Absence of H⁺-ATPase in Cortical Collecting Tubules of a Patient with Sjogren's Syndrome and Distal Renal Tubular Acidosis¹,²

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ABSTRACT

Distal urinary acidification abnormalities may arise from transepithelial voltage defects, permeability defects, or proton-secretory defects, but tests to determine the cellular mechanisms underlying secretory abnormalities have not previously been reported. A patient with Sjogren's syndrome and distal renal tubular acidosis due to a secretory defect is described, whose kidney biopsy was examined by fluorescent immunocytochemistry with an antibody to the Mr 31,000 subunit of the mammalian kidney vacuolar H⁺-ATPase and was compared with normal human kidney. Staining with the anti-H⁺-ATPase antibody in normal human kidney was detected in the brush border microvilli and subvillar invaginations of the proximal tubule and in intercalated cells in the collecting duct. A biopsy sample from the patient was devoid of any anti-H⁺-ATPase staining in the intercalated cells. Staining was also absent from the proximal tubule brush border microvilli but was present in the subvillar invaginations. Although autoantibodies to normal human kidney membrane proteins were detected in the serum by immunoblot analysis, no immunocytochemical evidence for anti-intercalated cell autoantibodies was observed in the patient's serum. This report demonstrates that the basis for the proton secretory defect in some patients with distal renal tubular acidosis is likely the absence of H⁺-ATPase in the intercalated cells. It also illustrates the potential diagnostic utility of anti-H⁺-ATPase antibodies in the classification of distal renal tubular acidoses.

Key Words: Proton pump, vacuolar H⁺-ATPase, distal renal tubular acidosis, Sjogren's syndrome

The syndrome of distal (Type I) renal tubular acidosis (dRTA) is characterized by an inappropriately alkaline urine (pH > 5.5) in the setting of systemic acidemia (pH < 7.35). In this disorder, the net rate of hydrogen ion secretion in the kidney collecting duct is insufficient to regenerate the bicarbonate consumed by daily nonvolatile acid production. The final regulation of urinary acidification is accomplished largely by the intercalated cells, a subpopulation of collecting duct cells that are specialized for proton transport. Intercalated cells transport hydrogen ion by means of proton pumps, belonging to the vacuolar class of H⁺-ATPases, that are maintained in dense arrays on the plasma membrane in a polarized distribution (1,2).

Normal distal acidification requires a lumen-negative potential difference in the cortical collecting duct, an impermeant luminal membrane that is able to sustain large pH gradients, and an adequate rate of hydrogen ion secretion by the intercalated cells of the cortical and medullary collecting ducts (3,4). Several diagnostic tests that examine these components of distal nephron acidification have been designed in order to distinguish derangements in collecting duct voltage, proton permeability, or proton secretory rates leading to dRTA. Proton secretory defects are characterized physiologically by an abnormally low urine-blood Pco₂ difference during bicarbonate administration and a blunted rate of hydrogen ion secretion in response to the administration of Na₂CO₃ or furosemide (3,5). A primary abnormality in proton secretion could arise, in principle, from an absence...
of or a decrease in the number of intercalated cells, from a decrease in the quantity of H⁺-ATPase in intercalated cells, from an abnormally slow rate of enzymatic activity of the H⁺-ATPase, or from an abnormal distribution of the enzyme with reduced amounts on the luminal membrane.

In this report, we describe the use of a monoclonal antibody to kidney vacuolar H⁺-ATPase to analyze the amount and distribution of the proton pump in normal human kidney and in a renal biopsy sample from a patient with Sjögren’s syndrome and dRTA due to a proton secretory defect. Intercalated cells were present in the biopsy sample but, in contrast to the normal kidney, were devoid of any detectable staining for H⁺-ATPase, suggesting that the distal acidification defect was caused by a lack of proton pumps in these cells. The findings illustrate that the immunocytochemical staining of kidney biopsy samples with antibody to H⁺-ATPase may be a useful test in determining the underlying abnormality in dRTA.

