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Original Article

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Immunohistochemical comparison of a case of inherited distal renal tubular acidosis (with a unique AE1 mutation) with an acquired case secondary to autoimmune disease

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Introduction

Distal renal tubular acidosis (dRTA) is a defect in urinary acidification characterized by an inability to acidify the urine to (conventionally) pH <5.5, even in the presence of systemic acidaemia; it is often associated with nephrocalcinosis, recurrent renal calculi and reduced bone mineral density. The underlying causes of this syndrome are several: it may be inherited or secondary to other conditions, such as autoimmune disease (notably Sjögren's syndrome), drug therapy or nephrocalcinosis itself [1].

Inherited dRTA can be recessive or autosomal dominant: the autosomal dominant form is invariably (and the recessive form occasionally) associated with mutations in the gene *SLC4A1*, which encodes for the membrane protein Anion Exchanger 1 (AE1). This transport protein in the kidney is expressed on the basolateral membrane of α-intercalated cells, where it plays a key role in linking apical H^+-ATPase-mediated proton secretion to basolateral bicarbonate efflux; ultimately controlling net urinary acidification and systemic acid–base balance. AE1 is highly expressed in erythrocytes and the kidney, and different mutations in this protein may cause red-cell defects (spherocytosis or Southeast Asian ovalocytosis) or dRTA. A number of dRTA-causing mutations of *SLC4A1* have now been described and characterized in several *in vitro* cell systems [2].

When dRTA-causing AE1 mutants are expressed in the non-polarized oocyte, they retain significant anion transport activity [2,3], in contrast to the poorly or non-functioning AE1 mutants associated with red-cell disorders. This finding led to the hypothesis that these mutations may cause a membrane trafficking, rather than a functional, defect in the AE1 protein. Recent *in vitro* studies seem to support this view, since various dRTA-associated AE1 mutants expressed in polarized renal epithelial cells in culture are either retained in the cytosol or mistargeted to the apical membrane [4–6]. However, little work has been possible on native human renal tissue, as only damaged kidney material from surgery or at post-mortem has been available, and a renal biopsy is not usually justified in the diagnosis or management of dRTA [7].

We have obtained tissue from a patient with a unique *SLC4A1* mutation, S613F [3] and tissue from another patient with autoimmune dRTA, probably as part of Sjögren's syndrome. In this report, we describe the clinical features of each patient and the immunohistochemical findings with antibodies directed against the AE1 protein or the vacuolar H^+-ATPase (vH^+-ATPase) proton pump.

Cases

Case 1, primary dRTA (S613F AE1 mutation)

A 70-year-old male patient underwent a native renal biopsy in February 2003. He had been referred aged 44 years with a 16-year history of recurrent nephrolithiasis. A plain abdominal X-ray at that time

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showed modest nephrocalcinosis and he was noted to have a plasma bicarbonate concentration in the low-normal range (~21 mmol/l). He had no proteinuria and his plasma creatinine concentration was 90 μmol/l. Distal RTA was suspected and he underwent a formal urinary acidification test: the standard short (8 h) oral ammonium chloride test, which was positive, confirming the diagnosis of type 1 dRTA.

Genetic screening as part of our earlier study of familial autosomal dominant dRTA showed that the patient and an affected daughter were heterozygous for a Ser to Phe substitution mutation at position 613 (S613F) in the *SLC4A1* gene encoding the anion exchanger AE1 [3]. His plasma creatinine concentration was 100 μmol/l in 1985 and deteriorated slowly to 139 μmol/l in 1995, when a ^{51}Cr-EDTA estimate of his glomerular filtration rate (GFR) was 46 ml/min/1.73 m^2. In 1991, he had been noted to have a faint IgG lambda paraprotein M band on protein electrophoresis, though too faint to quantify and not associated with any immune paresis or Bence–Jones proteinuria. He developed trace to 1+ dipstick proteinuria in 1997 (quantified at 0.23 g/l in a 24-h sample in 1999), which remained stable on dipstick testing thereafter. By 2002 his GFR had fallen to 32 ml/min/1.73 m^2, and together with his persistent and unexplained slight proteinuria in the context of a monoclonal gammopathy, it was decided that he should undergo an elective renal biopsy, to which he agreed. This was performed and two samples were obtained.

