Galectin-3 expression is induced in renal β-intercalated cells during metabolic acidosis

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Schwaderer, Andrew L., Soundarapandian Vijayakumar, Qais Al-Awqati, and George J. Schwartz. Galectin-3 expression is induced in renal β-intercalated cells during metabolic acidosis. Am J Physiol Renal Physiol 290: F148–F158, 2006. First published August 30, 2005; doi:10.1152/ajprenal.00244.2005.—The adaptation of the cortical collecting duct (CCD) to metabolic acidosis requires the polymerization and deposition in the extracellular matrix of the novel protein hensin. HCO3−-secreting β-intercalated cells remove apical Cl−:HCO3− exchangers and may reverse functional polarity to secrete protons. Using intercalated cells in culture, we found that galectin-3 facilitated hensin polymerization, thereby causing their differentiation into the H+−secreting cell phenotype. We examined the expression of galectin-3 in the rabbit kidney and its relationship to hensin during metabolic acidosis. In control kidneys, galectin-3 was expressed in the cortical and medullary collecting ducts. In the outer cortex 26 ± 3% of CCD cells expressed galectin-3 compared with 64 ± 3% of the cells of the inner cortex. In the CCD, galectin-3 was rarely expressed in β-intercalated cells, being primarily present in α-intercalated and principal cells. During metabolic acidosis, the intensity of cellular staining for galectin-3 increased and more cells began to express it; the percentage of CCD cells expressing galectin-3 increased from 26 ± 3 to 66 ± 3% in the outer cortex and from 64 ± 3 to 78 ± 4% in the inner cortex. This was particularly evident in β-intercalated cells where expression was found in only 8 ± 2% in control animals but in 75 ± 2% during metabolic acidosis in the outer cortex and similarly for the inner cortex (26 ± 6 to 90 ± 7%). Importantly, both galectin-3 and hensin were found in the extracellular matrix of microdissected CCDs; and during metabolic acidosis, many more cells exhibited this extracellular colocalization. Thus galectin-3 may play several important roles in the CCD, including mediating the adaptation of β-intercalated cells during metabolic acidosis.

hensin; extracellular matrix; cortical collecting duct; peanut agglutinin; aquaporin-2; anion exchanger-1

GALECTINS ARE A FAMILY of lectins that bind β-galactosides, 12 of which are present in the mammalian genome (13, 14). Galectins lack signal peptides but are often found outside cells, suggesting that they are secreted via the nonclassical pathway of protein transport. The functions ascribed to these proteins are remarkably diverse; they have been implicated in the control of cellular proliferation (47), apoptosis (15, 47), inflammation (16), differentiation (5, 46), and regulation of cell-to-matrix interactions (22). Galectin-3 (formerly known as Mac-2) contains an additional domain that likely mediates homooligomerization (14). It is expressed in a variety of epithelial cells (14). In the kidney, galectin-3 expression is primarily in the distal nephron (18, 21, 46). Galectin-3 was found to be upregulated in a variety of renal cells in acute renal failure induced by folic acid or ischemia (21), in glomeruli during diabetic nephropathy (18), and in cystic epithelium in autosomal recessive polycystic kidney disease (46).

Here, we studied the expression of galectin-3 in the collecting duct in the rabbit under normal conditions and during metabolic acidosis. In this tubule segment, intercalated cells, which mediate acid-base transport, have two canonical types, α-intercalated cells secrete H+ by an apical vacuolar type H+−ATPase and the basolateral Cl−:HCO3− exchanger AE1 (band 3) (4, 28). β-Intercalated cells secrete HCO3− by an apical Cl−:HCO3− exchanger, at least one of which is pendrin, and a basolateral H+−ATPase (25, 28). There are also noncanonical types of intercalated cells, which likely represent various stages in this spectrum (2, 7, 28, 29). Cortical collecting ducts (CCDs) from rabbits on a normal diet secrete HCO3− into the lumen and have an abundance of β-intercalated cells. Acid loading of the animals in vivo or exposure of isolated, perfused CCDs to acid media for 3 h in vitro results in conversion of HCO3− secretion to net H+ secretion (27, 30, 40). We believe that this dramatic change in flux is due to a change in phenotype or function of the intercalated cells (27, 30, 32).

Examination of single identified β-intercalated cells exposed to acid media showed that they lose their apical Cl−:HCO3− exchangers and within 3 h of exposure almost a third develop basolateral anion exchange (32). In more chronically treated animals, the number of β-intercalated cells decreases while that of α-intercalated cells increases by the same number in each CCD (30). Most compelling is the study of Bagnis et al. (1) who showed that chronic treatment of rats with acetazolamide (likely resulting in a metabolic acidosis) caused a large decrease in number of β-intercalated cells and a comparable increase in α-intercalated cells without any change in total number of intercalated cells in the CCD. The identified α-intercalated cells were tall and protruded into the lumen. These results have led us to the hypothesis that there is conversion of β-intercalated cells to phenotypical α-intercalated cells in response to metabolic acidosis.

