Immunocytochemistry of Band 3 Protein in Kidney and Other Tissues of Control and Cystic Fibrosis Patients

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ABSTRACT. Current evidence indicates that the underlying genetic defect in cystic fibrosis (CF) results in defective chloride transport, and more specifically, chloride impermeability. However, recent consideration has been given to a possible defect in band 3 protein at CF-affected sites. In an effort to determine a possible role for band 3, an anion exchange channel protein, a series of immunocytochemical localizations was performed. Immunocytochemical staining for the anion channel band 3, 43K, in normal and CF human kidney was confined to erythrocytes, glomerular podocytes, the basal region of half of the cells of the initial connecting segment of cortical collecting ducts, and a minority of cells in medullary ray-collecting ducts. Erythrocytes alone evidenced immunoreactivity for band 3 protein in human pancreas, submandibular gland, trachea, and lung. In all cases, specimens from patients with CF stained like those from control subjects for band 3. Abnormal Cl^- transport in CF tissues is not reflected in altered band 3, 43K, localizations. (*Pediatr Res* 21: 235-237, 1987)

Abbreviations

CF, cystic fibrosis
PBS, phosphate-buffered saline
SDS, sodium dodecyl sulfate
SDS-PAGE, SDS-polyacrylamide gel electrophoresis

Stemming from the diagnostically elevated Na^+ and Cl^- in CF sweat (1), the perception has emerged of deficient ion transport (2-5) and more specifically altered Cl^- permeability (3, 5) as the underlying genetic defect in this disease. Altered chloride transport in turn has prompted the consideration of the presence and/or abnormality of a band 3-mediated anion exchange in CF-affected tissues. In erythrocytes the band 3 anion channel facilitates rapid $\text{Cl}^-/\text{HCO}_3^-$ exchange where it makes up 25% of the total membrane protein of these cells (6). An effort was undertaken accordingly to localize band 3 protein immunocytochemically in sites of active ion transport with the aim of first determining whether sites affected in CF correspond with sites immunoreactive for band 3 protein and, second, comparing band 3-positive sites in normal and CF specimens.

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MATERIALS AND METHODS

Band 3 cytoplasmic domain (43K) purification. Band 3 cytoplasmic domain (43K) was isolated from whole human blood. After spectrin extraction (7) and extraction of ankyrin and protein 4.1 (8, 9), the cytoplasmic domain of band 3 was prepared from the high salt-extracted vesicles as described (10) by cleavage with α chymotrypsin, followed by chromatography on DEAE-cellulose (Watman, Clifton, NJ) and gel filtration on ultragel AcA 44 resin (LKB, Rockville, MD). Materials prepared by this method are referred to as 43K peptides, although such preparations appear more heterogeneous on SDS gels. Immunologic criteria described previously (10) demonstrated that the smaller peptides were derived from the dominant 43K product. In all purifications, fractions were analyzed by SDS-PAGE on 10% gels (11) and purified pools were concentrated by vacuum dialysis against 130 mM KCl, 20 mM NaCl, 10 mM Tris-HCl, pH 7.5, with 1 mM EDTA and 1 mM NaN_3 . Protein concentrations were determined by the methods of Lowry *et al.* (12) using bovine serum albumin standards. This method has been determined by amino acid analysis to reflect accurately the true protein concentration (13).

Antisera preparations. Sheep antiband 3 was generously provided by Dr. Vincent Marchesi and was prepared as described (14). This antiserum was prepared against the whole band 3 protein and was shown to react primarily with the cytoplasmic domain that is referred to as 43K. Rabbit anti-43K was prepared by immunizing a 7-kg New Zealand White female rabbit with 100 μg of 43K divided among sites in each hindquarter and each shoulder. For primary immunization the protein was diluted to 200 g/ml in PBS, then diluted 1:1 in complete Freund's adjuvant (Difco, Detroit, MI) which was mixed until a stable emulsion was formed. After intramuscular administration, the animal was boosted at 2 wk with 100 μg of antigen in incomplete Freund's adjuvant. Two weeks after boosting, the animal was bled from the ear veins, and the serum was tested for activity by an enzyme-linked immunosorbent assay using horseradish peroxidase-conjugated protein A. For subsequent bleedings, the rabbit was boosted as described, then bled 2 wk later.

Affinity purification of anti-43K antibodies. Rabbit anti-43K antibodies and sheep antiband 3 antibodies were affinity purified by passing the antisera through a 43K-Sepharose column as described (13). Briefly, 43K was conjugated to Sepharose CL-4B (Pharmacia, Piscataway, NJ) activated with CNBr (15). The column was washed with 300 mM NaCl, 100 mM Tris-HCl, 1 mM NaN_3 at pH 7.4, and eluted with the same buffer containing 6 M urea. The eluted antibody was concentrated by vacuum dialysis against PBS; some samples were lyophilized after concentration. Different lots of 43K were used to prepare the affinity column, to immunize, and to perform the inhibitions with antigen.

Tissue preparation. Human tissues were obtained from autopsies performed less than 6 h postmortem. Surgical specimens were also utilized and consisted of either normal tissues adjacent to tumors or organs prepared, but not used, for transplantation. The acquisition and use of human tissue was approved by the Internal Review Board for Human Research of the Medical University of South Carolina. Specimens of pancreas, submandibular gland, kidney, trachea, and lung were obtained from six CF patients and seven non-CF controls. Tissues were fixed 24 h at 4°C in Carnoy fixative and processed routinely into paraffin. Preliminary studies indicated that Carnoy fixative was superior to other routine fixatives in preserving the immunoreactivity of band 3 protein in human tissues.