CASE REPORT

A 37-yr-old black woman was referred in 1989 for the evaluation of hypokalemia and metabolic acidosis. She was well until 1982, when she experienced a limited episode of right carpal tunnel syndrome. From 1985 to 1990, she had intermittent neurologic symptoms, including dysphagia, dizziness, nausea, vomiting, right-sided monocular visual loss, left eye pain with decreased vision, right-sided headache, photophobia, and right-sided facial numbness. Her physical examinations during this time revealed relapsing and remitting neurologic defects that included: right-sided papilledema, hyperemic left optic disk, diplopia on far-left lateral gaze, left internuclear ophthalmoplegia, left facial droop, horizontal and vertical nystagmus, right-sided Marcus-Gunn pupil, left-sided hyperreflexia, slurred speech, and ataxic gait. She had a normal slit lamp examination on several occasions. She was initially given a diagnosis of multiple sclerosis and received short courses of prednisone at the times of neurologic exacerbation, with subjective improvement. Her laboratory tests over this 6-yr period showed an erythrocyte sedimentation rate of 23 to 50 mm/h, positive serum antinuclear antibodies with a titer of 1:80 and a speckled pattern, positive rheumatoid factor with a titer of 1:160 to 1:320, positive anti-Ro antibodies with a titer of 1:8, negative anti-La antibodies, negative antidual-stranded DNA antibodies, negative antisingle-stranded DNA antibodies, negative anti-Smith antibodies, negative cryoglobulins, negative lupus anticoagulant screen, and negative serum and urine protein electrophoresis and immunoelectrophoresis. Repeated cerebrospinal fluid studies were significant for two red blood cells/mm³; 40 to 88 white blood cells/mm³, of which 90 to 95% were lymphocytes and 4 to 5% were monocytes; protein of 0.24 to 0.39 g/L; glucose of 3.3 to 4.6 mmol/L; albumin of 0.17 to 0.25 g/L; and immunoglobulin G (IgG) of 0.05 to 0.08 g/L with a serum albumin of 40 to 48 g/L, a serum IgG of 18 to 22 g/L, and a cerebrospinal fluid IgG index of 0.74. Tests for cerebrospinal fluid myelin basic protein, oligoclonal bands, and VDRL were repeatedly normal. She had normal esophageal and chest roentgenograms. Repeated magnetic resonance imaging scans showed moderate cerebellar atrophy and isolated regions of high-signal intensity without mass effect adjacent to the fourth ventricle and in the medial aspect of right temporal lobe. She had normal four-vessel cerebral angiography.

In 1985, the serum electrolytes were: sodium, 130 mmol/L; potassium, 3.7 mmol/L; chloride, 100 mmol/L; and bicarbonate, 32 mmol/L. The serum pH ranged from 5.0 to 6.0. Subsequent measurements through 1987 showed a gradual decline in serum bicarbonate to 23 mmol/L with a urine pH of 6.5 and a gradual increase in serum creatinine from 53 to 97 μmol/L. In 1988, the patient was hospitalized with profound weakness and hypokalemia. At the time of admission, the serum sodium was 145 mmol/L, potassium was 1.6 mmol/L, chloride was 115 mmol/L, bicarbonate was 15 mmol/L, urea was 8.5 mmol/L, creatinine was 141 mmol/L, calcium was 2.46 mmol/L, phosphate was 0.65 mmol/L, and magnesium was 1.0 mmol/L. The admission urinalysis showed a pH of 7.0, specific gravity of 1.010, and 1+ protein. The urine sodium was 53 mmol/L, potassium was 10.7 mmol/L, chloride was 51 mmol/L, and creatinine was 3,898 μmol/L. Serum and urine cortisol levels were normal. The serum uric acid, calcium, and phosphate concentrations were repeatedly normal. There was no glycosuria or aminoaciduria, and the fractional excretion of phosphate ranged from 13 to 31%. The urine potassium excretion (U_k /V/GFR) was 0.89 to 1.16 mmol/L. A diagnosis of dRTA was made. Supplementation with potassium chloride and sodium-potassium citrate was begun, and the serum bicarbonate level increased to 20 mmol/L. Abdominal roentgenograms and renal ultrasound examinations showed no nephrocalcinosis. The serum creatinine level increased to 189 μmol/L, with 497 mg of protein in a 24-h urine sample. A left-sided percutaneous kidney biopsy was performed under ultrasound guidance. Routine histologic evaluation showed perivascular and periglomerular interstitial lymphocytic and plasmacytic infiltration without abnormalities of blood vessels. The immunofluorescence was negative. An electron microscopic examination showed lymphocytic infiltration between tubular epithelial cells associated with the focal degeneration of tubular epithelium and the accumulation of lysosomes within tubular epithelial cells.
On questioning, the patient recalled that a dry mouth had been present since the beginning of her neurologic illness. A Schirmer's test and rose bengal stain study were consistent with sicca syndrome. A lip biopsy was then performed and showed extensive interstitial lymphocytic infiltrates, nodular aggregates of lymphocytes in the glandular tissue, and focal lymphocytic infiltration of the salivary ducts, confirming the diagnosis of Sjogren's syndrome.