To provide a biochemical basis of this adaptation to metabolic acidosis, we immortalized rabbit β-intercalated cells (6, 42). When these cells are plated at low density, they appear similar in phenotype to β-intercalated cells, but when plated at high density, they have an α-intercalated cell phenotype, being columnar with an extensive apical cytoskeleton and projections into the lumen (37, 44). A necessary step in the development of the high-density phenotype is the deposition of a novel protein,
hensin, into the extracellular matrix (ECM) (42). Hensin is secreted as a monomer, converted into a dimer, and then is bundled into a polymer. Only the polymerized form of hensin is deposited into the ECM and is responsible for converting low-density cells to the high-density phenotype (11). Hensin polymerization was found to require another protein, which was identified as galectin-3. Galectin-3 and hensin specifically associate in the ECM, and removal of galectin-3 from the complex prevents the effect of polymerized hensin to induce the change in phenotype (12). Recent studies suggest that this conversion of low density (β-intercalated-like) to high density (α-intercalated-like) is a form of terminal differentiation where the latter form is a columnar epithelium (12, 44).

A similar phenomenon occurs in the rabbit CCD in vivo. Under conditions of metabolic acidosis, hensin is deposited in the ECM underneath adapting β-intercalated cells (32). Because this adaptation was prevented by hensin antibodies, it is likely that deposition of extracellular hensin was a necessary condition (32). Because hensin and galectin-3 are secreted into the media of cultured intercalated cells and associate with each other (12), we tested whether galectin-3 might have a similar role in mediating the adaptation to metabolic acidosis in vivo. To begin a systematic investigation into the role of galectin-3 in mediating the adaptation to metabolic acidosis, we examined the expression of galectin-3 in the kidney, its response to metabolic acidosis in vivo, and its association with extracellular hensin.

METHODS

Animals. Female New Zealand White rabbits weighing 1.5–2.5 kg were maintained on laboratory chow and tap water (40). Acid loading was accomplished by providing 75 mM NH₄Cl in 5% sucrose drinking solution and limiting food intake to 2% of body wt for 3 days (41). This treatment has previously been shown to reliably induce metabolic acidosis in rabbits (3, 33).

Immunohistochemistry. Rabbit, rat, and mouse kidneys were perfusion-fixed in periodate-lysine-paraformaldehyde (PLP) or a non-formaldehyde-based fixative (Prefer, Anatech, Battle Creek, MI). Tissue was cut into 1- to 2-mm slices perpendicular to the long axis and placed in the same fixative at 4°C overnight. After being rinsed with 70% ethanol, the sections were embedded in paraffin and 4-µm sections were placed on charged slides (Superfrost, VWR Scientific, Piscataway, NJ) (31). After deparaffinization with Prepar (Anatech) and hydration in a decreasing ethanol series, endogenous peroxidase was removed with 0.3% hydrogen peroxide and the membranes were permeabilized using 0.3% Triton X-100 (45). Various methods of antigen retrieval were used to maximize the observed signal, including microwave treatment and adjusting for pH and osmolality (34, 35). The best results were consistently obtained with 1 mmol Tris, 0.5 mmol EDTA, pH 6.5, heated in a microwave to 100°C for 10 min.

FBS (10%) was used as a block. Guinea pig anti-rabbit galectin-3 antibody was applied overnight at 4°C followed by biotin horse anti-goat secondary (Vector, Burlingame, CA) in 5% FBS. Avidin-biotinylated horseradish peroxidase (Vectastain Elite ABC kit Vector) was then applied, followed by a 5-min reaction with a substrate diaminobenzidine tetrahydrochloride kit (DAB; Vector) for brown color development (45). Sections were placed under coverslips using mounting medium (90% glycerol in PBS with 0.1% phenylenediamine to prevent quenching), examined at an objective magnification of ×4 using a Nikon E400 microscope, and digitally photographed using a Spot RT camera and software (Diagnostic Instruments, Sterling Heights, MI). Images were processed as for immunofluorescence.

Antibodies. Polyclonal anti-galectin-3 antibodies were generated as described in Hikita et al. (12). Briefly, rabbit galectin-3 was amplified by PCR from the total RNA fraction of immortalized rabbit β-intercalated cells (clone C). The PCR product was ligated into pcDNA2.1TOPO vector (Invitrogen). The fragment encoding galectin-3 was excised with BamHI and HindIII and was ligated to pQE30 (Qiagen, Valencia, CA) at the same sites yielding pQE-Gal, which encodes a His6-tag at the NH₂ terminus of galectin-3. His-tagged galectin-3 was purified from the bacterial lysate using nickel beads (Qiagen). After elution from the resin with 100 mM imidazole, the eluate was dialyzed against PBS at 4°C and stored at −80°C. One milligram of His-tagged galectin-3 was purified in both bacterial lysates and total cell extracts (12).

