Increased cystic fibrosis transmembrane conductance regulator (CFTR) expression in the human hydrosalpinx

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BACKGROUND: Hydrosalpinx (HSP), characterized by abnormal fluid accumulation in the Fallopian tube, is one of the main causes of infertility in women; however, the mechanism underlying the formation of hydrosalpinx fluid (HF) remains elusive. The present study investigated the possible involvement of cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-dependent chloride channel, in the pathogenesis of hydrosalpinx.

METHODS: Masson’s trichrome staining was used to characterize epithelial transformation in human HSP; RT–PCR, immunohistochemistry and immunofluorescence staining were used for CFTR expression and localization.

RESULTS: Masson’s trichrome staining showed areas of epithelial transformation, focally attenuated and pseudostratified. Immunostaining showed enhanced CFTR immunoreactivity in the focally attenuated and pseudostratified areas of HSP epithelium. RT–PCR revealed that CFTR expression in HSP was significantly greater than that in normal Fallopian tubes. CONCLUSIONS: These results indicate that HSP epithelium undergoes epithelial transformation with elevated CFTR expression, which may lead to increased transepithelial electrolyte and fluid secretion resulting in HF formation. The present findings may lead to the development of new treatment strategies for infertile patients with HSP.

Key words: CFTR/female reproductive tract/hydrosalpinx/hydrosalpinx fluid/infertility

Introduction

About 30% of infertile women seeking assisted reproductive treatments have hydrosalpinx (HSP) (Strandell et al., 1994; Blazar et al., 1997; Ng et al., 1997). Hydrosalpinx is detrimental to IVF outcome (Aboulghar et al., 1998; Strandell et al., 1998; Zeyneloglu et al., 1998; Camus et al., 1999). Hydrosalpinx fluid (HF) toxicity on mouse embryo development (Mukherjee et al., 1996; Bayler et al., 1997; Murray et al., 1997; Rawe et al., 1997; Sachdev et al., 1997; Koong et al., 1998; Spandorfer et al., 1999; Roberts et al., 1999; Carrasco et al., 2001; Arrighi et al., 2001) and human sperm (Ng et al., 2000) has also been documented. However, the molecular mechanism underlying the formation of HF is far from understood. In particular, the involvement of HSP epithelium has not been investigated although the secretory and absorptive activities of the epithelium have been considered to play a role (Ajonuma et al., 2002).

Movement of ions across the tubal epithelium is essential for the movement of fluid, which is not actively transported but moves in response to osmotic gradients largely established by the transport of ions across epithelium, particularly Cl− ions through ion channels such as the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-dependent Cl− channel located in the apical membrane and mainly responsible for Cl− secretion in many epithelia (Dickens and Leese, 1994; Quinton, 1999). CFTR has previously been shown to be expressed in the female reproductive tract of rodents (Rochwerger and Buchwald, 1993; Chan et al., 2002) and humans (Rochwerger and Buchwald, 1993; Tizzano et al., 1994). Previous studies from our laboratory have provided molecular and electrophysiological evidence of CFTR involvement in transepithelial fluid transport in the female reproductive tract of mice (Chan et al., 1999, 2002). Cyclic changes in uterine fluid volume have been explained by differential regulation of CFTR by ovarian hormones (Chan et al., 2002). The demonstration of cAMP-activated oviducal Cl− secretion in normal but not CFTR mutant mice (Leung et al., 1995) indicate a functional role of CFTR in oviductal secretory function. Therefore, it is likely that CFTR may play a crucial and important role in regulating fluid
balance in the Fallopian tubes and that abnormality of the tubal epithelium as in hydrosalpinx may lead to elevated CFTR expression, and thus HF formation. We undertook the present study to test this hypothesis by examining epithelial pathology and CFTR expression in the Fallopian tubes of infertile HSP patients seeking assisted reproduction treatments.

Materials and methods

Patients were recruited with ethical approval from the Ethics Committee of The Chinese University of Hong Kong. All patients gave written informed consent prior to their participation in this study.