Case 2, autoimmune dRTA

A 42-year-old female presented with severe hypokalaemia (1.3 mmol/l), proximal muscle weakness, low-plasma bicarbonate concentration (18 mmol/l), and a spot urine pH of 7.3. Her plasma creatinine concentration was 57 μmol/l; urea, calcium, phosphate and magnesium levels were all normal. She was found to be ANA, anti-Ro/SSA and anti-La/SSB positive and antiphospholipid antibody positive. She gave a history of a *sicca* syndrome with dry mouth and gritty eyes for some months. Thus, the clinical features were of hypokalemic dRTA due to autoimmune disease, probably Sjögren's syndrome. Repeat of her autoimmune screen showed her to be ANA positive, ENA (SSA and SSB) positive, and to have a raised polyclonal IgG level (22.3 g/l) and borderline low C3 level; there was mild nephrocalcinosis on a plain abdominal X-ray. However, because of some proteinuria (<0.5 g/24 h) and a concern that she may have SLE with antiphospholipid syndrome, a renal biopsy was performed.

Methods

Sections (2 μm) were cut from archived wax-embedded human kidney tissue using a microtome, placed on negatively charged coated slides (X-tra Microslides^{TM}, Surgipath, US) and dried overnight at 37°C. (Control tissue was obtained from an unsuitable donor transplant kidney.) The sections were subsequently de-waxed with histoclear (National Diagnostics, Hessle, UK) and rehydrated in decreasing concentrations of ethanol. Slides were heated in 10 mM citrate buffer in a microwave oven for 10 min and allowed to cool for 1 h, then washed 3 times for 5 min with excess phosphate buffered saline.

Non-specific binding was blocked by incubation with 10% normal horse serum (NHS) (Invitrogen Ltd, Renfrew, UK) for 30 min. Subsequently, the sections were incubated overnight at room temperature with the primary antibodies diluted in 10% NHS. The primary antibodies used were monoclonal: Bric 155 against the AE1 C-terminus, Bric 170 against the AE1 N-terminus [8] and E11 against the E subunit of the vH$^+$-ATPase (a gift from S. Gluck, UCSF, USA). Rabbit anti-aquaporin 2 (a gift from D. Marples, University of Leeds, UK) in a dilution of 1/1000 was used to positively identify collecting duct principal cells [9].

The following day slides were washed 3 times for 5 min, then incubated for 1 h with secondary antibodies [fluorescein isothiocyanate (FITC)-coupled anti-mouse IgG (green colour) (Jackson Immunoresearch, Luton, UK)] and Cy3 labelled anti-rabbit IgG (Abcam, Cambridge, UK) to detect bound primary antibodies. The specificity of all primary antibodies has been demonstrated previously [8–10]. There was no specific staining when the primary antibodies were omitted. Slides were washed, mounted in Citifluor (Citifluor Ltd, London, UK) and examined using a Zeiss Axioplan immunofluorescence microscope and photographs were taken with a Leica DC200 digital camera (Zeiss, Göttingen, Germany).

Results

Light microscopy

Case 1. Sections showed renal cortex with 10 glomeruli in total, all of which appeared normal. There was some mild tubular atrophy with a patchy lymphocytic infiltrate. Scattered foci of calcification were seen. No significant immuno-deposition was detected.

Case 2. Eight glomeruli were seen: one was sclerosed, but the others were normal. The tubules looked normal, but there was a patchy increase in interstitial fibrosis. There was no significant immuno-deposition and no features of SLE.

Immunohistochemistry

Control tissue. In control tissue all antibodies directed against AE1 demonstrated basolateral staining of cells in the connecting tubule (CNT), cortical (CCD) and medullary collecting duct. These cells were negative for the principal cell specific marker aquaporin 2 (AQP2) and were therefore identified as intercalated cells (arrows in Figure 1A and B). Notably, the AE1 antibodies also stained red blood cells present in the tissue (in peritubular capillaries). The distribution of the vH$^+$-ATPase along the collecting system was examined using an antibody (E11) against the