METHODS

Anti-H*-ATPase Immunocytochemistry

Tissue specimens from the patient and normal control human kidney were preserved in B5 fixative and embedded in paraffin, and 4-μm sections were cut. Sections were passed sequentially through incubations in xylene, decreasing concentrations of ethanol, Lugol's iodine solution, sodium thiosulfate, and phosphate-buffered saline (PBS). The sections were incubated in 20% calf serum–1% polyethylene glycol (Mw of 20,000) in PBS to block nonspecific binding (blocking solution 1). Sections were then incubated for 2 h with undiluted supernatant from hybridoma E11, a monoclonal antibody to the M, 31,000 subunit of the vacuolar H*-ATPase (6), and washed with PBS. Bound antibody was detected with fluorescein isothiocyanate–labeled goat anti-mouse IgG diluted 1:50 in blocking solution 1. The sections were mounted in a fresh mixture of 2 mg/mL of para-phenylene diamine in 50% glycerol (vol/vol) in PBS. The slides were viewed with an Axioptot microscope (Carl Zeiss, Thornwood, NY) with epifluorescent optics.

To test for the presence of endogenous anti-intercalated cell antibodies in the patient's serum, sections from the patient and normal control kidneys were passed serially through xylene, decreasing concentrations of ethanol, Lugol's iodine solution, sodium thiosulfate, and PBS. Sections were incubated first in 20% goat serum in PBS (blocking solution 2) to block nonspecific binding and then for 3 h in the patient's serum diluted 1:25 (vol/vol) in PBS. The sections were washed in PBS and incubated in blocking solution 2 for 30 min. Fluorescein isothiocyanate–labeled goat anti-human IgG, preabsorbed overnight at 4°C with a homogenate of normal human kidney, was diluted 1:50 in blocking solution 2 and applied to the sections for 20 min. The sections were washed and mounted as described above for immunofluorescence microscopy.

Immunoblot Analysis

Affinity-purified bovine kidney H*-ATPase was prepared with a monoclonal antibody affinity column as previously described (7). Normal human kidney microsomes were prepared by methods identical to those for the preparation of bovine kidney microsomes (7). Proteins were separated by electrophoresis on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel (either 50 μg per lane of human kidney microsomal protein or 20 μg per lane of affinity purified bovine kidney H*-ATPase) and transferred to nitrocellulose membranes as described previously (6,7). All subsequent steps in immunoblotting were performed at 23°C. The nitrocellulose membrane was stained in 1% naphthol blue black (amido black) in 45% methanol and 10% acetic acid solution for 1 min and was destained in the same solution without dye to reveal the transferred polypeptide bands. The membrane was cut into strips, incubated in 5% nonfat dry milk, 0.2% Tween-20, 5 mM sodium azide in PBS (blocking solution 3) for 1 h, then incubated for 2 h either in patient or normal human serum diluted 1:10 in blocking solution 3, in rabbit polyclonal anti-H*-ATPase antiserum (2) diluted 1:100 in blocking solution 3, or in undiluted culture supernatant from hybridoma E11 (6). The strips were then washed in PBS, incubated in the appropriate alkaline phosphatase-labeled anti-IgG (either anti-human, anti-rabbit, or anti-mouse) diluted 1:200 in blocking solution 3, washed in PBS, washed in substrate buffer (100 mM 2-amino-2-methyl-1-propanediol, 1 mM MgSO4, 1 mM levamisol; pH 9.6), and finally incubated with substrate solution (0.012 μM nitro blue tetrazolium, 0.0224 μM 5-bromo-4-chloro-3-indolyl phosphate in substrate buffer) to reveal membrane-bound antibody.

Physiologic Assessment of Urinary Acidification

Tests of urinary acidification were performed as described by Batlle et al. (5,8,9). These studies were performed with the approval of the Human Research Review Committee of the Medical College of Wisconsin.

RESULTS

The capacity of the patient to acidify the urine was tested after the oral administration of 6 g of ammonium chloride per day for 3 days. The lowest urine pH was 6.5, when the venous blood pH was 7.18. A simultaneous spot urine sodium was 68 mmol/L, urine potassium was 27 mmol/L, and urine chloride was 79 mmol/L; the urine anion gap was +16 mmol/L.

With IV bicarbonate loading, the fractional excretion of bicarbonate ranged from 0.7 to 1.5%. The maximal urine minus blood Pco2 difference was 9.8 mm Hg; the highest urine pH was 7.25 during this test, and at that time, the urine bicarbonate was 20.5 mmol/L, the urine Pco2 was 47.2 mm Hg, and the urine minus blood Pco2 difference was 7.6 mm Hg. With oral furosemide, the urine pH dropped from 7.0
to 6.4, consistent with a diagnosis of dRTA due to a proton secretory defect. During this test, the baseline ammonium excretion was 4.3 µEq/min and increased to 25.0 µEq/min after furosemide administration.