For double labeling of galectin-3 and hensin, a 360-bp fragment corresponding to the mouse CUB6 domain of hensin was amplified by PCR from a mouse kidney cDNA library. The purified fragments were ligated into pCR-Blunt and transformed into TOP10 cells. The plasmid DNA from positive transformants was isolated and digested with BamHI and HindIII. The digested product was then ligated into pQE30 and expressed in M15 bacteria. The His-tagged CUB6 domain was purified using a nickel column and was used to immunize rabbits (Pocono Rabbit Farm and Laboratory), as performed previously (38). The antisera generated from the CUB6 domain recognized a 220-kDa protein from the ECM of immortalized rabbit intercalated cells that was also recognized by SIRC6–7 hensin antibodies (36, 37, 44) (not shown).

Immunofluorescence. Immunofluorescence was used to obtain double labeling for galectin-3 and for the various cell types of the CCD. For immunofluorescent images, guinea pig anti-rabbit galectin-3 antibody was applied, followed by fluorescein secondary antibody (goat anti-guinea pig, Vector) (12) for rabbit sections. Rat anti-mouse galectin-3 (Cedarlane Laboratories, Hornby, Ontario, Canada) followed by fluorescein donkey anti-rat (Vector) was used on mouse sections and mouse anti-human galectin-3 (Abcam, Cambridge, MA) followed by fluorescein donkey anti-mouse (Vector) for rat sections. Sections were double labeled for principal cells with goat polyclonal anti-human aquaporin-2 (AQP-2) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and rhodamine polyclonal donkey anti-galectin-3 secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). α-Intercalated cells were labeled with mouse monoclonal AE1 antibody (provided by M. Jennings) and Texas red, goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories) (17). β-Intercalated cells were labeled with rhodamine peanut agglutinin (PNA; Vector Laboratories) (30, 32).

Sections obtained from three control and three acid-loaded rabbits were examined at ×1,100 magnification for fluorescein and Texas red or rhodamine fluorescence using a Nikon E400 microscope and photographed with the ×100 objective and a Spot RT camera and software (Diagnostic Instruments). The final images were processed with Adobe Photoshop software (Adobe Systems, San Jose, CA).

All primary antibodies were diluted 1:100 with 5% PBS in PBS and applied overnight at 4°C. Secondary antibodies were diluted 1:300 in 5% PBS in PBS and applied for 2 h at 20°C. For both light and immunofluorescent microscopy, negative controls were prepared using nonimmune guinea pig serum in the place of galectin-3 antibody.

Confocal fluorescence microscopy of galectin-labeled CCDs. CCDs were microdissected and labeled extracellularly with anti-galectin-3 antibody, followed by FITC-goat anti-guinea pig secondary antibody. For double labeling with hensin antibody, the above procedure was performed by simultaneously using polyclonal rabbit anti-mouse hensin CUB6 antibody (36) followed by rhodamine donkey anti-rabbit secondary (Jackson ImmunoResearch Laboratories). For extracellular labeling, the hensin and galectin-3 antibodies were diluted 1:100 with 1% BSA in PBS and applied to microdissected CCDs.
for 4 h at 4°C (32). The secondary antibodies were diluted 1:300 with 1% BSA in PBS and applied for 2 h at 4°C. After fixation in Prefer (Anatech) for 20 min, galectin-3-labeled tubules were, if desired, counterstained with 1:300 Texas red peanut agglutinin (United States Biological, Swampscott, MA) for 2 h at 20°C.

Each tubule was transferred to a slide in Gel/Mount, placed under coverslips, sealed, and examined at ×100 objective magnification using a dual-laser Leica TCS confocal system (Leica Microsystems Wetzlar, Wetzlar, Germany). Images of the two different fluorochromes were collected at 1-μm-thickness optical sections by using Leica software (Leica Microsystems Wetzlar). The final images were processed with Adobe Photoshop software (Adobe Systems) (32). Leica software was also used to create a two-dimensional cytofluorogram for evaluation of colocalization of galectin-3 and hensin.

Negative controls were prepared using nonimmune guinea pig serum in the place of galectin-3 antibody and rabbit serum in the place of the hensin antibody. At least five CCDs were evaluated for each staining combination to ensure consistent results.