HSP was obtained from six patients undergoing laparoscopic unilateral salpingectomy because of visible HSP shown on transvaginal scanning during IVF cycles of infertile women who had tubal factor infertility only. Normal Fallopian tubes (NFT) were obtained from three healthy women of reproductive age who agreed to have laparoscopic bilateral salpingectomy for tubal sterilization. Salpingectomy was done during mid–late proliferative stages (day 7–12) of their uterine cycles when CFTR expression is observed to be highest. Immediately after surgical removal, hydrosalpinges and NFT were collected into sterile tubes containing cold minimum essential medium (MEM; Life Technologies, Invitrogen, USA), placed on ice and transported to the laboratory.

Masson’s trichrome staining

HSP and NFT tissue pieces were fixed overnight in 4% paraformaldehyde (Fisher brand, Fisher Scientific, USA). Tissue samples were dehydrated in graded ethanol and embedded in paraffin wax. Sections were cut at 5 μm using a Reichert–Jung, Biocut Rotary Microtome 1130 (Germany), and dried unto Superfrost microscope slides (Fisher brand).

Slides were dewaxed in xylene and rehydrated in graded alcohol and stained in Celestine Blue for 30 min and washed, stained again in Mayer’s haematoxylin for another 30 min and washed in water. Slides were decolorized for a short time in acid alcohol and washed also in Scott’s tap water. Slides were then stained in 1% Xyline Ponceau in 0.5% HAC for 2 min and washed in water, and finally stained in 0.5% Fast Green for 20 s, blot dried, dehydrated in absolute alcohol, cleared and mounted using Permunt (Fisher Scientific Fair Lawn, USA). Observation was done under inverted microscope (Leica; DMR BE, Germany).

RNA extraction and RT–PCR

HSP and NFT were cut into small pieces after the removal of fatty tissues. Tissue pieces for RT–PCR were immediately snap-frozen in liquid nitrogen and later stored at −80°C until used.

RNA was isolated from HSP and NFT small pieces using TRIzol reagent (Gibco Invitrogen, USA). RT–PCR was performed and repeated three times using RNA obtained from small pieces of HSP and NFT. The specific oligonucleotide primers for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were: GTGGG-GGCGCCCCAGGCACCA (sense) and CTCCTTAATGTCACGCC-AGATTT (antisense), with expected cDNA of 515 base pairs (bp). The specific oligonucleotide primers for human CFTR were: AGCT-GGACCAGCCTTITTGAGAAA (sense) and CCACACGAAA-ATGTGCAAATGCAAGTCC (antisense), with expected cDNA of 554 bp. The conditions were: denaturation at 94°C for 45 s; annealing at 53°C, 58°C for 60 s; extension at 72°C for 60 s; 30, 33 cycles for GAPDH and CFTR respectively. Optimal amplification cycles are determined based on the linear relationship between the amount of PCR product detected and the number of amplification cycles. Due to the relatively small number of samples and in order to minimize unnecessary variables, RNA obtained from all the samples for each group were pooled together, reverse-transcribed and then used for PCR. The PCR products were analysed using 2% agarose gel electrophoresis stained with ethidium bromide and visualized in an UV-illuminated imager, Alpha Imager 2200 (Alpha Innotech Corporation, USA). The intensities of the bands of CFTR were normalized to that of GAPDH, which was amplified simultaneously and used as internal marker. Experiments in the absence of reverse transcriptase were conducted as negative control and experiments were repeated four times.

Niimmunohistochemistry

Tissue sections dried onto Superfrost microscope slides (Fisher brand) were deparaffinized in xylene and rehydrated in ethanol. Endogenous peroxidase activity was quenched using 3% hydrogen peroxide incubation for 30 min. The slides were then placed in a cooling jar with sodium citrate buffer (pH 6) for antigen retrieval for 5 min. After cooling, slides were rinsed in 1x phosphate-buffered saline (PBS) and incubated in normal blocking serum (Vectastain Elite ABC kit, Vector Laboratories, Inc., USA) for 30 min and washed with PBS. Slides were then incubated overnight with the primary antibody (CFTR, NeoMarkers, Lab Vision Corp., USA; 1:500 v/v in buffer) at 4°C overnight. Tissue sections were then washed with PBS and immunostaining was performed using a biotinylated secondary antibody, a horseradish peroxidase–H conjugate and a substrate–chromogen mixture (Vectastain Elite ABC reagent: ABC kit). Reaction was revealed by incubation with vasoactive intestinal peptide (VIP) substrate (Vector Laboratories, Inc., USA) for another 15 min, rinsed in water and counterstained with haematoxylin (Merck, Germany). Tissue sections were dehydrated in graded ethanol, mounted with Permunt (Fisher Scientific, USA) and observed under an Olympus epifluorescence microscope (Olympus IX-70, Japan). Intense purple color deposits indicated the site of positive immunostaining. The experiment was repeated three times and negative controls were applied for all tissue sections by using normal serum and omitting the primary antibody. Three people who never took part in slide preparations evaluated the slides individually. Interpersonal variability was <1%. CFTR immunostaining intensity values of NFT and HSP tissues were then analysed using Meta Morph image analysis software version 6.0 (Universal Imaging Corp., USA).