In prior studies with polyclonal antibodies (2,10) and monoclonal antibody E11 (6) to the kidney vacuolar H⁺-ATPase, we described the distribution of the proton pump in rat kidney. H⁺-ATPase staining was observed in the invaginations at the base of the microvilli (subvillar invaginations) in all proximal tubule segments, and prominent H⁺-ATPase staining was also present in the microvilli of the S1 and S2 segments. Intense staining of the intercalated cells was observed in both the cortical and the medullary collecting tubules. In the intercalated cells of the cortical collecting tubule, several morphologic patterns of H⁺-ATPase distribution were observed (6,10,11).

Immunocytochemical staining with the anti-H⁺-ATPase monoclonal antibody E11 was performed on both normal human kidney and the patient’s renal biopsy specimen, and the staining patterns were compared. The immunocytochemical distribution of H⁺-ATPase in two normal control human kidneys showed a pattern in the proximal tubules and cortical collecting tubules similar to that described in normal rat kidney (2,6) (Figure 1A). Prominent H⁺-ATPase staining of brush border microvilli and the subvillar invaginations was observed in all of the proximal convoluted tubules seen in the cortex. In the cortical collecting tubules, the intercalated cells stained intensely, comprising 42% of the cells, a value identical to that observed in the rat (2,6). The remaining cells, the principal cells, had no detectable staining for H⁺-ATPase. The intercalated cells in the normal human kidney cortical collecting tubule exhibited patterns of staining for H⁺-ATPase that were different from those described previously in rat kidney (6): well-polarized apically stained cells (WPA; stain limited to the apical region of the cell), poorly polarized apically stained cells (PPA; staining accentuated in the apical region although extending beyond the nucleus), cells with simultaneously distinct basolateral and apical pole staining (BA), poorly polarized basolaterally stained cells (PPB; staining accentuated in the basolateral region and extending beyond the nucleus), and cells with diffuse homogenous staining (D). The relative frequencies of the different subtypes of the intercalated cells in the cortical collecting tubules of the normal human kidney were: 13% WPA, 69% PPA, 3% PPB, 4% BA, and 11% D cells. No well-polarized basolaterally stained cells were found in the two specimens studied.

The staining for H⁺-ATPase in the patient’s biopsy sample differed greatly from that in the normal kidney. The most striking finding in the patient’s biopsy was the absence of any H⁺-ATPase staining in the cortical collecting tubules (Figure 1B). No intercalated cell H⁺-ATPase staining was apparent in the entire specimen. The brush border microvilli also lacked any staining, although bright staining was observed at the base of the microvilli, likely representing the subvillar invaginations. Hematoxylin and eosin-stained sections of the biopsy specimen (Figure 2) revealed 10 glomeruli, many proximal tubules, and several distal tubules. A diffuse interstitial infiltrate consisting of lymphocytes and plasma cells was present, and lymphocytes infiltrating the tubular epithelial cells were observed occasionally, diagnostic of a tubulitis.

The failure to detect H⁺-ATPase staining could indicate either that intercalated cells were absent from the collecting ducts or that intercalated cells were...
Proton Pump Deficiency in Distal RTA

Figure 2. Hematoxylin and eosin-stained section of the patient's biopsy. Tubules likely representing collecting tubules are indicated by arrows. Heavy mononuclear cell infiltration of the interstitium and invasion of the tubules are present. Magnification, ×400.

Figure 3. Electron micrograph of a collecting tubule from the patient's biopsy. Intercalated cells exhibiting the characteristic features of numerous mitochondria, dark cytoplasm, prominent tubulovesicular compartment, and numerous apical plasma membrane microprojections are present. L, lumen; IC, intercalated cell; PC, principal cell. Magnification, ×7,100.
not expressing immunocytochemically detectable H⁺-ATPase.