Analysis and statistics. One hundred cells each from the inner and outer cortex of three normal and three acid-loaded kidney sections were evaluated at ×1,000 magnification for galectin-3 and either peanut lectin, aquaporin-2, or AE1 staining. Data are presented as means ± SE. Standard paired and unpaired comparisons were performed using statistical software (Excel, Microsoft, Bellevue, WA). Statistical analyses were done either using raw data (before conversion to percentages) or using arcsin-transformed percentage data (10). Significance was asserted when P values were <0.05 as calculated by Student’s t-test (2-tailed, 2-sample equal variance, paired or unpaired).

RESULTS

Galectin-3 expression is limited to the distal nephron. Using immunohistochemistry and immunofluorescence, we found that galectin-3 was limited to the cortical and medullary collecting ducts of the rabbit distal nephron (Fig. 1). Collecting ducts were identified with aquaporin-2 (AQP2) colabeling. Galectin-3 was expressed in collecting ducts to varying degrees from the outer cortex to the inner medulla (Fig. 2). A minority of cells in the outer cortex were positive for galectin-3. In the inner cortex, both the number of galectin-3-positive cells and the intensity of cellular expression increased. The outer and inner stripes of the outer medulla were characterized by con-

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**Fig. 1.** Immunofluorescent images of FITC galectin-3 staining in the rabbit, mouse, and rat kidney. The staining is limited to the collecting ducts (arrows) in the rabbit cortex, rabbit medulla, mouse cortex, mouse medulla, rat cortex, and rat medulla. Magnification ×20.
Almost every cell was positive for galectin-3 throughout the inner medulla showed an intense apical speckled pattern. Of the inner medulla, but collecting duct cells in the inner half of the inner medulla showed an intense apical speckled pattern. Almost every cell was positive for galectin-3 throughout the inner medullary collecting duct. A similar pattern of expression was seen in mouse and rat kidneys where galectin-3 was seen in the distal nephron (Fig. 1). In all three species, the expression of galectin-3 was primarily cytoplasmic in both the outer and inner cortex, although occasional nuclear staining was noted. The inner third of the inner medulla had apical speckled staining, while the staining was cytoplasmic in the remainder of the medulla. Other nephron segments did not show galectin-3 staining in any species. Staining was not observed when nonimmune serum was used as a negative control.

Cell type-specific expression of galectin-3 in CCD. Expression of galectin-3 in individual cell types of the rabbit CCD was performed using markers of principal (AQP2), α-intercalated (AE1), and β-intercalated cells (PNA). In the rabbit outer cortex, 35 ± 2% of the cells had apical PNA labeling (β-ICs), 58 ± 4% were principal cells with apical or cytoplasmic AQP2 expression, and 19 ± 2% had basolateral or cytoplasmic AE1 labeling (α-ICs) (Table 1). In the inner cortex, 19 ± 2, 67 ± 3, and 26 ± 3 of cells were PNA, AQP2, and AE1 positive, respectively. Overall, the percentage of cells that expressed galectin-3 was less in the outer cortex (26 ± 3%) than the inner cortex (64 ± 3%, P < 0.001).

While all subtypes of cells in the CCD expressed galectin-3, the expression varied greatly among cell subtypes and between the outer and inner cortex (Fig. 3 and Table 1). In the outer cortex, only 8 ± 2% of PNA-positive cells in the outer cortex and 26 ± 6% in the inner cortex expressed galectin-3. For AQP2-positive cells, 30 ± 12% (outer cortex) and 62 ± 9% (inner cortex) expressed galectin-3, and for AE-1-positive cells 43 ± 9% (outer cortex) and 54 ± 3% (inner cortex) expressed galectin-3. Note also that the predominant staining pattern for AE1 in the cortex of the normal rabbit was cytoplasmic, rather than basolateral, as shown previously by Verlander et al. (43). Thus galectin-3 expression predominated in α-intercalated cells and principal cells of normal rabbits, suggesting that this protein might be involved in some of the unique functions of these cells.

Extracellular galectin-3 colocalizes with hensin. Monomeric hensin secreted from principal cells of normal rabbits, suggesting that this protein might be involved in some of the unique functions of these cells.

Table 1. Percentage of Gal-3, PNA, AQP2, AE1, and double labeled cells

<table>
<thead>
<tr>
<th></th>
<th>Normal Outer Cortex</th>
<th>Normal Inner Cortex</th>
<th>Acid-Loaded Outer Cortex</th>
<th>Acid-Loaded Inner Cortex</th>
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<tbody>
<tr>
<td>% of Total Cells</td>
<td>% Gal-3+ of total cells 26±3</td>
<td>64±3</td>
<td>66±3*</td>
<td>78±4*</td>
</tr>
<tr>
<td></td>
<td>% PNA+ of total cells  35±2</td>
<td>19±2</td>
<td>29±3</td>
<td>12±2*</td>
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<tr>
<td></td>
<td>% AQP2+ of total cells   58±4</td>
<td>67±3</td>
<td>51±3</td>
<td>52±3*</td>
</tr>
<tr>
<td></td>
<td>% AE1+ of total cells    19±2</td>
<td>26±3</td>
<td>23±3</td>
<td>34±3</td>
</tr>
<tr>
<td>% of Identified Cells</td>
<td>% Gal-3+/PNA+ cells    8±2</td>
<td>26±6</td>
<td>75±2*</td>
<td>90±7*</td>
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<tr>
<td></td>
<td>% Gal-3+/AQP2+ cells    30±12</td>
<td>62±9</td>
<td>72±5*</td>
<td>82±4</td>
</tr>
<tr>
<td></td>
<td>% Gal-3+/AE1+ cells     43±9</td>
<td>54±3</td>
<td>73±8</td>
<td>69±12</td>
</tr>
</tbody>
</table>