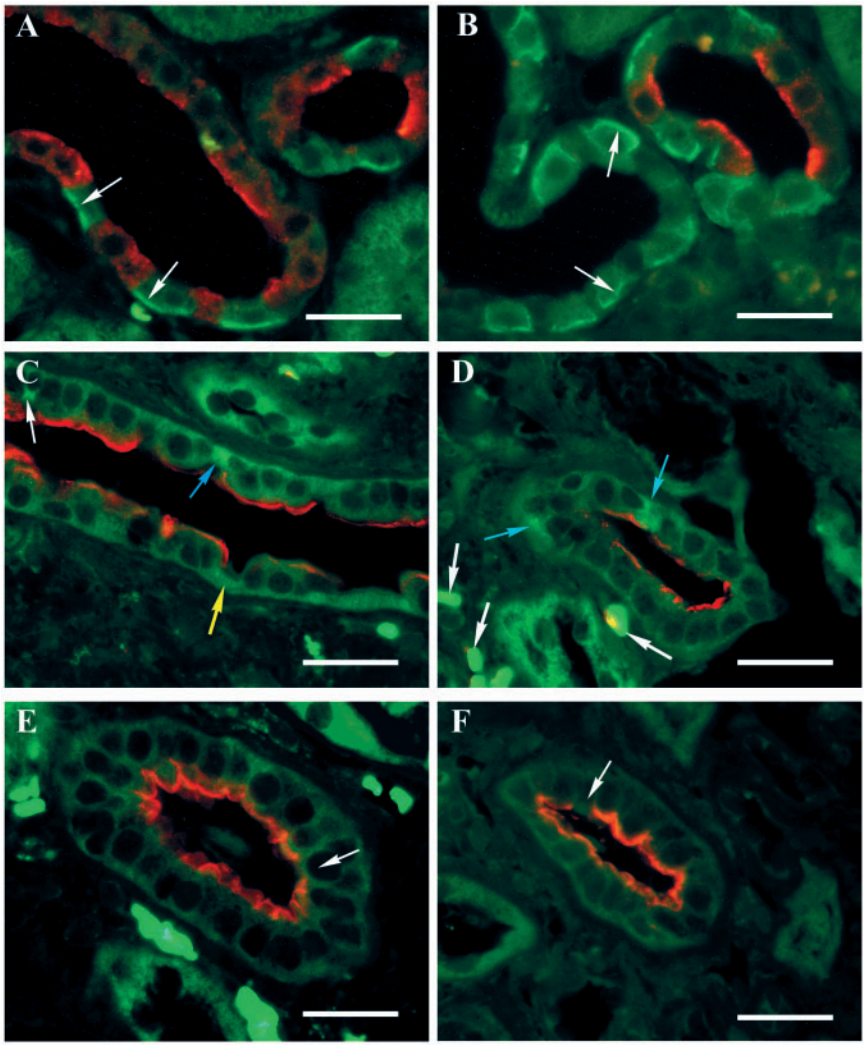

Fig. 1. In all photomicrographs AQP2 immunostaining (red) identifies principal cells of the collecting duct. All scale bars are 50 μm. (**A**) Control human kidney showing Bric 155 antibody immunostaining basolateral kAE1 in the cortical collecting duct. (**B**) Control human kidney showing Bric 170 antibody immunostaining basolateral kAE1 in the connecting segment. (**C**) S613F mutation patient Bric 155 antibody showing kAE1 either cytoplasmic (blue and white arrows) or absent (yellow arrow) in cortical collecting duct. (**D**) S613F mutation patient Bric 170 antibody showing cytoplasmic kAE1 (blue arrow) and also strong AE1 expression in coincident erythrocytes (white arrows) in cortical collecting duct. (**E**) Sjögren's patient Bric 155 antibody shows no kAE1 expression in α-intercalated cells. Again, bright imunofluorescence demonstrates normal AE1 expression on coincident erythrocytes. (**F**) Sjögren's patient Bric 170 antibody shows no kAE1 expression in α-intercalated cells.

31 kDa E subunit. Staining was predominantly apical and sub-apical in most intercalated cells in the CNT and CCD, and in all intercalated cells along the OMCD and IMCD (Figure 2A and B). Thus, in control tissue the majority of intercalated cells showed positive immunostaining for basolateral AE1 and apical and sub-apical vH^+-ATPase.

Case 1. Renal tissue from the patient with the S613F mutation (Figure 1C and D) showed an overall decrease in the intensity of AE1 immunoreactivity (compared with control) and marked cytoplasmic staining (arrows Figure 1C), but no clear basolateral localization. AE1 staining of red blood cells was also seen (white arrows Figure 1D). The S613F mutant tissue showed decreased staining and apparent cytosolic localization of vH^+-ATPase in type α-intercalated cells (identified as being negative for AQP2 staining), which appeared to be smaller in size and fewer in number when compared with control tissue (Figure 2D and E). Shayakul *et al.* [7] also described reduced expression of vH^+-ATPase in α-intercalated cells that were fewer in number.