Immunofluorescence staining

Tissue sections dried onto Superfrost microscope slides (Fisher brand) were deparaffinized in xylene, rehydrated in graded ethanol and rinsed in tap water. Incubating slides in a solution of 0.3% hydrogen peroxide in water for 30 min quenched endogenous peroxidase activity. Then slides were washed in water and incubated in normal blocking serum (Vectastain Elite ABC kit) for another 30 min to block non-specific sites. Excess serum was removed from the slides by blotting and incubated overnight in primary antibody (CFTR1: 500 V/V in buffer, Neo Markers; Lab Vision Corp., USA) at 4°C overnight. Tissue sections were then washed with PBS and incubated for 30 min in a diluted biotinylated secondary antibody solution (Vectastain Elite ABC kit) for another 30 min to block non-specific sites. Excess serum was removed from the slides by blotting and incubated overnight in primary antibody (CFTR1: 500 V/V in buffer, Neo Markers; Lab Vision Corp., USA) at 4°C overnight. Tissue sections were washed for 5 min three times with PBS and incubated for 30 min in a diluted biotinylated secondary antibody solution (Vectastain Elite ABC kit). Tissue sections were again washed for 5 min three times in PBS and incubated for 20–30 min in FITC-conjugated anti-mouse antibody (Vector Laboratories, Inc.,) for 30 min. Slides were washed in PBS, mounted with glycerol and observed under an Olympus epifluorescence microscope (Olympus IX-70, Japan). Three people who never took part in the slide preparation also evaluated the slides. Intensity values of immunofluores-
cence staining of NFT and HSP tissues were also analysed using Meta Morph image analysis software version 6.0 (Universal Imaging Corp., USA).

**Statistical analysis**

Data presented are mean (SEM) for the ratio of CFTR/GAPDH expression intensities as analysed using 2% agarose gel electrophoresis. Differences between groups were assessed using Mann–Whitney U-test and analysis of variance (ANOVA) with Newman–Keuls multiple comparison test for immunohistochemical staining intensity and immunofluorescence staining intensity values. \( P \leq 0.05 \) was considered significant. Statistical analysis was done using GraphPad Prism (GraphPad Software Inc., USA).

**Results**

**Masson’s trichome staining**

Masson’s trichrome staining allows one to distinguish clearly between muscles, connective tissue and epithelium. Masson’s trichrome staining of HSP tissues showed extensive epithelial loss, areas of epithelial transformation (flattening of epithelial cells and cuboidal transformation), different degrees of focal attenuation (Figure 1a–c) and pseudostratification (Figure 1d) of HSP epithelium.

**Immunolocalization of CFTR in hydrosalpinx**

We further compared the immunolocalization of CFTR protein in HSP and NFT. Immunoreactive CFTR, obtained using CFTR antibody and a VIP substrate kit, was detected strongly in the focally attenuated and pseudostratified areas of HSP epithelium (Figure 2). In the healthy Fallopian tubes, CFTR was localized on the apical side of luminal epithelium only (Figure 2b), while in hydrosalpinx it was found in both apical and basolateral surfaces of the pseudostratified (Figure 2c, d) and focally attenuated (Figure 2e, f) epithelium. Staining intensity of HSP was significantly different from those of NFT and controls \( (P < 0.0001, \) Figure 2g). Immunofluorescence staining further confirmed enhanced CFTR expression and abnormal localization in HSP (Figure 3). Densitometric measurements showed that the immunoreactive intensity in HSP was significantly different from that in NFT \( (P < 0.0001, \) Figure 3e).