Immunocytochemistry. Sections from all specimens were rehydrated and treated with 3% hydrogen peroxide to eliminate endogenous peroxidase activity prior to overnight exposure at 4°C to the band 3 antibody diluted over a range of 20 to 200 µg/ml to achieve an optimal concentration. Sections of kidney serial to those stained for band 3 were stained similarly with commercial antisera to Tamm-Horsfall protein (Cooper Biomedical, Inc., Malvern, PA) and human epidermal keratin (Dako Laboratories, Westbury, NY) to identify renal segments with band 3 reactive cells. To visualize sites of antibody binding, the Vectastain ABC-HRP Kit (Vector Laboratories, Inc., Burlingame, CA) was employed (16). Sections were incubated with a DAB-H₂O₂ substrate, dehydrated, and mounted for viewing. Controls consisted of substituting the primary antibody in the staining sequence with either affinity purified IgG from a nonimmune rabbit or the specific band 3 antibody dilution absorbed with a second band 3 protein preparation for 24 h at 4°C.

RESULTS

Immunoreactivity for band 3 anion channel protein was observed in selected cells in the kidney but was not evident in any cells other than erythrocytes in the submandibular gland, pancreas, trachea, and lung.

In the kidney immunostaining was confined to glomerular podocytes and the cortical collecting tubules. The band 3-reactive segments were identified further by their staining for epidermal keratin but not for Tamm-Horsfall protein in serial sections. Thick ascending limbs, identified by Tamm-Horsfall reactivity, and distal convoluted tubules, characterized as negative for Tamm-Horsfall protein and keratin, lacked band 3 immunoreactivity. Intense immunostaining for band 3 protein was localized in intermittent cells comprising half of the cells lining the initial connecting segment of the cortical collecting tubules (Figs. 1 and 2). Band 3-immunoreactive cells comprised only a small minority of cells in the medullary ray portion of the collecting ducts (Fig. 3) and were seldom observed in the medullary collecting tubules. Patterns and intensity of immunoreactivity were the same in autopsy and surgical specimens.

Podocytes exhibited diffuse cytoplasmic staining (Fig. 1). In contrast, the immunoreactivity in the tubule epithelium appeared restricted mainly to the basal and the basolateral plasmalemma. Erythrocytes in vessels revealed intense membrane surface staining in the kidney.

In the CF specimens, band 3 immunoreactivity was identical to that seen in controls. No difference was discernible between the two groups in the number or location of positive cells or in the intensity or location of their cytological staining (Fig. 4).

Sections exposed to a comparable dilution of antigen-absorbed antibody or affinity purified IgG from nonimmune sera in place of the primary band 3 antibody revealed no immunostaining at these sites. In addition, identical staining patterns were observed using the sheep and rabbit antiband 3 antibodies.

DISCUSSION

The unaltered pattern of immunostaining observed in CF kidney and erythrocytes extends previous observations of com-

Fig. 1. Immunostaining for band 3 protein in control human kidney cortex is restricted to glomerular podocytes and collecting tubules (arrows). The inset shows immunoreactivity for band 3 in a podocyte (arrow), $\times 180$. Inset $\times 1900$.

Fig. 2. Band 3 protein in the initial (or connecting) segment of normal cortical collecting tubules (arrow) is confined to the basal region of some cells, $\times 900$.

Fig. 3. Band 3 protein in the medullary ray segment of normal collecting ducts is demonstrable in only a few cells, $\times 450$.

Fig. 4. Immunoreactivity for band 3 protein in a collecting tubule from a CF patient is identical to that observed in control kidneys. Erythrocytes, upper center, stain strongly, $\times 950$.

parable band 3 immunoreactivity in superficial duct cells of CF and normal sweat glands (17). These findings do not support a postulated abnormal band 3 protein in CF. While it is possible that an immunologically unaltered but functionally deficient band 3 protein may exist in the CF-affected sites, the present failure to detect band 3 anion channels in primary target sites of pathological change such as lung, pancreas, and salivary glands discredits more conclusively a general band 3 involvement in CF. It seems unlikely that false negatives would be obtained in all of these sites given the strong immunoreactivity in identically and concurrently processed and stained human kidney as reported herein. Stained erythrocytes, moreover, provided a positive control in all specimens.

In this light, the study provides little evidence with which to question reports of Cl^- impermeability in CF-affected sites (3, 5) and certainly offers no support for the alternative involvement of a neutral anion exchange mechanism in the CF defect. These results are not entirely surprising in view of the lack of either altered chloride transport in erythrocytes (18) or altered function of the kidneys of CF patients, both demonstrated sites of band 3 protein.

The basolateral membrane localization of band 3 protein in select cells of the cortical collecting duct is interesting, however, as it relates to human renal function and the regulation of acid/base balance. Band 3 anion channels so situated in the collecting duct epithelium could mediate the exchange of serosal Cl^- for cytosolic HCO_3^- , thereby facilitating serosal secretion of HCO_3^- generated by cytosolic carbonic anhydrase. Indeed, evidence exists for two types of intercalated cells in the cortical collecting tubule (20, 21). One intercalated cell type apparently secretes H^+ while the other secretes HCO_3^- . Basolateral band 3 channels localized in H^+ -secreting intercalated cells could function to allow the exchange of serosal Cl^- for intracellular HCO_3^- and, therefore, facilitate HCO_3^- resorption by the kidney cell. A recent report appearing at completion of this study described staining for band 3 protein in intercalated cells of rat renal collecting tubules but not in glomerular podocytes (22). The rat band 3-positive cells were identified as intercalated cells by the high density of mitochondria as visualized by antibodies to mitochondrial aldehyde dehydrogenase. Ultrastructural verification of intercalated cell characteristics in the band 3-positive cells of the human kidney will be necessary.

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