Mispolarization of desmosomal proteins and altered intercellular adhesion in autosomal dominant polycystic kidney disease

Melina Silberberg,1 Audra J. Charron,1 Robert Bacallao, and Angela Wandinger-Ness1

1Department of Pathology, University of New Mexico, Albuquerque, New Mexico; and 2Department of Nephrology, University of Indianapolis, Indianapolis, Indiana

Submitted 12 January 2005; accepted in final form 1 February 2005

Silberberg, Melina, Audra J. Charron, Robert Bacallao, and Angela Wandinger-Ness. Mispolarization of desmosomal proteins and altered intercellular adhesion in autosomal dominant polycystic kidney disease. Am J Physiol Renal Physiol 288: F1153–F1163, 2005.—Polycystin-1, the product of the major gene mutated in autosomal dominant polycystic kidney disease (ADPKD), has been shown to associate with multiple epithelial cell junctions. Our hypothesis is that polycystin-1 is an important protein for the initial establishment of cell-cell junctions and maturation of the cell and that polycystin-1 localization is dependent on the degree of cell polarization. Using laser-scanning confocal microscopy and two models of cell polarization, polycystin-1 and desmosomes were found to colocalize during the initial establishment of cell-cell contact when junctions were forming. However, colocalization was lost in confluent monolayers. Parallel morphological and biochemical evaluations revealed a profound mispolarization of desmosomal components to both the apical and basolateral domains in primary ADPKD cells and tissue. Studies of the intermediate filament network associated with desmosomes showed that there is a decrease in cytokeratin levels and an abnormal expression of the mesenchymal protein vimentin in the disease. Moreover, we show for the first time that the structural alterations seen in adherens and desmosomal junctions have a functional impact, leaving the ADPKD cells with weakened cell-cell adhesion. In conclusion, in this paper we show that polycystin-1 transiently colocalizes with desmosomes and that desmosomal proteins are mislocalized as a consequence of polycystin-1 mutation.

Polycystin; dispase assay; polarity; cytoskeleton; calcium switch

AUTSOMAL DOMINANT POLYCYSTIC kidney disease (ADPKD) is a common, life-threatening genetic disease that principally affects the kidney but may also have extrarenal manifestations such as liver cysts and cardiovascular defects. ADPKD has a dominant mode of inheritance and afflicts more individuals than cystic fibrosis, sickle cell anemia, hemophilia, muscular dystrophy, Down’s syndrome, and Huntington’s disease combined. The disease at a gross anatomic level is characterized by the progressive appearance of multiple fluid-filled cysts in both kidneys, which gradually compromise normal function and ultimately result in end-stage renal failure (2).

Cysts develop following inheritance of a germline mutation in one of two genes, PKD1 (80% of all cases) (4, 5, 21, 22a) or PKD2 (24), and acquisition of subsequent somatic mutations in the normal PKD1 or PKD2 alleles of a tubular epithelial cell (40, 49). Thus the disease appears recessive at the cellular level. Multiple functions and localizations have been ascribed to polycystin-1 and polycystin-2; the products of PKD1 and PKD2, respectively, and they appear to participate in a variety of protein-protein complexes (3, 26, 45). Polycystin-1 is a large transmembrane protein with extracellular domains typical of proteins that mediate cell-cell and cell-extracellular matrix interactions (21, 28) and intracellular domains implicated in the transduction of signals (44). Polycystin-1 interacts via its COOH terminus with polycystin-2, a protein that forms a calcium-permeable channel (18, 25, 34, 42). The composite localization and functional studies to date show the polycystins to play an important role in mechanosensory signal transduction, as well as cell adhesion (9, 11, 19, 22, 29, 36, 38, 47, 50).

However, there is no clear consensus as to how the mutant polycystins incite or contribute to cyst formation.

The importance of the polycystins in cell-cell adhesion is evidenced by observations that polycystins are associated with intercellular adhesive junctions including both adherens junctions and desmosomes (19, 36, 38). Desmosomes constitute a key adhesive junction at the basolateral plasma membrane and are critical for stabilizing the epithelial sheet through connections to intermediate filaments. The desmosomal cadherins desmoglein (Dsg) and desmocollin (Dsc) constitute the core transmembrane proteins that interact with desmosomal cadherins on adjacent cells via their luminal domains and associate with desmosomal plaque constituents via their cytosolic domains. The desmosomal plaque is constituted of plakoglobin (Pg), plakophilins, and desmoplakin (DP), which bind to the intermediate filament cytoskeleton. By immunoelectron microscopy, polycystin-1 is known to be intimately associated with desmosomes in subconfluent Madin-Darby canine kidney (MDCK) cells (38). This raised the question as to whether desmosomal adhesion was abnormal in ADPKD.

In this study, we examine the status of desmosomal junction and intermediate filament protein assemblies and test functional cell-cell adhesion in ADPKD cells. Temporal analyses of desmosome assembly in normal kidney cells show polycystin-1 associated with desmosomes only at early time points. Our findings suggest a role for polycystin-1 in the formation of normal desmosomes at the lateral plasma membrane.