Values are means ± SE. Gal-3, galectin-3; PNA, peanut agglutinin; AQP2, aquaporin-2; AE1, anion exchanger 1; +, positive staining. The sums of the PNA, AQP2, and AE1-positive cells or the total of Gal-3-positive cells do not total 100 due to the variability of staining percentages across multiple sections.

*Statistical significance compared with change from normal rabbit kidney of same cortical section.
to metabolic acidosis (32). Deposition of polymerized hensin has been demonstrated to cause β-intercalated cells to convert from HCO₃⁻-secreting cells to H⁺-secreting cells both in culture (42) and in the CCD (32). Extracellular expression and colocalization of galectin-3 with hensin in the ECM would indicate a possible role for galectin-3 in the conversion of β-intercalated cells into a phenotype resembling α-intercalated cells.

To examine for the presence of extracellular proteins, non-permeabilized rabbit CCDs were stained for galectin-3 and/or hensin and examined by confocal microscopy. Occasional cell outlines positive with a speckled staining pattern of galectin-3 were identified (Fig. 4). Similar results were seen when rabbit CCDs were stained for hensin (32). When CCDs were simultaneously stained for both proteins, there was colocalization at the cellular outlines (Fig. 4, arrows). Using Leica colocalization software, we generated a two-dimensional cytofluorogram and found a significant proportion of yellow value intensity pairs, a pattern indicating colocalization of galectin-3 with hensin (Fig. 4, bottom right).

Metabolic acidosis induces protein expression of galectin-3. To test for the effect of metabolic acidosis, we compared galectin-3 expression in the cell subtypes of the acid-loaded and the normal rabbits. In kidney sections, the intensity of staining for galectin-3 in the distal nephron increased with metabolic acidosis, with the most notable increase occurring in the outer cortex (Fig. 5). The percentage of cells expressing galectin-3 increased from 26 ± 3 to 66 ± 3% (P < 0.001) in the outer cortex and from 64 ± 3 to 78 ± 4% (P < 0.05) in the inner cortex following acid loading. The percentage of PNA-positive cells also positive for galectin-3 sharply increased during metabolic acidosis in the outer cortex (8 ± 2 to 75 ± 2%, P < 0.001) and inner cortex (26 ± 6 to 90 ± 7%, P < 0.01; Fig. 6). The percentage of AQP-2-labeled cells positive for galectin-3 significantly increased in the outer cortex (30 ± 12 to 72 ± 5%, P < 0.05) but not in the inner cortex (62 ± 9 to 82 ± 4%, P = 0.1). The acidosis-induced increase in percentage of AE1-labeled cells positive for galectin-3 was not statistically significant in either outer cortex (43 ± 9 to 73 ±
Galectin-3 labeling of AQP2 and AE1 identified cells was variable in the normal kidney cortex: labeling was present in some but not all AQP2- and AE1-stained cells. However, in the outer and inner cortex of acid-loaded rabbits, galectin-3 expression was present in most of the AQP2- and AE1-labeled cells. As expected, AQP2 maintained an apical location and AE1 assumed a basolateral location with increased intensity after acidosis (Fig. 7).

Confocal microscopy provided the best images for extracellular labeling of galectin-3 in identified CCD cells (Fig. 8). The normal CCD (top left) had only occasional cells that labeled for extracellular galectin-3. However, after acid loading (top right), both the number of cells and intensity of galectin-3 expression increased. Unlike PNA-positive cells in the normal CCD (arrows, left middle and bottom), PNA-positive cells in the acid-loaded CCD (arrowheads, right middle and bottom) were often colabeled with galectin-3. These results demonstrate that β-intercalated cells respond to metabolic acidosis by increasing the synthesis of galectin-3 as well as by secreting it to the ECM.

