Alterations in cell-adhesive and migratory properties of proximal tubule and collecting duct cells from bcl-2 −/− mice

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Ziehr, Jacqueline, Nader Sheibani, and Christine M. Sorenson. Alterations in cell-adhesive and migratory properties of proximal tubule and collecting duct cells from bcl-2 −/− mice. Am J Physiol Renal Physiol 287: F1154–F1163, 2004. First published August 3, 2004; doi:10.1152/ajprenal.00129.2004.—Bcl-2 protects cells from apoptosis initiated by a variety of stimuli including loss of cell adhesion. Bcl-2 −/− mice develop renal hypoplastic/cystic dysplasia with renal cyst formation coinciding with renal maturation in normal mice. To gain a better understanding of the role cell-adhesive mechanisms play during renal maturation, we generated proximal tubule and collecting duct cell lines from postnatal day 10 (P10) and P20 bcl-2 +/+ and bcl-2 −/− mice. Very little is known about the role cell-adhesive and migratory mechanisms play during renal maturation. We observed that modulation of cell-adhesive properties, which normally occur in a nephron segment-specific manner during renal maturation, and cell migration were altered in cells from bcl-2 −/− mice. Enhanced migration of bcl-2 −/− proximal tubule cells in a scratch wound assay was completely inhibited by incubation with PP1 (Src inhibitor) and moderately affected by incubation with SB-203580 (p38 inhibitor). These cells expressed increased levels of fibronectin and had numerous central focal adhesions. P20 bcl-2 −/− proximal tubule cells adhered to fibronectin but adhered poorly to collagen, vitronectin, or laminin. Collecting duct cells, similar to proximal tubule cells from bcl-2 −/− mice, demonstrated enhanced migration in a scratch wound assay that was inhibited by incubation with PP1. Migration of these cells was moderately affected by incubation with PD-98059 (MEK inhibitor) or LY-294002 (PI3 kinase inhibitor), whereas incubation with SB-203580 had no effect. P10 bcl-2 −/− collecting duct cells also expressed increased levels of fibronectin but decreased levels of thrombospondin-1 and demonstrated precocious binding to fibronectin and vitronectin compared with bcl-2 +/+ cells. The ability of P20 bcl-2 −/− collecting duct cells to adhere to fibronectin and vitronectin corresponded with a decline in thrombospondin-1 expression. Therefore, alterations in cell-adhesive and migratory characteristics may be an early indicator of aberrant renal epithelial cell differentiation.

thrombospondin-1; fibronectin; focal adhesion; renal maturation

CELL MIGRATION, PROLIFERATION, differentiation, and apoptosis play integral roles during kidney development. The formation of nephron segments requires that a complex series of interactions occur to allow proper migration and positioning of specific cell types. After induction of the metanephros and condensation of the metanephric blastema, tubulogenesis proceeds through a histologically defined sequence of stages during which developing nephrons assume comma and S-shapes (2, 12). The rodent is born with immature kidneys in which nephrons continue to develop from the nephrogenic zone located at the periphery of the kidney. This period of time is termed renal maturation and is complete by postnatal day 16 (P16) (7). However, the molecular and cellular processes that are essential during renal maturation remain largely unknown.

Proper regulation of apoptosis is essential for normal kidney development and to maintain normal renal function in the adult. Bcl-2 plays an important role in regulating apoptosis during kidney development. Bcl-2 is highly expressed in the ureteric bud and metanephric mesenchyme during early development and gradually declines to low levels in the postnatal kidney (10). The physiological role bcl-2 plays during kidney development is not well understood. We have shown that cystic kidneys from bcl-2 −/− mice demonstrate nuclear localization of β-catenin, loss of apical brush-border actin staining, and sustained activation of focal adhesion kinase (FAK) and paxillin, suggesting that altered cell-adhesive properties may contribute to the cystic process (15, 18). We also showed that bcl-2 interacts with paxillin and FAK in metanephroi from normal mice (18). The physiological consequences of these interactions and the effect of loss of bcl-2 has on such interactions, perhaps contributing to renal cyst formation, require further investigation. We presently lack essential information as to what steps are critical for cyst formation and how to modulate this process. The loss of bcl-2 during early development affects the environment in which renal epithelial cells are required to survive, differentiate, and mature. Thus bcl-2-deficient renal epithelial cells may not receive the appropriate signals, form appropriate signaling complexes, or have such an excessive loss of progenitor cells that initiation of terminal differentiation may not be achieved.