MATERIALS AND METHODS

Antibodies and reagents. All reagents were from Sigma (St. Louis, MO) unless otherwise indicated. The following commercial antibodies against desmosomal components were used in immunofluorescence: mouse mAb directed against Dsg-2 (1:25, clone 6D8, Zymed Laboratories, South San Francisco, CA), mouse mAb directed against Pg (1:100, clone PG-11E4, Zymed Laboratories), and mouse mAb...
directed against DP (1:2, DP 1 and 2 clone 2.15, 2.17, 2.20, Progen Biotechnik, Heidelberg, Germany). Dr. K. J. Green (Dept. Pathology, Northwestern University, Chicago, IL) kindly provided a rabbit pAb directed against DP (NW161) and a mouse mAb directed against Dsc-2 (clone 7G6). Antibodies against cytoskeletal proteins were as follows: mouse mAb directed against vimentin (1:100, clone Vim 3B4, DakoCytonmation, Carpinteria, CA) and mouse mAb directed against cytokeratin 18 (1:100, clone DC10, DAKO). A pAb directed against polycystin-1 (1:100, NM005) was obtained from rabbits injected with the COOH-terminal region of the protein (exon 46; 4070–4302 aa) and purified using a DEAE-matrix in our laboratory (36). Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA): FITC-conjugated donkey anti-mouse and rhodamine-conjugated donkey anti-rabbit (used for tissue sections) or Cy5-conjugated donkey anti-rabbit (used for cultured cells). Nuclei were stained with Hoechst 33258 (Eastman Kodak, Rochester, NY) or propidium iodide. Cysts and tubule glyco- calyx were labeled with FITC-linked peanut lectin. Sulfo-NHS-LC- Biotin was purchased from Pierce Biotechnology (Rockford, IL). Cell culture medium was from Gibco (Grand Island, NY), and serum was from Hyclone (South Logan, UT).

Cell culture. Primary normal epithelial kidney cells were isolated from kidneys of donors that were medically unsuitable for transplant and therefore procured for research through the National Disease Research Interchange. Cyst-lining epithelial cells were initially derived by Dr. F. Carone and S. Nakamura and subsequently by dissection in our laboratory of kidneys from patients with ADPKD that were removed for clinically indicated reasons. Briefly, on arrival, normal kidneys were placed on ice; the cortex was excised and minced into fine pieces that were incubated in Enzyme Solution (collagenase, hyaluronidase, DNase I, soybean trypsin inhibitor and desmosomal mispolarization in ADPKD

Immunofluorescence staining of cells and tissue. Briefly, immunofluorescence staining of cells grown on coverslips or filters at full confluence was performed as follows. Cells were rinsed with PBS+ (PBS containing 1 mM each MgCl2 and CaCl2), preextracted with 0.01–2% saponin (depending on normal/pathological, passage number, sparse/confluent cells) in PIPES solution (80 mM PIPES, 5 mM EGTA, and 1 mM MgCl2) for 3–5 min. Saponin preextraction is a well-described method for selectively permeabilizing the plasma membrane and allowing small soluble cytosolic proteins to be extracted, thereby improving membrane protein staining. Following preextraction, cells were fixed with 3% paraformaldehyde in PBS+ for 20 min, quenched with NH4Cl for 10 min, and permeabilized with 0.1% Triton X-100 (vol/vol) for 5 min after which samples were blocked with 0.4% fish skin for 10 min and washed. Filters were excised from the filter support and cut in two to four pieces before incubation with primary antibody. After incubation with secondary antibody plus propidium iodide, cells were postfixed with 3% paraformaldehyde, quenched, and mounted with ProLong or SlowFade (Molecular Probes, Eugene, OR). Nuclei were stained with Hoechst (1:1,000) or propidium iodide (1 μg/ml) plus RNase A (20 μg/ml). Filters were placed on a microscope slide with mounting media cell side up in the center of four nail polish posts and covered carefully with a coverslip.

Bioreactivation of plasma membrane proteins was done as follows. Briefly, cells were placed on ice and labeled with Sulfo-NHS-LC-Biotin (Pierce Biotechnology) twice for 15 min. Free biotin was quenched with cold 50 mM NH4Cl twice for 15 min, and the cells were subjected to saponin preextraction and fixation as described above.

For cytoskeleton labeling, cells were fixed in methanol at −20°C for 5 min and processed for immunofluorescence staining. All samples were imaged with a Zeiss LSM 510.