**CFTR expression by RT–PCR**

To further compare the expression of CFTR mRNA in HSP and NFT, semi-quantitative RT–PCR was performed using GAPDH (515 bp) as internal marker. The PCR products obtained were the expected molecular weight sizes of human CFTR (554 bp). CFTR expression, as shown by CFTR/GAPDH ratio, in HSP was significantly greater than that in NFT \( (P = 0.0022, \) Figure 4).

**Discussion**

We have previously proposed that abnormalities in the trans-epithelial ion transport across HSP epithelium may be one of the important factors in the pathophysiology of post-infectious HF formation (Ajonuma et al., 2001). The present study has indeed confirmed epithelial transformation and up-regulation of CFTR in HSP. Although extensive damage and loss of epithelial lining have been previously observed in HSP, areas of epithelial lining showing flattened and transformed epithelial cells have also been reported (David et al., 1969), consistent with our present observation. We have further demonstrated abnormality in CFTR expression and localization associated with the transformed epithelium in HSP. Instead of apical expression in the NFT, CFTR was predominantly found in the focally attenuated and pseudostratified areas of HSP epithelium. Elevated CFTR mRNA expression in HSP was also demonstrated by semi-quantitative RT–PCR. These data support the notion

![Figure 1. Masson’s trichrome staining of hydrosalpinx (HSP) tissues showing extensive epithelial loss, areas of epithelial transformation (flattening of epithelial cells and cuboidal transformation), different degrees of focal attenuation (arrow, A–C) and pseudostratification (arrows, D) of HSP epithelium. Magnification × 40, bar = 30 μm. L = luminal epithelium.](image-url)
that CFTR, as a Cl$^-$ channel, may play an important role in HF formation.

Ion channels are membrane proteins that act as gated pathways for the movement of ions across cell membranes. Fluid movements across secretory epithelia are secondary to ion movements, particularly Cl$^-$ . The importance of ion movements across epithelia lies in their coupling with the movement of water, which is not actively transported but moves in response to osmotic gradients established by the transport of ions (Leese, 1988). CFTR expression has been reported in the reproductive tissues of rodents (Rochwerger and Buchwald, 1993; Chan et al., 2002) and humans (Rochwerger and Buchwald, 1993; Tizzano et al., 1994); its regulation by ovarian hormones has been well documented (Rochwerger and Buchwald, 1993; Mularoni et al., 1995) and attributed to the cyclic changes in uterine fluid volume.

Figure 2. Immunohistochemical staining for cystic fibrosis transmembrane conductance regulator (CFTR) immunoreactivity on paraffin sections of normal Fallopian tubes (NFT) and hydrosalpinx (HSP). (A) Negative control, (B) apical localization of CFTR protein in NFT luminal epithelium (arrow). CFTR protein localization on the pseudostratified (C, D) and focally attenuated (E, F) HSP epithelium. Magnification × 40, bar = 30 μm. Staining intensity measurements were done using Meta Morph image analysis software version 6.0 (Universal Imaging Corp., USA) showed that the CFTR immunoactive intensity in HSP was significantly different from that of NFT and control (*P < 0.0001, Newman–Keuls, G). L = luminal epithelium; CONT. = negative control.
(Chan et al., 2002) since expression of CFTR is expected to lead to significant increase in the osmotic water permeability (Kunzelmann and Mall, 2002). Therefore, transformed epithelial cells with increased expression of CFTR in HSP, as observed in this study, may be one of the underlying mechanisms for abnormal fluid secretion and accumulation in the HSP lumen. Increased CFTR expression in HSP, significantly more than the expression in NFT during mid–late proliferative stage of the uterine cycle, when CFTR expression is maximal, is also suggestive of increased fluid secretion. The abnormal fluid accumulation in HSP may also result from the indirect effect of CFTR as a regulator of other epithelial channels and transporters, such as water channels, protein (aquaporin AQP) and epithelial sodium channels (ENaC). Up-regulation of water channel (Kunzelmann and Schreiber, 1997; Kunzelmann, 1999) and inhibition of ENaC function, and thus fluid reabsorption (Stutts et al., 1995) by CFTR could both lead to a net accumulation of fluid in HSP.