Increased extracellular colocalization of hensin and galectin-3 during metabolic acidosis. We previously found that during metabolic acidosis, there is increased extracellular localization of hensin (32). Because we found that galectin-3 colocalizes with hensin in the normal CCD and exhibits increased expression during metabolic acidosis, we tested whether it, too, is secreted and localizes to the ECM. CCDs from acid-loaded rabbits were microdissected and stained extracellularly for galectin-3 and hensin without permeabilization. Using confocal microscopy (Fig. 9, arrows), we found that most cells had extracellular galectin (green) and hensin (red), with nearly all the cells showing colocalization (yellow in the merged images). This is in stark contrast to the normal CCD, which shows only patchy areas of colocalized hensin and galectin-3 on the cell outlines (Fig. 4). A two-dimensional cytofluorogram demonstrated a large increase in colocalized value intensity pairs, as well as increased intensity of the fluorescence in the acid-loaded CCD (Fig. 9) compared with the normal CCD (Fig. 4).

8%, \( P = 0.06 \) or inner cortex (54 ± 3 to 69 ± 12%, \( P = 0.3 \)). However, the intensity of the galectin-3-labeled α-intercalated cells appeared to increase, and the predominant AE1 staining in the cortex changed from cytoplasmic to basolaterally polarized (Fig. 7).

Immunofluorescence microscopy images of PNA-positive cells exhibited an increase in galectin-3 protein expression with acidosis (Fig. 7). Galectin-3 expression was infrequently found in the PNA-labeled cells in the outer and inner cortex of normal rabbits, and this was confirmed by confocal microscopy (Fig. 7, row 2). After acid loading, galectin-3 labeling was usually present in the PNA-labeled cells, and often intense in both the outer and inner cortex. The peanut lectin labeling was apical and became thinner and more internalized in the acid-loaded cortex, as seen previously (32).
Fig. 7. Immunofluorescence microscopy of normal and acid-loaded outer cortex and normal and acid inner cortex labeled with FITC galectin-3 and rhodamine PNA, AQP2, or AE1. Magnification ×100. Cells with only galectin-3 staining are labeled with arrows; cells with PNA, AQP2, or AE1 and galectin-3 expression are labeled with arrowheads. Row 2 exhibits 1-μm optical sections examined by confocal microscopy.

Fig. 8. Confocal immunofluorescence microscopy of normal and acid-loaded rabbit CCDs (from outer cortex) labeled with extracellular FITC galectin-3 and rhodamine PNA. Magnification ×100. Cells with only apical PNA labeling are marked with arrows. Cells with galectin-3 and PNA labeling are marked with arrowheads. XY and XZ axis of PNA-labeled cells of normal and acid-loaded rabbits demonstrate extracellular galectin-3 staining in the acid-loaded but not in the normal rabbit CCD.
DISCUSSION

Expression of galectin-3 in the kidney. Galectin-3 is a lectin that is an important component in a cell culture model used to simulate the kidney’s response to metabolic acidosis. To systematically investigate the role of galectin-3 in mediating the renal adaptation to metabolic acidosis, we examined the expression of galectin-3 in cell subtypes of the normal rabbit kidney, its response to metabolic acidosis in vivo, and its association with extracellular hensin. We found that renal galectin-3 expression is limited to the distal nephron, consistent with the observations of previous investigations in rats (21) and humans (18). Galectin-3 was previously reported to be expressed by collecting duct \( /H9251 \)-intercalated cells (identified by AE1 labeling) in the human kidney (46). We demonstrated variable galectin-3 expression by all cell subtypes of the CCD, but much more commonly by \( /H9251 \)-intercalated cells and principal cells than by \( /H9252 \)-intercalated cells. The previous conclusion that galectin-3 expression occurs only in \( /H9251 \)-intercalated cells might have resulted from the use of inadequate markers for principal cells and \( /H9252 \)-intercalated cells (46). We were able to use excellent cell-specific markers for the CCD to identify those cells expressing galectin-3. Although cytoplasmic expression of galectin-3 in the distal nephron has been previously described (46), and confirmed by us in this study, extracellular expression of galectin-3 in the CCD is a novel finding and suggests that galectin-3 is involved in interactions with the ECM in the kidney.

Expression of galectin-3 in immortalized intercalated cells. When we generated an immortalized cell line from the \( /H9252 \)-intercalated cell, our hope was that it would form a discovery system to allow us to identify new proteins involved in the complex mechanism of regulation of intercalated cell function during metabolic acidosis. No cell culture model, least of all immortalized cells, reproduces the function of cells in situ. However, their convenient use can lead to identification of proteins and genes necessary for physiological function. Our immortalized cells did not respond to metabolic acidosis in culture, raising the question that a factor produced by other cells is needed to initiate the cascade of events that result in the change in phenotype from \( /H9252 \)-intercalated cells to \( /H9251 \)-intercalated cells. However, we found that seeding density reproduced the change in phenotype: high-density cells resembled \( /H9251 \)-intercalated cells, while low density cells were similar to \( /H9252 \)-intercalated cells (42). The transition between the two phenotypes was due to the deposition of hensin in the ECM. This fibrillar protein is produced in monomeric soluble form by the low-density cells and polymerized by a complex series of steps into the insoluble fiber form that functions to change the phenotype of the cells. In microdissected CCDs, we discovered that \( /H9251 \)-intercalated cells expressed hensin in situ and that metabolic acidosis led to the deposition of hensin in the ECM of adapting \( /H9252 \)-intercalated cells (32). Furthermore, antibodies to hensin blocked the change in phenotype induced by metabolic acidosis. These studies confirmed the efficacy of the immortalized intercalated cells to act as a surrogate assay system for identification of proteins needed for acid-base regulation in the CCD.