Frozen normal or ADPKD tissues were cryosectioned (4-μm thickness) using a Reichert Ultracut S ultramicrotome with Leica EMFCS cryochamber. Sections were collected on Fisherbrand Super Frost+/Plus microscope slides (Fisher Scientific, Pittsburgh, PA), fixed with 3% paraformaldehyde, and blocked/permeabilized with 10% newborn calf serum/0.1% Triton X-100 (vol/vol) for 40 min. Tissue samples were stained with primary antibodies for 2 h and secondary antibodies for 30 min, all at 37°C in a humidified chamber. Nuclei were stained with propidium iodide (1 μg/ml). Tissue was washed three times for 10 min each with PBS/0.1% Triton X-100 (vol/vol) and mounted in Slow Fade mounting medium. A Zeiss LSM 510 was used to obtain high-resolution images of stained tissue sections. Distal tubule glyco- calyx staining was done with FITC-labeled peanut lectin (Arachis hypogaea) for 1 h at room temperature (RT) after incubation with secondary antibodies. For labeling of cytoskeletal components, tissue sections were fixed in methanol at −20°C for 5 min, after which, they were blocked in 10% newborn calf serum and incubated with the appropriate antibodies.

Calcium switch assay. The procedure followed was as described previously (30) with minor modifications to suit our primary cultures. Cells were seeded on coverslips or filters at full confluence in normal calcium DMEM/F-12 supplemented with 10% FCS and antibiotics and left for 2 h to allow attachment to the substratum. Next, cells were rinsed five times in SMEM (low calcium media, <5 μM Ca2+) and incubated overnight in SMEM with 5% dialyzed FCS at 37°C. The following day, cells were switched to normal calcium DMEM/F-12 and left for different lengths of time ranging from 0 min to 24 h, after which cells were fixed in 4% paraformaldehyde or preextracted with 0.01% saponin (wt/vol) and fixed. After this, cells were quenched with 50 mM NH4Cl, permeabilized with 0.075% saponin (wt/vol) in PBS (PBS-S), and then incubated with primary antibodies dissolved in 2% BSA for 1 h at RT. Cells were washed three times in PBS-S and incubated for 1 h at RT with secondary antibodies. After being washed for 20 min with PBS-S, cells were mounted on slides with Slow Fade media.

Cell surface biotinylation and streptavidin affinity precipitation. Confluent filter-grown cells were selected based on TEER measurements of ~250 Ω/cm², indicative of intact tight junctions. Confluent filter cultures were washed with ice-cold PBS+ and
incubated with ice-cold sulfo-NHS-SS-biotin at 0.6 mg/ml prepared in PBS+ pH 8.0. Either the apical (1 ml volume) or basolateral (1.5 ml volume) surface was biotinylated twice in succession for 15 min each, using fresh biotinylation reagent each time. The biotinylation reaction was terminated by replacing the second biotin solution with the same volume of ice-cold 50 mM NH4Cl in PBS+ for 30 min on ice. Following biotinylation, cells were lysed in 100 μl of 1% SDS (wt/vol), 15 mM Tris-HCl pH 8.0, 4 mM EDTA, 1 μM chymostatin, leupeptin, antipain, and pepstatin A (CLAP), and 1 μM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), boiled for 5 min, and diluted in 900 μl of Trition X-100 [0.5% Triton X-100 (vol/vol), 15 mM Tris-HCl pH 8.0, 150 mM NaCl, 4 mM EDTA, CLAP, and AEBSF] containing 40 μl of streptavidin-agarose (Pierce Biotechnology). The sample was rocked at 4°C for 1 h, and the beads were washed and boiled for 5 min in 40 μl of 2X SDS sample buffer containing 50 mM dithiothreitol.

Western blot analysis. Proteins released from streptavidin-agarose beads or whole SDS lysates were separated on 10 or 12.5% SDS Criterion precast polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose (Hybond-C Extra, Amer sham Biosciences UK) or polyvinylidene difluoride membranes (Im mobilon-P, Millipore, Billerica, MA). Non-specific binding sites were blocked by 1-h incubation at RT with 5% nonfat dried milk (wt/vol) in PBS containing 0.1% Tween 20 (vol/vol; TBS-T). Blots were washed twice for 15 min in TBS-T and incubated with the appropriate dilution of primary antibody for 1 h at RT. After two more 15-min washes, blots were probed with horseradish peroxidase-conjugated secondary antibody for 40 min at RT, washed twice for 15 min with TBS-T, and bound antibodies were detected using ECL SuperSignal (Pierce Biotechnology). For quantification of protein loading, before protein blocking of the membrane, it was stained with Coomassie blue, imaged with a Fluor-S, Bio-Rad Imager and the optical density was analyzed with Quantity One software. After developing the membrane with ECL, specific vimentin and cytokeratin bands were quantified with the same instrument. The ratio of vimentin OD to cytokeratin to total protein was used for normalization purposes.

Dispase assay. A dispase assay for measuring cell-cell adhesive function (8) was modified for our purposes and performed as follows. Cells grown on collagen IV-coated 35-mm dishes (BD BioCoat, Bedford, MA) were incubated with dispase enzyme (2.4 U/ml; Roche Applied Science, Indianapolis, IN) at 34°C for 15-30 min after which they were carefully scraped to separate the monolayer from the dish. The sheets of cells were gently transferred with a wide-tipped transfer pipette into a snap cup tube where they were subjected to shaking on a shaker (model M65125, Integrated Separation Systems Enprotech) at 10 rpm for 3 min. Subsequently, cells were left to settle for 1 min, and 50 μl of the supernatant were taken in duplicate for cell counting in a cell counter (Z2 Coulter Counter, Beckman, Fullerton, CA). The remaining cells were pelleted and incubated with trypsin at 37°C for 10 min after which they were counted in the cell counter along with the samples taken just after shaking.