Previous work from this laboratory demonstrated significant cyst formation in both proximal tubules and collecting ducts in P20 bcl-2 −/− mice (16). In the studies described here, we investigated cell-adhesive and migratory properties of proximal tubule and collecting duct cells from P10 (before renal maturation) and P20 (following renal maturation in normal mice) bcl-2 +/+ and bcl-2 −/− mice. Very little is known about the processes required for renal maturation. We demonstrate that modulation of cell-adhesive properties occurs in a nephron segment-specific manner and is aberrant as early as P10 in bcl-2 −/− mice. This is the earliest indication of renal maturation gone awry in bcl-2 −/− mice. We observed increased secretion of fibronectin, decreased secretion of thrombospondin-1, increased migration in a scratch wound assay, altered adhesion to extracellular matrices, and integrin expression. The Src kinase inhibitor, PP1, effectively inhibited mi-
igration of bcl-2 −/− proximal tubule and collecting duct cells. Therefore, altered cell-adhesive and migratory properties may be an earlier indicator of aberrant differentiation leading to renal cyst formation.

**MATERIALS AND METHODS**

**Cell preparation.** Immortomice expressing a temperature-sensitive SV40 large T antigen were obtained from Charles River Laboratories (Wilmington, MA). Bcl-2 −/+ mice were crossed with the Immortomouse and screened as previously described (16). The kidneys from P10 and P20 bcl-2 +/+ and bcl-2 −/− Immortomice were harvested from two to three mice of each genotype and processed individually. The experiments described here were performed with two separate isolations of cells with similar results.

The tissue was minced into small pieces in a 60-mm tissue culture dish using sterile razor blades and digested in 5 ml of collagenase type I (1 mg/ml in serum-free DMEM; Worthington, Lakewood, NJ) for 30–45 min at 37°C. After digestion, DMEM containing 10% FBS was added, and the cells were pelleted and resuspended in DMEM containing 10% FBS. The cells were resuspended in growth medium containing 10% FBS. The cells were maintained in growth medium containing 0.04% EDTA and incubated with 3 ml of dissociation inhibitor; 10 μg/ml; g/ml; Sigma, St. Louis, MO), 50 μg/ml streptomycin (Sigma, St. Louis, MO), 50 μg/ml gentamicin (Invitrogen), and 50 U/ml nystatin (Sigma) and plated on a 100-mm dish precoated with Matrigel (1:400 in serum-free DMEM:F-12 medium). The cells were plated and grown to near confluence. One 100-mm dish of cells was harvested by incubation with 2 mM EDTA in Tris-buffered saline (TBS) containing 0.05% BSA for 10 min and scraping. The cells were rinsed with serum-free DMEM:F-12 and incubated with magnetic beads (Dynex, Biotech, Brown Deer, WI) precoated with Dolichos biflorus agglutinin (DBA) or Lotus tetragonolobus agglutinin (LTA). After being bound, the magnetic beads were washed six times with DMEM containing 10% FBS and the bound cells were plated into a single well of a 24-well plate precoated with Matrigel (1:400 in growth medium). The cells were maintained at 33°C with 5% CO2. Cells were progressively passed to larger plates, maintained, and propagated on Matrigel (1:400)-coated 60-mm plates. The selection process was repeated twice.

**Western blot analysis.** For fibronectin and thrombospondin-1 analysis, cells were plated at 2 × 10⁴ per 60 mm on Matrigel-coated dishes and allowed to reach ~90% confluence in 2 days. The cells were then rinsed once with serum-free medium and incubated with serum-free growth medium (complete medium without serum) for 2 days. Then, conditioned medium was collected and centrifuged. The samples were mixed with appropriate volume of 6X SDS buffer and analyzed by 4–20% SDS-PAGE (Invitrogen). The proteins were transferred to a nitrocellulose membrane, and the membrane was incubated with a rabbit anti-rat fibronectin (Invitrogen) or anti-thrombospondin-1 (Clone A61.1, Neo Marker, Fremont, CA). The blot was washed, incubated with appropriate secondary antibody, and developed using ECL (Amersham, Piscataway, NJ).