Galectin-3 and hensin polymerization. Previous work (12) suggested that galectin-3 facilitates the polymerization and deposition of hensin in the ECM. The combination of polymeric hensin and galectin-3, but not hensin or galectin-3 individually, induced apical endocytosis. We demonstrated Fig. 9. Extracellular labeling of an acid-loaded rabbit CCD (outer cortex) for galectin-3 and hensin evaluated by confocal microscopy. Magnification \( \times 100 \). Extracellular labeling for galectin-3 (FITC) and hensin (rhodamine) is demonstrated by continuous staining of the majority of cell outlines (arrowheads). An occasional cell outline demonstrates mostly galectin-3 staining (arrows). High amounts of colocalization (yellow) can be seen on the merged image and 2-dimensional cytofluorogram.
that addition of galectin-3 to monomeric hensin results in the formation of dimers. Furthermore, dissociation of fibrillar (insoluble) hensin using the reversible chemical-modifying agent dimethyl maleic anhydride released galectin-3, and the resultant hensin loses its ability to induce the conversion of low-density cells (resembling β-intercalated cells) to the high-density phenotype (resembling α-intercalated cells) in vitro. Removal of the dimethyl maleic anhydride in the presence of excess galectin-3 restores the functional ability of fibrillar hensin. These results demonstrate the necessity of galectin-3 for polymerization of hensin in vitro. Here, we demonstrated that galectin-3 is secreted extracellularly where it likely interacts with the ECM and that in this role it colocalizes with hensin. Given the role of galectin-3 in the aforementioned cell culture model, its expression during metabolic acidosis in vivo warranted further evaluation. In the immortalized cells, we found increased expression and secretion of galectin-3 when the cells were seeded at high density (12). In the kidney, we also found that metabolic acidosis increases the expression of galectin-3. The induction of galectin-3 expression in the CCD is distinct from the induction of galectin-3 expression in the glomeruli with diabetic nephropathy (18) or in proximal tubules and thick ascending limbs during acute renal failure (21), suggesting that galectin-3 may have a unique function related to the adaptation to metabolic acidosis, as opposed to that occurring as a nonspecific response to cellular insult or injury. More significantly, we found that metabolic acidosis was associated with enhanced localization of galectin-3 to the extracellular basolateral domain. In that site, metabolic acidosis clearly results in increased colocalization with hensin. The most intriguing finding is that galectin-3 was only minimally expressed in the β-intercalated cells of normal rabbits but its expression increased dramatically in these cells when they were adapting to metabolic acidosis. Indeed, galectin-3 appeared in the ECM of adapting β-intercalated cells. This induction of galectin-3 expression in β-intercalated cells is another example of the development of a characteristic more typical of α-intercalated cells in response to metabolic acidosis. A similar phenomenon has been noted for the expression of extracellular hensin by adapting β-intercalated cells (32).

Metabolic acidosis and AE1 expression by intercalated cells. It is a matter of debate whether in response to metabolic acidosis β-intercalated cells develop all of the phenotypic characteristics of α-intercalated cells or whether they simply become dormant. In response to 3 h of acid exposure, all identified β-intercalated cells removed apical Cl: HCO₃⁻ exchangers, and at least a third of them developed basolateral Cl: HCO₃⁻ exchanger activity (32). Moreover, acid-incubated CCDs exhibited an increase in proton secretory flux, in addition to the decrease in bicarbonate secretory flux (32). Dissociated CCD cells from acid-loaded rabbits expressed 4.5 times greater levels of AE1 mRNA than cells from alkali-loaded rabbits, and CCD lysates from acidicot rabbits showed much higher expression of AE1 protein than did CCD lysates from normal rabbits (8). Bagnis et al. (1) reported that chronic treatment of rats with acetazolamide (likely resulting in a metabolic acidosis) caused a 50% decrease in the number of canonical β-intercalated cells and a 30% increase in α-intercalated cells without any change in the total number of intercalated cells in the CCD, suggesting that β-intercalated cells are converted into actual α-intercalated cells. However, Sabolic et al. (26) demonstrated only an increased intensity of AE1 staining during acute (6 h) metabolic acidosis without any increase in the fraction of AE1-positive cells. Verlander et al. (43) observed that AE1 was mainly confined to multivesicular bodies and cytoplasmic vesicles in controls, but during chronic metabolic acidosis AE1 was localized to the basolateral membrane and appeared much more intense.