The Human Research Review Committee of the University of New Mexico granted exempt status approval under Human Health Services regulations and applicable Food and Drug Administration regulations 21CFR50.56 and 45CFR46.110 (b)(2).

RESULTS

Desmosomal proteins are mislocalized to the apical domain in ADPKD. It has been postulated that adherens junctions are necessary for desmosomal formation and that when E-cadherin is not expressed, desmosome assembly is delayed or blocked (1, 27). In ADPKD, E-cadherin is depleted from the lateral membrane of disease cells and found intracellularly in vesicles (10). Furthermore, polyclin-1 has been reported to colocalize with desmosomes in subconfluent MDCK cells (38). Taken together, these observations raised questions regarding the status of desmosomal junctions in the disease, which were addressed by examining primary human cells in culture and cystic epithelium in situ.

Endogenous desmosomal structures were evaluated by immunolabeling normal and cystic primary human kidney epithelial cells grown on filters (Fig. 1, A and B). We discovered that while desmosomal proteins are still present at the lateral plasma membrane (Fig. 1A, xy view), they are also found at the apical plasma membrane of ADPKD cells (Fig. 1A, xz view). Labeling for both cytoplasmic desmosomal components, such as DP and Pg (Fig. 1A) or for the integral membrane desmosomal cadherins such as Dsg (Fig. 1B), consistently revealed an apical staining in all ADPKD samples evaluated. The reported analyses throughout were performed on three ADPKD patient samples and comparatively evaluated against three independent normal patient samples. To confirm that our PKD cells have normal functioning tight junctions, we measured tritiated intrulin flux across the monolayer and found the flux across both ADPKD and normal kidney cell monolayers to be similar and significantly below the values seen for blank filters (Table 1). The TEEs of ADPKD and NK cells were >200 and >300 Ω/cm², respectively. To distinguish whether the apical desmosomal staining in ADPKD cells was due to apical plasma membrane expression or due to an intracellular subapical pool, we labeled the apical plasma membrane with LC-biotin followed by streptavidin-Cy5 and coimmunostained for desmosomal protein (Fig. 1B). Apical Dsg was found completely colocalized with biotin at the apical membrane, confirming the apical plasma membrane expression of desmosomal components. In addition, the biotin labeling was selectively confined to the apical plasma membrane and no overlap with the basolateral desmosomal protein pool was observed, excluding the possibility that altered tight junction function might account for desmosomal proteins at the apical cell domain. This is also in agreement with our previously published studies showing that ADPKD cells have morphologically normal tight junctions and retain the capacity to polarize most plasma membrane proteins (10).

To further substantiate the extent of desmosomal cadherin mislocalization, PKD monolayers with TEER >250 Ω/cm² were subjected to domain-selective apical or basolateral biotinylation. The biotin-labeled proteins were precipitated with streptavidin-agarose beads and the distribution of apical vs. basolateral Dsg in normal and ADPKD cells was evaluated by immunoblotting (Fig. 1C). In fully polarized normal kidney cells, Dsg was exclusively basolateral, whereas in ADPKD cells Dsg was equally distributed between the apical and basolateral domains of ADPKD cells. The aberrant polarization of desmosomal components was also evident in cystic tissue as revealed by immunofluorescence staining of normal and ADPKD tissue cryosections (Fig. 1, D and E). Colocalization with peanut lectin was used to establish the apical plasma membrane expression of DP in situ (Fig. 1E). Lectins only showed an overlapping signal with DP in ADPKD tissue sections. Although some DP staining in normal tissue sections gave the impression of being apical in nontangential areas of the section, an apical localization was excluded as no colocal-
ization with lectins was apparent. Together, the data demonstrate that in ADPKD desmosomal proteins are mislocalized in situ and primary ADPKD cells in culture recapitulate this phenotype.

Table 1. **Inulin flux measurements**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Inulin Flux, μl/cm²min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDCK</td>
<td>-0.04</td>
</tr>
<tr>
<td>Primary normal human kidney cells</td>
<td>0.15±0.10</td>
</tr>
<tr>
<td>Primary human ADPKD cells</td>
<td>0.20±0.10</td>
</tr>
<tr>
<td>Blank filter</td>
<td>125±2</td>
</tr>
</tbody>
</table>

Values are means ± SE. MDCK, Madin-Darby canine kidney cells; ADPKD, autosomal dominant polycystic kidney disease.