**Indirect immunofluorescence staining.** Proximal tubule or collecting duct cells were plated on chamber slides (CC1:Falcon, Franklin Lakes, NJ) coated with Matrigel as described above. Cells were then rinsed with PBS, fixed with 3% of paraformaldehyde for 10 min on ice, washed two times with PBS, and incubated with anti-vinculin (1:100; Sigma), paxillin (1:200; BD Biosciences), β-catenin (1:600; Sigma), aquaporin-1 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), aquaporin-2 (1:100; Santa Cruz Biotechnology), calbindin (1:100; Santa Cruz Biotechnology), DBA-FITC (1:40; Vector, Burlingame, CA), or LTA-FITC (1:40; Vector) for 30 min at 37°C. After being washed three times with TBS, cells were incubated with appropriate CY3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) at 37°C for 30 min. Cells were washed three times with TBS, mounted, and photographed using a Zeiss fluorescence microscope (Axioskop, Zeiss, Germany) equipped with a digital camera.

**Scratch wound assays.** The migration of cells was assessed in scratch wound assays. Confluent monolayers of proximal tubule or collecting duct cells were wounded using a micropipette tip (1-ml blue tip), rinsed with growth medium, and wound closure was monitored for 48 h and photographed using a digital camera. The cells were grown on Matrigel- or fibronectin (2 μg/ml)-coated plates. Similar assays were performed in the presence of 5-fluorouracil (10 μg/ml; Sigma) to rule out the potential contribution of differences in cell proliferation. These experiments were repeated at least twice with similar results using different isolations of cells. In some cases, the cells were wounded and incubated in medium containing DMSO (control), PP1 (Src inhibitor; 10 μM), PD-98059 (MEK inhibitor; 50 μM), LY-294002 (PI3 kinase inhibitor; 3.25 mM), or SB-203580 (p38 inhibitor; 10 μM) for 48 h (all from Alexis, San Diego, CA).

**Cell adhesion assays.** Varying concentrations of fibronectin, laminin, vitronectin, and type I collagen (all from BD BioSciences) were coated on 96-well plates. As a control, wells were coated with BSA (1% BSA) overnight at 4°C. After being blocked with 1% BSA, 5 × 10⁴ cells were removed by EDTA, washed in serum-free medium, added to each well, and the cells were allowed to adhere to the plate for 1–2 h at 37°C. The plate was washed with PBS containing 1 mM Ca²⁺ and 1 mM Mg²⁺ until no cells remained in the wells coated with BSA. The number of cells in each well was quantitated by measuring the cellular phosphatase activity as previously described (11). All samples were done in triplicate.

**FACScan analysis.** FACScan analysis was performed essentially as previously described (11). The cells were washed once with PBS containing 0.04% EDTA and incubated with 3 mM of dissociation solution (Sigma) to remove the cells from the plate. The cells were washed with PBS, blocked in PBS containing 1% goat serum on ice for 20 min, and incubated with the appropriate dilution of primary antibody, anti-α (AB1920; Chemicon, Temecula, CA), α (AB1949; Chemicon), α (MAB1378; Chemicon), β (SC-8978; Santa Cruz Biotechnology), or control IgG (Chemicon). The cells were washed with PBS containing 1% BSA and then incubated with the appropriate secondary antibody on ice for 30 min. After the incubation, the cells were washed twice with PBS containing 1% BSA and resuspended in 0.5 ml of PBS containing 1% BSA. FACScan analysis was performed on a FACScan caliber flow cytomter (Becton-Dickinson, Franklin Lakes, NJ).

**Immunohistochemistry.** As previously described (18), fresh frozen kidney sections were fixed in acetone, blocked, and incubated with anti-rat fibronectin (1:500; Invitrogen). The sections were then incubated with indocarbocyanin (CY3)-labeled secondary antibody (Jackson ImmunoResearch). An equal timed photograph was then taken of the sections. Photographs were taken using a Zeiss fluorescence microscope (Axioskop, Zeiss) equipped with a digital camera.

