SLC26A3 in the ED, which probably results in the inhibition of NHE3 and the modulation of CFTR. This leads to poor reabsorption of water and NaCl, an aberrant secretion of HCO₃⁻, a further tendency to form spermatoceles, and acidic Cl⁻ rich seminal plasma, which could result in the clinical phenotype with hypo-osmotic fluid of the epididymal lumen and poor sperm motility. Disruption of SLC26A3 in the AMRC of the epididymis and in the seminal vesicle, perhaps together with CFTR, may similarly affect sperm maturation and motility. The normal testicular histology of the patients with CLD (Höglund et al., in press) is likely to make the utilization of artificial reproductive technologies to treat infertility possible.

CLD-associated male subfertility and the diverse functional properties of the SLC26A3 protein will provide interesting prospects to further studies of the pathogenesis of male infertility. Furthermore, it would be important to study males with CLD and other mutations of the SLC26A3 gene than V317del to search for possible phenotypic variants which have not been observed for the intestinal phenotype. More than 30 different mutations have been shown to be associated with the diarrhoeal component of CLD (Mäkelä et al., 2002), and it is fascinating to speculate whether milder forms of CLD or heterogeneous mutations of SLC26A3 could be manifested as only male subfertility similar to that observed for some CFTR mutations (van der Ven et al., 1996).

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SLC26A3, CFTR and NHE3 in male reproductive tract


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