**Abnormal intermediate filament protein profile in ADPKD.** Desmosomal junctions are normally anchored to a keratin intermediate filament network in epithelia that gives strength to intercellular adhesion and protects the monolayer to shear stress. Vimentin is an intermediate filament protein that is characteristic of fibroblasts, endothelial cells, and mesenchymal cells. In vivo, there are few examples of coexpression of desmosomal proteins and intermediate filaments of the vimentin type (23). To analyze the system of desmosomal junctions and its attached cytoskeleton, ADPKD tissue sections were immunolabeled for vimentin and cytokeratin and compared with normal kidney tissue sections (Fig. 2). Although cytokeratin staining could still be seen in the cyst-lining epithelia, the signal was rather

![Fig. 1. Desmosomes are mispolarized in autosomal dominant polycystic kidney disease (ADPKD) cells in vitro and in situ. A: confocal images of filter cultures of normal (NK) and ADPKD cells (PKD) labeled for the desmosomal components (green) desmoplakin (DP) and plakoglobin (Pg). Top: xy view to show desmosomal appearance at the lateral membrane. Bottom: xz view to show desmosomal distribution between apical (arrows) and lateral cell domains. Nuclear staining, used as structural reference, is seen in blue. Bars = 20 μm. B: apical plasma membrane localization of desmosomal (desm) cadherin in PKD cells. The apical domain of filter grown normal human kidney cells was labeled in vivo with biotin (biot) and then fixed and processed for immunofluorescence. The overlay Dsg labeling (green) and biotin-Cy5-streptavidin are shown in a xy view at the level of apical cell domains (note that because of filter wrinkling, more of the lateral domain and even the filter appear at the top left corner, whereas the apical surface of the cells gradually fades out in the bottom right corner) and the 3 separate channels are seen in a xz view. Bars = 20 μm. C: Western blot of streptavidin-precipitated, biotinylated proteins, blotted with anti-Dsg antibody. Dsg was exclusively basolateral (bl) in normal kidney cells (N), whereas basolateral and apical (ap) Dsg was seen in ADPKD cells (P). D: confocal image of cryosections of normal and polycystic tissue. Labeling against desmoplakin shows apical staining of the cyst-lining epithelia in polycystic kidney tissue (arrowheads) while only lateral signal can be observed in normal tubules (arrows). Bars = 30 μm. E: apical plasma membrane localization of desmosomal proteins was evidenced using peanut lectins that bind to the apical cell glycocalyx (green) and antibodies against DP (red). Shown in overlay images (desm+lectins) are normal kidney tubules and cyst-lining epithelial tissue sections (arrows denote colocalization seen only in PKD tissue). Bars = 20 μm.
expression was predominant and vimentin expression was minimal in normal kidney epithelial cells (Fig. 3A, NK). In contrast, vimentin remained highly expressed in ADPKD cells even after long-term culture and exhibited a conspicuous filamentous pattern throughout the cytoplasm of ADPKD cells (Fig. 3A, PKD, bottom). PKD cells expressed some cytokeratin, although the levels appeared to be significantly lower than in NK cells (Fig. 3A, PKD, top). To quantify vimentin and cytokeratin levels, lysates of NK and ADPKD cells were prepared 7 days postconfluence and analyzed by immunoblotting (Fig. 3B). Quantification revealed that PKD cells express five times more vimentin and four times less cytokeratin than normal cells (Fig. 3B). These results are in agreement with the cytoskeletal alterations found in vivo and demonstrate that the normal desmosome-intermediate filament system is disrupted in ADPKD.

**Abnormal cell-cell adhesion in polycystic kidney cells.** The structural abnormalities of desmosomal anchoring junctions and the cytoskeletal network found in ADPKD in these studies prompted us to examine the impact on ADPKD cell-cell adhesion at a functional level. Cell-cell adhesion was monitored by releasing monolayers with dispase, a neutral protease that cleaves fibronectin and type IV collagen but degrades type I collagen only minimally (39). After dispase treatment, the monolayer was subjected to shear stress using a published protocol modified to suit our primary cells (8, 20). The intercellular adhesions between cells from ADPKD patients were significantly more fragile, and cells were largely disaggregated after shaking as monitored by microscopy and cell counting (Fig. 4). The differences in the status of cell-cell adhesion were clearly evident by microscopic inspection even immediately after release of the monolayers with dispase and became even more pronounced after shaking (Fig. 4A). The numbers of single or doublet cells after shaking are expressed as a percentage of the total number of individual cells released after trypsinization. ADPKD cells were $83.4 \pm 2.2\%$ single or doublet cells after shaking while only $40.3 \pm 3.95\%$ of NK cells were present as single or doublet cells after the same procedure (Fig. 4B). These data demonstrate that PKD cells harbor a profound defect in cell-cell adhesion and that structural deficits in desmosomal junctions have a negative impact on the integrity and stability of the ADPKD epithelial sheet.

**Polycystin-1 colocalizes with desmosomal proteins when cells are subconfluent but not when fully polarized.** The observed mispolarization of desmosomal components in ADPKD described above and the report by other investigators (38) that polycystin-1 colocalizes with DP in MDCK cells cultured on plastic surfaces prompted us to analyze the relationship between polycystin-1 and desmosomal components in our primary human cells grown on filters. No colocalization between polycystin-1 and desmosomal proteins could be seen when desmosomes were fully mature, seen as a regular punctate pattern. In most stretches of the plasma membrane, polycystin-1 and desmosomal staining appeared mutually exclusive. Representative examples of DP and Dsg staining are shown in Fig. 5A. Pg, which is normally also present in adherens junctions, was occasionally seen to overlap with polycystin-1, mainly at cell corners, in fully confluent monolayers (data not shown).