The present study showed an increase in the percentage of AE1 cells with acidosis, but the increases in the outer and inner cortex did not reach statistical significance. A possible explanation is that our cell counts were directed toward counting galectin-3-positive cells, which were colabeled with AE1, PNA, and AQP2, and therefore insufficient numbers of AE1-positive cells were counted in the cortex to provide adequate statistical power (Table 1). If we were to have examined more intercalated cells in more sections, we might have shown significant increases in AE1-positive cells. In any case, we clearly confirmed that acidosis induced basolateral polarization and heightened intensity of AE1 in cortical intercalated cells (Fig. 7).

It should be pointed out that the cytoplasmic AE1 staining, which was more characteristic of the normal cortex, may not be indicative of proton-secreting α-intercalated cells, because such a location may not facilitate bicarbonate exit from a proton-secreting cell. During acidosis, there was a predominant change to basolateral staining of most AE1-labeled intercalated cells in the cortex, and thereby to a phenotype more capable of secreting protons (43) (Fig. 7). Because we have not been able to measure proton secretion by individually identified α-intercalated cells, we are unable to clearly determine whether this change in AE1 staining results in more proton-secreting intercalated cells in response to metabolic acidosis. Nevertheless, we demonstrated in this paper that during metabolic acidosis β-intercalated cells developed some characteristics of α-intercalated cells, particularly the deposition of the proteins galectin-3 and hensin into the ECM.

Galectin-3 and the principal cell. Interestingly, galectin-3 expression increased in principal cells (AQP2 positive) during metabolic acidosis. The role of galectin-3 expression in principal cells is not known. Principal cell-mediated sodium reabsorption creates lumen electronegativity that facilitates α-intercalated cell proton secretion (24). Principal cells are known to express hensin (36), and we demonstrated a significant increase in galectin-3 expression during metabolic acidosis. It is possible that galectin-3 and hensin are part of a mechanism by which principal cells adjust their function to optimize the renal tubular environment for acid secretion during metabolic acidosis.

Function of galectin-3. The function of galectins remains a vexing issue. Galectin-3 added to developing kidneys in organ culture had a clear effect to inhibit branching morphogenesis, suggesting its involvement in nephrogenesis (5). Similar studies have been performed in other systems. Among the rigorous criteria for identification of the function of a protein is genetic deletion of such proteins. Deletion of galectin-3 results in fertile and viable mice with no obvious defects in kidney structure or function. The conclusion that a protein is “redundant,” often stated when genetic deletions result in no obvious defects, potentially reflects the possibility that the appropriate “stress-testing” had not been performed. For instance when they were rendered diabetic, accelerated diabetic glomerulopa-
thy with increased proteinuria, ECM gene expression, and mesangial expansion was noted in the galectin-3 knockout mice compared with wild-type mice (23). Of course, redundancy is a legitimate concern when one is dealing with a gene family of 12 proteins, each of which binds to the same or similar sugars and is often multiply expressed. Despite this, new lines of evidence must be developed in such circumstances and the experimental system needs to be subjected to different types of studies to demonstrate the importance of these proteins in functions that may neither be life-threatening nor associated with obvious structural changes.

The functions of galectin-3 in the collecting duct are not known; we suggested that it facilitates the polymerization and deposition of hensin in the ECM and that this matrix complex initiates or mediates the adaptation of β-intercalated cells to metabolic acidosis. Hensin is a large protein composed of many domains including scavenger receptor cysteine (SRCR) domains-rich (38). The SRCR domains appear to be critical for hensin function in that antibodies to these domains can block the function of hensin (32, 37). A large number of proteins with SRCR domains exist and they include both secreted proteins (such as hensin) and membrane receptors. One intriguing finding is that a protein identified as a tumor-associated antigen and which was found to bind to Mac-2 (i.e., galectin-3) was identified to be an SRCR domain protein (9, 39). Indeed, the NH2-terminal and the intervening proline-, tyrosine-, and glycine-rich repeat (R) domains may enable galectin-3 to aggregate and cross-link ligands (19, 20, 22). Hence, it is possible that one domain of galectin-3 (possibly the COOH-terminal carbohydrate recognition domain or the repeating R domains) is a potential site of interaction with hensin. Studies to examine how galectin-3 interacts with ECM proteins and what its possible function is in the β-intercalated cells’ adaptation to metabolic acidosis would require an in-depth evaluation of galectin-3-deficient mice exposed to perturbations of acid-base homeostasis.

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