The patient's serum was analyzed by immunoblotting for antibodies either to human kidney membrane antigens or to the mammalian vacuolar H⁺-ATPase (Figure 4). The serum was incubated first with nitrocellulose strips containing polypeptides from normal human kidney microsomal membranes separated by electrophoresis (Figure 4A). Alkaline phosphatase–labeled goat anti-human immunoglobulin was then used to detect any antibodies from the patient's serum that were bound to the membrane strips. Two faint polypeptide bands at Mr values of 50,000 and 28,000 were detected. Antibody binding to these polypeptides was not present when strips with the same kidney proteins were incubated with normal human serum. A second immunoblot analysis was performed to determine if autoantibodies to the mammalian vacuolar H⁺-ATPase were present in the patient's serum (Figure 4B). The serum was incubated with strips of nitrocellulose containing electrophoretically separated polypeptides of the purified bovine kidney vacuolar H⁺-ATPase, and antibody binding was detected with a second, alkaline phosphatase–labeled antibody as described above. No anti–H⁺-ATPase antibodies were detected in the patient's serum. As a control, the Mr 31,000 subunit of the H⁺-ATPase was detected with alkaline phosphatase–labeled goat anti-mouse immunoglobulin.

Because one previous report has implicated anti-intercalated cell antibodies as a cause of the acidification defect in patients with autoimmune diseases (13), we performed an analysis for anti-intercalated cell antibodies in our patient's serum. Patient and control kidney tissue samples were incubated with the patient's serum and then with fluorescein isothiocyanate–labeled goat anti-human IgG to detect any bound human antibody. No staining was observed in any part of the kidney in either specimen (data not shown). Hence, no immunocytochemically detectable autoantibodies against proximal tubule or collecting duct cells were present in the patient's serum.

**DISCUSSION**

Györy in 1968 (14) and Halperin et al. in 1974 (3) postulated that an abnormally low rate of hydrogen ion secretion by the collecting duct could produce dRTA. battle later provided additional physiologic evidence for a proton secretory defect in five patients with dRTA (5). From these and other studies, it is clear that proton secretory defects comprise one subset of patients with dRTA but the cellular basis for such defects has remained obscure.

The patient's acidification abnormality fulfilled the clinical criteria for classic dRTA. The urine was inappropriately alkaline, and the measured urinary cations exceeded the measured urinary anions (denoting a positive urinary anion gap) during spontaneous systemic acidosis and after ammonium chloride administration. This suggested that the patient had a low urinary ammonium excretion, which is characteristic of distal tubular proton secretory defects (15,16) and which was confirmed by a direct measurement of the patient's ammonium excretion rate. Proximal tubular function appeared intact, as evidenced by the lack of aminoaciduria, glycosuria, or elevated fractional excretion of bicarbonate. The physiologic studies indicated that the patient's dRTA was the result of a proton secretory defect. The urine-blood Pco₂ difference was abnormally low during bicarbonate administration, the urine pH changed...
The significance of these kidney autoantibodies is distal nephron may be a cause of acidification defects in some patients (21), but there are currently no available data on patients with dRTA to determine whether such a mechanism has a role in clinical disease.

The patient's clinical course fulfills all but one of the criteria for Sjögren's syndrome (22), which is an immunologic disorder involving the lacrimal and salivary glands, the central nervous system, and the kidney. Dry eyes and mouth are the most common clinical manifestations, but dRTA and neurologic symptoms have been reported in a number of patients (23–25). Although the patient's neurologic illness closely mimicked multiple sclerosis, the coincident xerostomia, xerophthalmia, a typical lip biopsy, and RTA are most consistent with primary Sjögren's syndrome and neither serum anti-Ro antibodies nor RTA have been reported in multiple sclerosis.

Increasing evidence suggests that Sjögren's syndrome is a systemic immunologic disorder. Humoral autoimmunity may be involved in the pathogenesis of the disease. Salivary gland immunoglobulin and rheumatoid factor synthesis and intrathecal IgG synthesis have been observed in some patients (25,26). Serum samples from a substantial group of patients with primary Sjögren's syndrome contain free monoclonal lambda light chains, pointing to a generalized abnormality in immunoglobulin production (27). The infiltration of salivary glands (28) and kidney (29,30) by T-lymphocytes supports a role for cellular autoimmunity. The interstitial nephritis in Sjögren's syndrome is characterized by an infiltration of predominantly T4 (helper/inducer) lymphocytes between tubular epithelial cells (29,30). The infiltration of the renal interstitium with predominantly T4 cells is also observed in renal transplant rejection (31), another disorder frequently associated with distal acidification defects (32). In contrast, T8 (cytotoxic/suppressor) lymphocytes appear to predominate in the interstitial nephritis associated with nonsteroidal anti-inflammatory agents (33) and urinary acidification defects have not been described in that disorder.

The selective defect in acidification and the absence of H*-ATPase in the intercalated cells in the cortical collecting tubules of our patient suggest that the immunologic reaction in the kidney may be directed specifically at intercalated cells in Sjögren's syndrome with dRTA. Future investigations on how the immune system might suppress the expression of the H*-ATPase in the kidney may lead to new therapeutic strategies for this disorder.

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