Puzzled as to how our findings could be reconciled with previous ultrastructural studies showing polycystin-1 associated with desmosomes, we noted that the previous studies were...
performed on subconfluent MDCK cells (6, 38). This prompted us to repeat the immunolocalization studies on filter-grown normal human kidney cells at subconfluence. At subconfluence, polycystin-1 was significantly colocalized with desmosomal proteins as documented in MDCK cells (Fig. 5B). A homogenous, overlapping staining pattern for polycystin-1 and desmosomal proteins was seen at the plasma membrane, at sites of early cell-cell contact.

Fig. 3. ADPKD cells express an immature intermediate filament network even when fully confluent. A: confocal images of cultured primary normal kidney cells (NK) and ADPKD cells (PKD) labeled for cytokeratin intermediate filaments (CK). Bottom: confocal images of normal and polycystic kidney cells in culture, labeled for vimentin intermediate filaments (VIM). Bars = 20 μm. B: immunoblots of lysates from 2 different samples each of normal (NK) and ADPKD (PKD) kidney cells probed for CK or VIM and quantification of CK and VIM protein expression levels (blots show representative samples). The y-axis represents the intensity of the CK or the VIM band (OD CK) normalized according to the total protein loaded per lane as detected by Coomassie blue staining (OD com). CK graph: *P = 0.01. VIM graph: P = 0.057.

Fig. 4. Cell-cell adhesion is compromised in ADPKD cells. NK and PKD cell monolayers were incubated with dispase, released from the substrate, and briefly shaken for a fixed time. Two different ADPKD and two normal samples were analyzed in triplicate. A: cells were imaged with a Zeiss inverted light microscope. Top: NK and PKD cells forming confluent monolayers (×10 objective). Middle: normal kidney cells in large, interconnected sheets (NK cells), whereas ADPKD cells are in small aggregates (PKD) after incubation with dispase enzyme, but before shaking. Bottom: same samples after shaking, where the difference is further increased. B: quantification of the numbers of single cells in suspension after shaking was performed using a Beckman Coulter counter. The cell fraction between 10 and 20 μm in size was analyzed (single cells or doublets). Plotted are the percentages of single or doublet cells after shaking as a function of the total number of cells assayed by trypsinization (**P = 0.005).
Polycystin-1 colocalizes with desmosomes transiently during the establishment of cell-cell contacts in a calcium switch assay. To further evaluate the temporal relationship of polycystin-1 and desmosomal protein interactions, we subjected our primary normal human kidney epithelial cells to the well-established “calcium switch” assay (30). This assay involves incubating cells at full confluence in low-calcium medium to prevent cell junction assembly and then switching to high-calcium media to trigger synchronous junction assembly. Normal primary kidney cells were immunostained to localize polycystin-1 and various desmosomal components at different time points up to 24 h after raising the media calcium levels. Interestingly, at time 0 (immediately before returning cells to normal calcium), polycystin-1 was observed at the plasma membrane at points of intimate cell-cell contact (Fig. 6, 0 h DP). Despite the close cell-cell contact, desmosomal proteins were completely absent from the plasma membrane and were entirely contained in intracellular vesicles. In low calcium, cells that could not make contact with other cells harbored polycystin-1 in vesicles immediately under the plasma membrane, whereas desmosomal proteins were located deeper within the cell and not overlapping with polycystin-1 (data not shown). The data indicate that polycystin-1 arrives at the plasma membrane before desmosomal proteins and that polycystin-1 localization to sites of cell-cell contact is not calcium dependent. At intermediate time points, plasma membrane regions enriched in desmosomal staining or polycystin-1 staining were observed in a mutually exclusive pattern, as seen in confluent monolayers (Fig. 6, 2–6 h DP and 2–6 h Dsg). No significant difference in the state of cell polarization was seen between 2 to 6 h after the switch. Only sparse colocalization between polycystin-1 and desmosomes could be seen at this stage, suggesting a transient association (Fig. 7, 4 h DP and 4 h Dsg). Twenty-four hours after the calcium switch, almost all cells harbored desmosomes at the plasma membrane, and polycystin-1 was excluded from desmosomal positive structures (Fig. 6, 24 h DP and 24 h Dsg). Low levels of plasma membrane polycystin-1 at the level of desmosomal structures 24 h after the calcium switch may be due to the redistribution of polycystin-1 to other sites that are outside the confocal image slice.

The desmosomal and adherens junction plaque protein Pg showed different behavior. Pg was conspicuously colocalized with polycystin-1 early after restoration of normal calcium (Fig. 7B, 4 h Pg) but continued to colocalize with polycystin-1 even after 24 h (Fig. 7B, 24 h Pg). This finding is in agreement with published studies demonstrating an association between Pg and polycystin-1 in fully confluent cell lines through coimmunoprecipitation assays (43).