Case 2. The renal tissue from the patient with probable Sjögren's syndrome showed complete absence of immunoreactivity for AE1 in intercalated cells (Figure 1E and F), whereas red cells in the tissue sections showed strong immunoreactivity (Figure 1E). The vH^+-ATPase staining of intercalated cells in

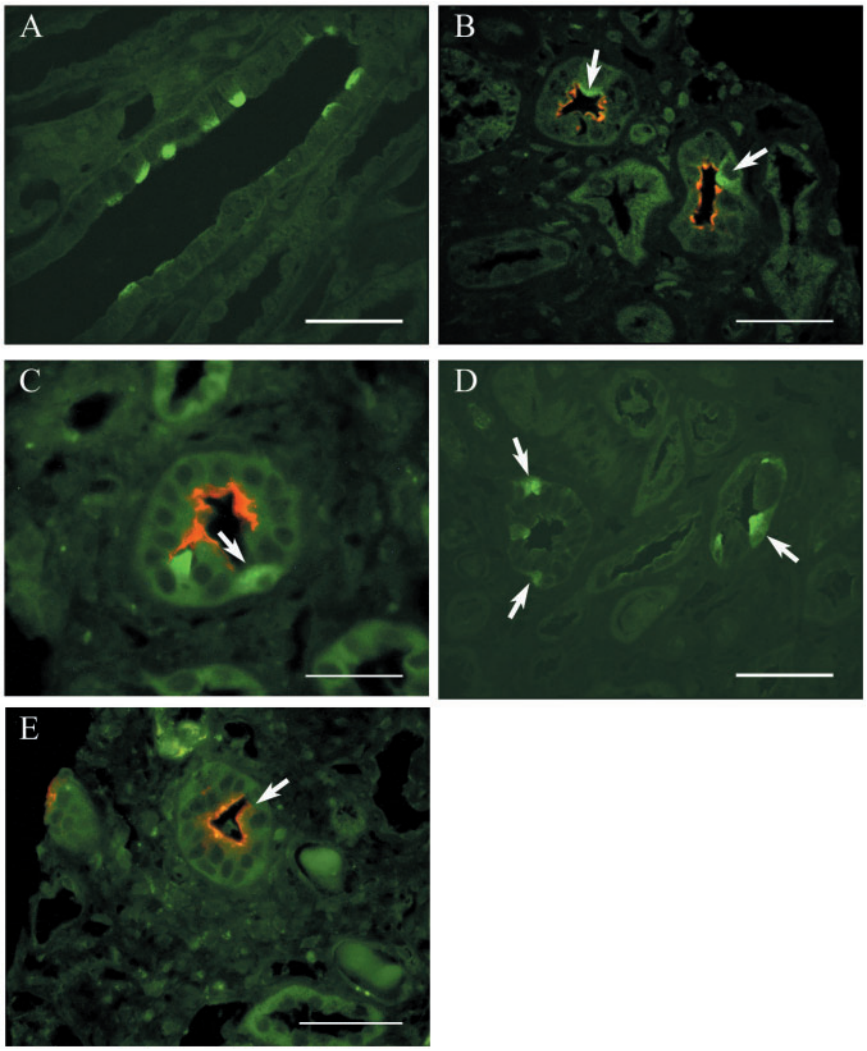

Fig. 2. In Figures 2**B**, **C** and **E**, red immunostaining with anti-AQP2 identifies principal cells in the collecting ducts. Scale bars are 50 μm. (**A**) Control human kidney showing apical immunostaining of vH^+-ATPase in α-intercalated cells in the cortical collecting duct. (**B**) Control human kidney showing apical immunostaining of vH^+-ATPase in α-intercalated cells in the cortical collecting duct. (**C**) S613F mutation patient vH^+-ATPase expression is cytoplasmic in the α-intercalated cells (arrows) in the cortical collecting duct. (**D**) S613F mutation patient vH^+-ATPase expression is cytoplasmic in the α-intercalated cells (arrows) in the cortical collecting duct. (**E**) Sjogren's patient vH^+-ATPase expression is absent (arrows).

the CCD was also negative, demonstrating loss of immunoreactivity for both kAE1 and vH^+-ATPase (Figure 2E), consistent with previous findings in renal tissue from patients with Sjögren's syndrome [7,11–13].