**RESULTS**

**Bcl-2 −/− proximal tubule and collecting duct cells secrete increased amounts of fibronectin.** The cell migration and adhesion characteristics of renal epithelial cells during postnatal renal maturation remain largely unknown. Here, we used cell lines from bcl-2 +/+ and bcl-2 −/− mice to examine their cell-adhesive and migratory properties. We compared and contrasted proximal tubule and collecting duct cells prepared from P10 (before renal maturation) and P20 (following renal maturation) mice. Proximal tubule and collecting duct cells were prepared as described in MATERIALS AND METHODS. The identity of the proximal tubule cells was verified by positive staining for LTA and aquaporin-1, whereas collecting duct cells were positive for DBA, aquaporin-2, and calbindin (data not shown).
One characteristic of dedifferentiated cells or cells that have not completed terminal differentiation is that they tend to display increased expression of fibronectin compared with their differentiated counterparts (20). Serum-free medium was placed on P10 and P20 proximal tubule and collecting duct cells and collected 48 h later. Figure 1A is a Western blot of the conditioned medium blotted for fibronectin. We observed that proximal tubule and collecting duct cells from P10 and P20 bcl-2 −/− mice secrete increased amounts of fibronectin into the medium compared with bcl-2 +/+ cells.

We next examined deposition of fibronectin in kidneys from P20 bcl-2 +/+ and bcl-2 −/− mice. Kidneys from bcl-2 +/+ display faint fibronectin staining in the cortex (Fig. 1B). The arrow points to the renal capsule. In contrast, kidneys from bcl-2 −/− mice display increased deposition of fibronectin (Fig. 1B). We observed similar increased fibronectin staining in kidneys from bcl-2 −/− mice regardless of the degree of cyst formation (data not shown). Therefore, increased production of fibronectin also occurs in kidneys from bcl-2 −/− mice.

Collecting duct cells secrete a decreased amount of thrombospondin-1. Thrombospondin-1 is a secreted extracellular matrix protein that can modulate cell-adhesive and migratory properties through its interactions with a number of cell-surface receptors and cytokines (1, 13). Serum-free medium was placed on P10 and P20 proximal tubule and collecting duct cells and collected 48 h later. Figure 2 is a Western blot of the conditioned medium blotted for thrombospondin-1. Both bcl-2 +/+ and bcl-2 −/− P10 proximal tubule cells secreted a large amount of thrombospondin-1. This level decreased substantially in P20 proximal tubule cells.

The regulation of thrombospondin-1 in proximal tubule and collecting duct cells was not identical. P10 bcl-2 +/+ collecting duct cells secrete substantial amounts of thrombospondin-1, which declines to low levels at P20. In contrast, bcl-2 −/− collecting duct cells secreted consistently lower levels of thrombospondin-1 at the times examined. Thus thrombospondin-1 is inappropriately downregulated in P10 bcl-2 −/− collecting duct cells. These data suggest that cell adhesion and migratory properties may be affected in cells from bcl-2 −/− mice.