The data show that polycystin-1 is present at the plasma membrane of cell-cell contacts before cell-cell junction assembly and that there is a transient proximity between polycystin-1 and desmosomes during the early steps of junctional assembly, which is lost once desmosomal maturity is achieved.
In this study, we demonstrate the mispolarization of desmosomal proteins to the apical domain in ADPKD cells in culture and in cystic tissue. The desmosomal protein rearrangements are paralleled by an abnormal intermediate filament network in the disease cells with decreased cytokeratin expression and abnormal persistence of vimentin expression. The observed structural alterations in the adhesive junctions also contributed to functionally weakened cell-cell adhesion that made PKD epithelia increasingly vulnerable to dissociation in response to shear stress.

E-cadherin, which is intracellular in disease cells (10), has been demonstrated to be necessary for desmosomal assembly (27). In ADPKD cells, N-cadherin has been found to substitute for the loss of E-cadherin (36). N-cadherin, a mesenchymal cadherin, has proven as effective as E-cadherin in its capacity to stimulate desmosome assembly (33) and may account for the presence of desmosomal puncta at the lateral membrane of ADPKD cells. For this reason, it is unlikely that alterations in adherens junction assembly alone can account for the observed alterations in desmosome assembly in ADPKD.

Fig. 6. PC1 marks cell-cell contacts before junction assembly in a calcium switch assay. Cells were subjected to a calcium switch assay and processed at different time points after restoring normal calcium to the growth media. Desmosomal proteins (desm) were colabeled with PC1 and the overlay (merge) image is shown at right. DP, Dsg, and PC1 were labeled in cells fixed at time 0 (0 h), 2–6 h, or 24 h after the calcium switch. At 0 h, arrows denote PC1 at the plasma membrane in regions of cell-cell contact. At 2–6 h DP, arrowhead indicates plasma membrane region where desmosomes are already present and arrow denotes a less-developed plasma membrane zone, which is enriched in PC1. At 2–6 h Dsg, arrows denote the mutually exclusive staining pattern of desmosomes and PC1. Samples were imaged on Zeiss LSM510. Bars = 10 μm (0 h DP, bar = 20 μm).
Select cell surface molecules are well known to be mislocalized in ADPKD cell epithelia. \( \text{Na}^+\text{K}^+\text{-ATPase} \) and epidermal growth factor receptor are apically mislocalized in ADPKD (14, 48). This localization is normal in the developing kidney but not in the adult kidney where these proteins are principally at the basolateral membrane. In the case of the \( \text{Na}^+\text{K}^+\text{-ATPase} \), its mispolarization has been attributed to the continued expression of a fetal isoform of the \( \beta \)-subunit, which contains apical targeting information (46). \( \text{Pg} \), a protein shared by adherens junctions and desmosomes, also exhibits an apical localization during kidney development (19). Furthermore, in polarizing MDCK cells, the desmosomal cadherins Dsg and Dsc-2 are initially inserted at low levels over the entire cell surface, where the half-desmosomes are continually removed by endocytosis until stable cell-cell contact is established (13, 15). At the point of cell-cell contact, cells switch to a second stage of desmosome assembly that involves morphologically complex vesicles and selective targeting to nucleation sites on the basolateral membrane (7). Epithelial cells that undergo dedifferentiation in the course of tumorigenesis often express N-cadherin in lieu of E-cadherin and have elevated expression of vimentin just like ADPKD cells. However, in tumor cells, in contrast to ADPKD cells, desmosomes disappear completely from the plasma membrane and are found exclusively intracellularly. Therefore, the apical mislocalization of desmosomes and expression of an immature intermediate filament cytoskeleton in ADPKD are more consistent with the idea that ADPKD epithelia regress to a fetal epithelial phenotype in response to the expression of mutant polycystins (2) rather than a complete transition to a mesenchymal phenotype.

The loss of normal desmosome assembly in ADPKD cells raised the question as to whether polycystin-1 might be involved in desmosome assembly. Although other investigators have examined desmosome-polycystin-1 interactions and found them to colocalize ultrastructurally, the state of cell polarization was not taken into account (6, 38). Therefore, we evaluated desmosome assembly in relation to polycystin-1 from a dynamic point of view, both during the transition from subconfluent to fully confluent monolayers and in a calcium switch model. In subconfluent cells and immediately after restoring normal calcium, polycystin-1 and desmosomal pro-
teins were extensively colocalized at newly forming cell-cell contacts. As early as 2 to 6 h after returning cells to normal calcium, polycystin-1 and desmosomal proteins began to exhibit mutually exclusive localizations approaching the pattern seen in fully polarized cells. Only one desmosomal protein Pg remained partially colocalized with polycystin-1 in fully confluent monolayers. This may be due to a pool of Pg associated with adherens junctions or even a junction-independent pool. Owing to the high degree of desmosomal protein insolubility, even at early stages of desmosome formation, it was not possible to monitor their association with polycystin-1 by communoprecipitation beyond an association with Pg, as reported previously (19). Nevertheless, our data are in agreement with those of Scheffers et al. (38), who showed by electron microscopy intimate proximity between polycystin-1 and DP in MDCK cells. Based on the published literature and the present findings, we conclude that polycystin-1 is probably required at the lateral membrane early in the establishment of cell polarity, where it transiently associates with desmosomal components and may contribute to their proper assembly.