Discussion

The most commonly reported dRTA-causing AE1 mutations in Caucasians produce a substitution of arginine at residue 589 (R589H, R589S and R589C). The other reported dominant forms are S613F, A858D, G609D and two truncations at the C-terminus, R901X (Band 3 Walton) and a 23 amino-acid deletion [1]. These mutations have substantial transport activity when expressed in *Xenopus Laevis* oocytes [2]. They are expressed normally in the red cell membrane, in part because of the presence of glycophorin A, which can also increase their surface expression in oocytes [3]. These findings suggested that the kAE1 mutations do not cause a reduction of transport capacity, raising the possibility that the mutant protein is either mistargeted to the apical membrane, or retained in the cell, which is consistent with the results of *in vitro* expression studies in various cell models (Madin–Darby Canine Kidney cells and Human Embryonic Kidney-293 cells); moreover, coexpression of autosomal dominant dRTA mutants and wildtype (wt) kAE1 show retention of wt kAE1 in the cytoplasm, probably by hetero-oligomer formation and thus a dominant-negative effect [4,6].

Therefore, the cell model of autosomal dominant dRTA is one of functionally normal (or near normal) mutant kAE1 molecules forming hetero-oligomers with wt kAE1 and causing retention in the endoplasmic reticulum (ER), with perhaps in some cases apical mistargeting [4]. However, in native renal tissue the evidence for cytosolic retention or aberrant targeting in familial dRTA has been less clear. A recent report using an antibody against both AE1 and AE2 could not detect cell membrane-associated AE1 staining in vH^+-ATPase positive cortical collecting duct cells in native renal tissue from a patient with the R589H mutation [7]. The increased AE1 immunoreactivity that was seen did not correspond to cell boundaries and the authors concluded that this was an artefact capturing the peroxidase product in areas of nephrocalcinosis. Thus, information on AE1 targeting was uncertain because of the quality of the biopsy material: the renal tissue had chronic scarring from pyelonephritis, nephrocalcinosis and abscesses.

In contrast, the renal biopsy sample from our patient with inherited dRTA had no major tissue abnormalities and allowed a more detailed investigation of AE1 localization. In agreement with the *in vitro* data for the S613F mutation [6], we found cytosolic staining for AE1 in α-intercalated cells, but no clear basolateral or apical staining. Our data confirm that the S613F AE1 protein is retained intracellularly *in vivo* and that it seems to prevent wt AE1 from reaching the basolateral membrane. Furthermore, we also observed a reduction in apical vH^+-ATPase staining for the ubiquitous E subunit present in α-intercalated cells, whereas the weaker staining normally seen in the proximal tubule was unaffected. In addition, the α-intercalated cells seemed to be reduced in number and smaller in size. Interestingly, a decline in α-intercalated cell number has also been observed in rats chronically treated with acetazolamide and in the carbonic anhydrase 2 knockout mouse [14].

A Korean study [15] reported vH^+-ATPase and AE1 immunostaining in 11 patients with dRTA of differing aetiology (idiopathic, tubulointerstitial and autoimmune) and found that all dRTA renal biopsy specimens had reduced or absent immunoreactivity to vH^+-ATPase, and that approximately half had negative AE1 immunoreactivity. Unfortunately, no information was given about the aetiology in those patients with absent AE1 staining, and no family history or genetic information was available for those patients with 'idiopathic' dRTA.

As well as occurring as a primary genetic disorder, dRTA can also be secondary to other, often autoimmune, conditions like Sjögren's syndrome. Secondary dRTA in Sjögren's is common and human tissue has been examined previously in three published studies [11–13]. Cohen *et al.* [12] demonstrated a lack of vH^+-ATPase immunostaining in the cortical collecting ducts of renal biopsy tissue from a patient with Sjögren's and Defranco *et al.* [13] also found absent vH^+-ATPase and kAE1 immunostaining. These authors examined the serum of their patients and found no evidence of anti-H^+-ATPase or anti-intercalated cell antibodies, though Bastani and colleagues [11] in another report detected autoantibodies against vH^+-ATPase. These findings and ours suggest that different cellular events can underlie dRTA. In our patient with inherited dRTA, altered expression and location of AE1 and vH^+-ATPase was observed, perhaps indicating some form of 'coupling' between the two proteins in their intracellular regulation; whereas in the patient with autoimmune dRTA, neither protein could be detected. The aberrant localization and/or absence of these two major α-intercalated cell acid–base transporters explains the underlying acidification defect in both patients, and highlights the differences in their pathogenesis. However, there is still much to learn about the interrelationship between these key transporter proteins.

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Conflict of interest statement. None declared.

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