Bcl-2 −/− proximal tubule cells demonstrate increased migration in a scratch wound assay. We examined migration of proximal tubule and collecting duct cells using a scratch wound assay. Proximal tubule cells were wounded and grown in culture for 48 h (Fig. 3). At 48 h, only a few P10 bcl-2 +/+ cells had migrated into the wound (note arrows). In contrast,
These experiments were repeated 3 times with similar results. Therefore, bcl-2 −/− cells were more migratory. We observed that P20 bcl-2 −/− proximal tubule cells secreted increased amounts of fibronectin compared with their wild-type counterparts (Fig. 1). We next examined whether plating proximal tubule cells on fibronectin (2 μg/ml)-coated plates would influence their migration in a scratch wound assay. Figure 3B is a scratch wound assay of P20 bcl-2 +/+ and bcl-2 −/− proximal tubule cells plated on Matrigel- or fibronectin-coated plates. As observed previously, little if any migration of P20 bcl-2 +/+ proximal tubule cells plated on Matrigel-coated plates was noted (Fig. 3, A and B). However, when these same cells are seeded on fibronectin-coated plates, P20 bcl-2 +/+ proximal tubule cells nearly fill the wound in 48 h. P20 bcl-2 −/− cells plated on fibronectin behave similarly in a scratch wound assay to those plated on Matrigel with wound closure within 48 h. Thus the presence of fibronectin may influence cell migration. Several signaling pathways including PI3 kinase, Src, Erks, and p38 MAPK have been implicated in cell migration (8, 14). Altered signaling through these pathways could contribute to renal cyst formation due, in part, to sustained migration and altered adhesion. We next examined whether inhibitors of these signaling pathways could block migration of P20 bcl-2 −/− proximal tubule cells using the scratch wound assay. P20 bcl-2 −/− proximal tubule cells were wounded and grown in culture for 48 h in medium containing DMSO (control; data not shown), PP1 (Src inhibitor; 10 μM), PD-98059 (MEK inhibitor; 10 μM), LY-294002 (PI3 kinase inhibitor; 3.25 mM), or SB-203580 (p38 inhibitor; 10 μM). P20 bcl-2 −/− proximal tubule cells incubated with DMSO (control), LY-294002 (PI3 kinase inhibitor), or PD-98059 (MEK inhibitor) readily migrated into and closed the wound (Fig. 3C). In contrast, cells incubated with PP1 (Src inhibitor) exhibited little to no migration into the wound, whereas incubation with SB-203580 (p38 inhibitor) had a moderate effect on migration (Fig. 3C).

Bcl-2 −/− collecting duct cells demonstrate increased migration in a scratch wound assay. Migration of collecting duct cells was analyzed using a scratch wound assay. Similar to proximal tubule cells, collecting duct cells from P10 and P20 bcl-2 +/+ mice demonstrated little migration into the wounded area. Collecting duct cells from P10 and P20 bcl-2 −/− mice spread readily across the wound to fill the area (Fig. 4A).

We next incubated collecting duct cells with PI3 kinase, MEK, Src, and p38 MAPK inhibitors to determine whether these signaling pathways were involved in migration of P20 bcl-2 −/− collecting duct cells. The P20 bcl-2 −/− collecting duct cells were wounded and grown in culture for 48 h in medium containing DMSO (control; data not shown), PP1 (Src inhibitor; 10 μM), PD-98059 (MEK inhibitor; 50 μM), LY-294002 (PI3 kinase inhibitor; 3.25 mM), or SB-203580 (p38 inhibitor; 10 μM). P20 bcl-2 −/− collecting duct cells incubated with DMSO (control; data not shown), and SB-203580 readily migrated into and closed the wound (Fig. 4B). In contrast, addition of PP1 inhibited migration into the wound while decreased migration into the wound was observed in collecting duct cells incubated with PD-98059 and LY-294002 (Fig. 4B). These data suggest that differences in signal transduction pathway preferences may exist between tubular segments. This could indicate that adhesive characteristics may be unique to specific tubular segments at different stages of development.

Adhesion to extracellular matrices is development and nephron specific. The differences in migration observed in the proximal tubule and collecting duct cells suggested that altered adhesive mechanisms may exist. Adhesion to collagen, fibronectin, laminin, and vitronectin was assessed in proximal tubule and collecting duct cells from P10 and P20 bcl-2 +/+ and bcl-2 −/− mice (Fig. 5A). P10 proximal tubule cells did not adhere to collagen even at 10 μg/ml (Fig. 5A). After renal maturation, P20 bcl-2 +/+ proximal tubule cells adhere to collagen with saturation occurring at 1–2 μg/ml of collagen. No significant adhesion of P20 bcl-2 −/− proximal tubule cells to collagen was observed. Similarly, P10 bcl-2 +/+ and bcl-2 −/− proximal tubule cells do not adhere well to laminin, although modest adhesion was noted at the highest concentration of laminin (10 μg/ml) in P10 bcl-2 −/− cells. In contrast, P20 bcl-2 +/+ proximal tubule cells adhere well to laminin, whereas P20 bcl-2 −/− cells did not adhere. Therefore, adhesion to collagen and laminin increases following renal maturation (P20) in bcl-2 +/+ proximal tubule cells.