Although the reason for the increased expression of CFTR observed in HSP remains to be investigated further, one possible cause may be infection. Salpingitis and pelvic inflammatory disease (PID) precede HSP and our previous study demonstrated evidence of chronic ongoing low-grade infection in HSP (L.C.Ajonuma et al., unpublished data). It has been reported that bacterial infections up-regulate CFTR expression (Resta-Lenert and Barrett, 2002). Cytokines produced during infection, suggested to be contributory to the adverse effects of HF (David et al., 1969) such as interleukin 1 beta (IL-1β) are potent modulators of CFTR (Cafferata et al., 2000). During infection, bacteria binding may stimulate receptor signalling, leading to protein tyrosine phosphorylation, and finally increased CFTR expression. The significance of the presence of focally attenuated and pseudostratified epithelium in HSP is not clear at the moment. However, it has been reported that Chlamydial infection alters the transcription of host cell genes including those for cell differentiation, transcription factors and inhibition of apoptosis (Xia et al., 2003). Proliferating and dedifferentiating epithelial cells may show changes in their ion transport or channel expression. Recently, it has been demonstrated that enhanced cAMP-activated Cl− secretion in the hyperproliferative colonic mucosa was caused by elevated CFTR.

**Figure 3.** Immunofluorescence staining of hydrosalpinx (HSP) paraffin sections showing cystic fibrosis transmembrane conductance regulator (CFTR) protein localization on the pseudostratified and focally attenuated HSP epithelium (A–B); (C) apical localization of CFTR on normal Fallopian tubes (NFT) epithelium and D, negative control in which normal serum was used and CFTR antibody was omitted. Magnification × 20, bar = 30 μm. Densitometric measurements using Meta Morph image analysis software version 6.0 showed that CFTR immuoreactive intensity in HSP was significantly different from that in NFT (P < 0.0001, Figure 3E). L = luminal epithelium.
expression (Umar et al., 2000). The epithelial changes seen in this study, possibly induced by Chlamydial chronic infection, may have contributed at least in part to the increased CFTR expression. It has also been demonstrated that C. trachomatis infection results in increased tyrosine phosphorylation of several host proteins including those involved in signal transduction pathways (Bliska et al., 1993; Birkeland et al., 1994; Fawaz et al., 1997; Xia et al., 2003). In fact, our preliminary data showed that C. trachomatis inoculated into healthy Sprague–Dawley rat uteri induced uterine infection, massive uterine fluid accumulation (as in HSP) and increased CFTR mRNA expression (Ajonuma et al., 2004), supporting the notion that infection may lead to up-regulation of CFTR with concomitant changes in ion flux across the apical membrane including hypersecretion of chloride ions and inhibition of sodium reabsorption, together contributing to the net increase in electrolytes in the Fallopian tube accompanied with increased fluid accumulation producing HSP.

Accumulation of HF in the Fallopian tubes and its regurgitation into the uterine cavity may be a contributing factor for infertility observed in HSP patients, and impaired implantation or endometrium receptivity of transferred embryos during IVF (Mansour et al., 1991; Meyer et al., 1997). This is consistent with the present finding that CFTR is up-regulated in HSP. In fact, the observed improved IVF outcome in HSP patients pretreated with antibiotics (Sharara et al., 1996; Hurst et al., 2001) may be due to the return of CFTR expression back to the normal level when infection had subsided, leading to reduced HF reflux into the uterine cavity. Salpingectomy prior to IVF has been reported to be beneficial (Strandell et al., 1999, 2001; Johnson et al., 2002), and currently, salpingectomy for large hydrosalpinges prior to IVF is an accepted practice. However, most patients are reluctant to consent to this procedure, which is also reflected in the small sample numbers in our study. Whether this represents the general HSP, most or a particular HSP histological type remains to be evaluated further. If treatment with CFTR specific inhibitors in conjunction with antibiotics improves IVF and embryo transfer outcome in a clinical trial, it will certainly be a more attractive option to most patients than salpingectomy. Therefore, clinical applicability of CFTR-specific inhibitors with antibiotics as potential adjunct treatment for HSP should be evaluated.

In summary, the present study has demonstrated for the first time epithelial transformation with increased CFTR expression in human HSP, thus providing a possible molecular mechanism of HF formation in HSP and infertility. These findings may lead to the development of a new treatment strategy for tubal factor infertile patients with HSP.

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