A paradox arises from the fact that ADPKD is an adult-onset disease, impacting tubular epithelia after the nephrons are already developed and kidney epithelial cells normally do not divide in the adult kidney. Furthermore, in fully confluent polarized epithelial cells, polycystin-1, although still present in small amounts at the lateral membrane, no longer colocalizes with desmosomes. At this stage, it was shown to accumulate in the apical primary cilium (Fig. 8) (50). In the cilia polycystin-1 and polycystin-2 serve in mechanosensory transduction and may play a role in the maintenance of cell polarity (29, 31). How then could mutant polycystin-1 lead to the desmosomal derangements found in this study? We speculate that as kidney epithelial cells start proliferating in response to a second site mutation in the PKD1 or PKD2 genes, it would dictate the need for new cell-cell junctions. At this moment, the requirement for polycystin-1 function to stabilize normal desmosomal localization would first become evident.

The mechanism whereby polycystin-1 might contribute to the proper polarization of desmosomal proteins at the basolateral domain remains a matter of speculation. We hypothesize that during normal development of cell-cell junctions, polycystin-1 serves as a surface sensor that signals initial cell-cell contact and then stabilizes desmosomes at the lateral membrane by promoting cells to switch to the second stage of desmosome assembly as defined by Burdett and Sullivan (7) (Fig. 8, schematic model).

It is also of interest to consider how improper desmosomal localization and cytoskeleton alterations contribute to disease pathogenesis in ADPKD. We envisage that it may contribute at several levels. First, the alterations in desmosomes (shown here) and in adherens junctions (shown previously in Refs. 10, 36) most likely account for the weakened cell-cell adhesion evidenced in the disase assay. This weakened cell-cell contact may gradually allow the increasingly migratory N-cadherin-positive cells to be extruded into the parenchyma as the normal cells attempt to reestablish a fully polarized, contiguous tubular epithelium by closing off the cyst. Second, the increased sensitivity of cystic epithelia to shear stress may also limit tolerance to cyst fluid pressure and cause breaks in the monolayer that predispose to infection and bleeding into the cyst. Third, improper desmosome location and linkage to the cytoskeleton may lead to abnormal growth control. Desmosomes, like adherens junctions, serve to anchor cytosolic plaque proteins with dual functions in cytoskeletal attachment and in cellular growth control (16, 17, 37, 41). It is possible that the derangement of desmosomes interferes with the complete sequestration of desmosomal plaque proteins, leading to an enhanced cytosolic and nuclear pool of Pg. Pg has the capacity to bind to the TCF/LEF transcription factor and inhibit β-catenin-mediated transcription. Pg can also compete with β-catenin for the proteasome-mediated degradative APC complex in the cytoplasm. Thus Pg mislocalization precipitated by incomplete or improper desmosome assembly could impact cell proliferation in ADPKD.

In summary, in this paper we show that normal polycystin-1 function is required for the exclusive lateral desmosome localization and formation of a mature cytoskeleton and its mutation leads to defects in cell-cell adhesion that may have significant consequences in disease progression.

ACKNOWLEDGMENTS

We are indebted to the anonymous patients and families who donated tissue through the Polycystic Kidney Disease Foundation (PKDF) and the National Disease Research Interchange to make this research possible. We gratefully acknowledge E. Romero for expert technical assistance and V. Bivins for administrative support. We thank M. Grady for help with the scanning of the film negatives. We also thank Dr. F. Bolognani for helpful discussions throughout the project. Immunofluorescence images in this paper were generated in the Fluorescence Microscopy Facility, which received support from the National Center for Research Resources (Grants P20-RR-11830, S10-RR-14668, S10-RR-016918), National Science Foundation Grant MCB-9982161, National Cancer Institute Grant R24-CA-88339, the University of New Mexico Health Sciences Center, and the University of New Mexico Cancer Center. We thank Dr. R. Lee and G. Phillips for expert technical support in the maintenance and operation of the Cancer Center Microscopy Facility.

GRANTS

Studies were supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant R01–50141, PKDF Grant 12A2R–12C2R, and American Heart Association Grant 0040211N to A. Wandinger-Ness and subcontracts to R. Bacallaio. M. Silberberg is supported by PKDF fellowship 72a2.

DISCLOSURES

Present address of A. Charron: Dept. of Molecular Microbiology, Washington University, St. Louis, MO 63104.

REFERENCES

7. Burdett ID and Sullivan KH. Desmosome assembly in MDCK cells: transport of precursors to the cell surface occurs by two phases of vesicular