We examined the ability of proximal tubule cells to adhere to fibronectin and found that both bcl-2 +/+ and bcl-2 −/− proximal tubule cells adhere to fibronectin (Fig. 5A). Bcl-2 +/+ P10 and P20 proximal tubule cells demonstrated similar adhesion to fibronectin. Although P20 bcl-2 −/− proximal tubule cells retain their ability to adhere to fibronectin, it is significantly lower than that observed in P10 cells. A slightly different trend is observed for adhesion to vitronectin. P10 bcl-2 +/+ cells exhibited moderate adhesion to vitronectin with P10 bcl-2 −/− cells being even
Fig. 3. Proximal tubule cells from bcl-2 −/− mice displayed increased migration in a scratch wound assay. A: P10 and P20 proximal tubule cells were grown to confluence and wounded with a P1000 pipet tip. Photomicrographs were taken after 48 h. Under these conditions, bcl-2 −/− proximal tubule cells migrate to close the wound during the 48 h in culture. B: P20 proximal tubule cells were plated on Matrigel- or fibronectin (2 μg/ml)-coated plates. The cells were grown to confluence and wounded. Photomicrographs were taken after 48 h. Please note migration of bcl-2 +/+ proximal tubule cells on fibronectin-coated plates. Note arrow points to wound. C: P20 bcl-2 −/− proximal tubule cells were wounded and incubated with PP1 (Src inhibitor; 10 μM), PD-98059 (MEK inhibitor; 50 μM), LY-294002 (PI3 kinase inhibitor; 3.25 mM), or SB-203580 (p38 inhibitor; 10 μM). Photomicrographs were taken following 48 h. These experiments were repeated 3 times with similar results.
more adherent to vitronectin. However, a decline in the ability of P20 bcl-2+/− proximal tubule cells occurred with significant adhesion only noted at the highest concentration of vitronectin (10 μg/ml).

We next examined how adhesion to various matrices was affected in postnatal collecting duct cells (Fig. 5B). P10 bcl-2+/+ collecting duct cells did not adhere well to collagen, laminin, fibronectin, or vitronectin. Although P10 bcl-2−/− cells did not adhere well to collagen or laminin, these cells did adhere to vitronectin and fibronectin. P20 bcl-2+/+ and bcl-2−/− collecting duct cells demonstrated similar adhesive characteristics. Neither P20 bcl-2+/+ or bcl-2−/− cells adhered well to collagen or laminin, although both cells displayed a similar ability to adhere to fibronectin and vitronectin. The level of adhesion to fibronectin and vitronectin in P20 collecting duct cells was similar to that observed in the P10 bcl-2−/− collecting duct cells.

Aberrant integrin expression in P20 bcl-2−/− proximal tubule cells, P20 bcl-2−/− proximal tubule cells exhibit lack of adhesion to collagen and laminin. We next examined integrin expression of the α3-, α5-, α6-, and β1-subunits in proximal tubules by FACScan analysis (Fig. 6). Bcl-2+/- proximal tubule cells did not express detectable levels of α5-integrin; however, bcl-2−/− proximal tubule cells demonstrate significant α5-integrin expression. Similar expression of α3- and α6-integrin as well as a slight decrease in β1-integrin expression were observed in bcl-2−/− cells compared with their wild-type counter parts.

**Fig. 4.** Collecting duct cells from bcl-2−/− mice display increased migration. A: P10 and P20 collecting duct cells were grown to confluence and wounded. Photomicrographs were taken after 48 h. Under these conditions, bcl-2−/− collecting duct cells migrate to close the wound during the 48 h in culture. B: P20 bcl-2−/− collecting duct cells were wounded and incubated with PP1 (10 μM), PD-98059 (50 μM), LY-294002 (3.25 mM), or SB-203580 (10 μM). Photomicrographs were taken following 48 h. These experiments were repeated 3 times with similar results.
Formation of focal adhesions and cell-cell contacts. Vinculin staining was used to visualize focal adhesions in proximal tubule and collecting duct cells from P10 and P20 bcl-2+/+ and bcl-2−/− mice. A: adhesion of proximal tubule cells to matrices. B: adhesion of collecting duct cells to the various matrices. In all cases, ● represents bcl-2+/+ cells and ■ represents bcl-2−/− cells (means ± SD). These adhesion assays were repeated twice with similar results. All samples were performed in triplicate. OD, outer diameter.

DISCUSSION

Cell migration, proliferation, differentiation, and apoptosis play integral roles during nephrogenesis. Unfortunately, examining cell migration and adhesive mechanisms in an intact organ can be a difficult undertaking. Preparation of cell lines was an important step in understanding the cell-adhesive characteristics of proximal tubule and collecting duct cells during renal maturation and how altered regulation of these processes may contribute to renal cystic disease. Other laboratories have prepared immortalized kidney cell lines in this manner (9, 21) that have adhesive mechanisms similar to nonimmortalized
primary kidney cell lines (23, 24). Very little is known about regulation of cell-adhesive and migratory properties during renal maturation. However, our data emphasize the need to use appropriate age-matched and nephron segment-matched controls when studying normal and diseased kidneys. We observed that modulation of cell-adhesive properties which normally occur in a segment-specific manner was altered in renal epithelial cells from bcl-2−/− mice. Here, we show that bcl-2−/− proximal tubule cells demonstrated 1) enhanced migration in a scratch wound assay that was inhibited by incubation with PP1 (Src inhibitor) and moderately affected by SB-203580 (p38 inhibitor) (Fig. 3C); 2) increased expression of fibronectin and numerous central focal adhesions; and 3) decreased ability of P20 bcl-2−/− proximal tubule cells to adhere to fibronectin and an inability to adhere to collagen or laminin. Collecting duct cells from bcl-2−/− mice demonstrated 1) enhanced migration in a scratch wound assay that was inhibited by incubation with PP1 (Src kinase inhibitor) and slowed by incubation with PD-98059 (MEK inhibitor) or LY-294002 (PI3 kinase inhibitor); 2) increased levels of fibronectin and central focal adhesions; and 3) decreased ability to adhere to collagen or laminin. Collecting duct cells from bcl-2−/− mice demonstrated 1) enhanced migration in a scratch wound assay that was inhibited by incubation with PP1 (Src kinase inhibitor) and slowed by incubation with PD-98059 (MEK inhibitor) or LY-294002 (PI3 kinase inhibitor); 2) increased levels of fibronectin and central focal adhesions; and 3) decreased ability to adhere to collagen or laminin.
bronectin and decreased levels of thrombospondin-1 expression; and 3) precocious binding to fibronectin and vitronectin at P10. The ability of collecting duct cells to bind to fibronectin and vitronectin corresponded with a decline in thrombospondin-1 levels in these cells. This is consistent with anti-adhesive activity associated with thrombospondin-1 (5).

Bcl-2 plays an important role in regulating apoptosis during nephrogenesis. Bcl-2 expression is highest during early nephrogenesis and gradually declines to low levels in the postnatal kidney (3, 10). In the proximal tubule and collecting duct cell lines used in the studies presented here, bcl-2 protein expression was at undetectable levels. The important role bcl-2 plays during early nephrogenesis is underscored by the excessive apoptosis at embryonic day 12 (E12) in bcl-2 -/- mice (17) and resulting renal hypoplasia. It is likely that the lack of terminal differentiation in kidneys from bcl-2 -/- mice results from changes that occurred in an earlier stage of development. Loss of bcl-2 during early development would dramatically affect the environment in which renal epithelial cells are required to survive, differentiate, and mature. It is tempting to speculate that different cell types may adapt differently to these adverse circumstances. Thus loss of bcl-2 early during nephrogenesis may affect the ability of these kidneys to differentiate/mature perhaps as a result of inappropriate signal transduction in the postnatal kidney.

Renal cyst formation has been described as a benign neoplasm (6). As seen in many disease states, renal cysts typically contain dedifferentiated epithelial cells. Understanding the process of development including postnatal renal maturation is essential to understanding various renal diseases. Here, we demonstrate that cell-adhesive properties are impacted in a nephron and developmental stage-specific fashion. After renal maturation, bcl-2 +/+ proximal tubule cells acquire the ability to adhere to collagen and laminin. We also observed that following renal maturation, proximal tubule cells (bcl-2 +/+ and bcl-2 -/-) lose their ability to adhere to vitronectin. In contrast, P20 bcl-2 -/- proximal tubule cells demonstrate an inability to adhere to collagen and laminin.

Collecting duct cells demonstrate a different pattern of adhesion to these extracellular matrices. Before renal maturation (P10), bcl-2 +/+ collecting duct cells did not adhere to collagen, fibronectin, laminin, or vitronectin. These cells also expressed very low levels of fibronectin and significant levels of thrombospondin-1. After renal maturation, bcl-2 +/+ collecting duct cells gain the ability to adhere to fibronectin and vitronectin. This corresponded with a decline in thrombospondin-1 levels and a modest increase in fibronectin expression. Interestingly, both P10 and P20 bcl-2 -/- collecting duct cells adhere to fibronectin and vitronectin but not collagen or laminin. These cells also expressed greater amounts of fibronectin but low levels of thrombospondin-1. In addition, altered adhesion to extracellular matrices was observed in cells from postnatal polycystic kidneys from cpk mice (22). Therefore, gaining a better understanding of the processes required for normal renal maturation will give us important insight into which deregulated processes contribute to renal cyst formation.
Proximal tubule and collecting duct cells from postnatal bcl-2−/− mice migrate more rapidly into a wound than their wild-type counterparts and secrete more fibronectin. We also observed upregulation of α5-integrin in P20 bcl-2−/− proximal tubule cells. It is tempting to speculate that fibronectin plays a role in the enhanced migration because P20 bcl-2+/+ proximal tubule cells plated on fibronectin-coated plates lose their nonmigratory phenotype and instead migrate to fill a wound within 48 h (Fig. 3B). Although the trigger for increased secretion of fibronectin is elusive, excessive fibronectin may play an important role in a less dedifferentiated phenotype. An environment where fibronectin expression is high could favor focal adhesion formation disrupting cell-cell interactions. In addition, bcl-2−/− collecting duct cells express low levels of thrombospondin-1. Thrombospondin-1 levels are high at P10 and decrease to low levels at P20 in bcl-2+/+ collecting duct cells. Increased levels of thrombospondin-1 at P10 could increase transforming growth factor-β levels promoting differentiation and leading to a decrease in proliferation (4). Thus the appropriate expression of fibronectin and thrombospondin-1 in the postnatal kidney may be an essential part of renal epithelial cell differentiation.

Our previous work in intact cystic kidneys from P20 bcl-2−/− mice demonstrated sustained activation of Src and Erks but no alteration of p38 MAPK or PI3 kinase activation (19). Migration of bcl-2−/− proximal tubule and collecting duct cells is inhibited by the Src kinase inhibitor PP1. The inhibitor studies presented here also suggest that differences in signaling pathways usage may exist between different nephron segments in cystic kidneys from bcl-2−/− mice. Work from other laboratories suggests that Src activity correlates with migration (8, 14), whereas activation of the MAP kinase/Erks pathway correlates with cell survival and proliferation (8). Although this appears to be the case in proximal tubule cells, collecting duct cells from bcl-2−/− mice behave somewhat differently. Migration of P20 bcl-2−/− collecting duct cells was inhibited by PD-98059 (MEK inhibitor) and LY-294002 (PI3 kinase inhibitor), suggesting that migration in this segment may be more tightly regulated.

In summary, modulation of cell-adhesive properties occurs in nephron segment-specific manner. Although both bcl-2−/− proximal tubule and collecting duct cells demonstrated similar alterations in migration and secretion of fibronectin, differences in adhesion to extracellular matrices were consistent with their nephron segment of origin. These data imply that treatment of a renal disease in which multiple nephron segments are involved may be a complex undertaking. Therefore, understanding the processes involved during terminal differentiation of renal epithelial cells may allow us to identify their trigger(s), pointing the way for effective treatments of renal cystic disease.

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