β_4 Integrin and Laminin 5 Are Aberrantly Expressed in Polycystic Kidney Disease

Role in Increased Cell Adhesion and Migration

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Extracellular matrix alterations have been suggested to be part of the early events occurring in Autosomal Dominant Polycystic Kidney Disease (ADPKD), a disease characterized by formation of renal cysts and progressive renal failure. Here we report that cDNA array analysis identified β_4 integrin aberrant expression in ADPKD cells. Furthermore, laminin 5 (Ln-5), the main $\alpha_6\beta_4$ integrin ligand, was also found to be abnormally expressed in ADPKD. Studies performed with ADPKD cyst-lining epithelial cells (CC) by comparison with normal tubular cells indicate that integrin $\alpha_6\beta_4$ -Ln-5 interactions are involved in cellular events of potential importance for cystogenesis: 1) laminin 5 is a preferential adhesion substrate for CC, mainly through $\alpha_6\beta_4$ interaction, 2) CC increased haptotactic and chemotactic motility depends on the presence of Ln-5 and requires integrin $\alpha_3\beta_1$ cooperation, and 3) CC haptotactic or chemotactic migration is specifically increased by mAb-mediated β_4 integrin ligation, through an $\alpha_3\beta_1$ integrin-dependent and independent pathway, respectively. These results highlight the role of Ln-5 and $\alpha_6\beta_4$ integrin in adhesive and motility properties of cyst-lining epithelial cells, and further suggest that integrins and extracellular matrix modifications may be of general relevance to kidney epithelial cell cyst formation. (*Am J Pathol* 2003, 163:1791–1800)

Autosomal dominant polycystic kidney disease (ADPKD), the most frequently inherited kidney disease, is due to germline and somatic PKD1 or PKD2 gene mutations. The main clinical features is the progressive appearance and growth of multiple renal cysts, resulting in frequent end-stage renal failure. The proteins encoded by PKD1

and PKD2, polycystins 1 (Pc-1) and 2 (Pc-2), are long transmembrane proteins located in the primary cilia that interact and function together as a non-selective cation channel.¹ The long extracellular domain of Pc-1 may be involved in cell-cell and cell-matrix interactions. Signal transduction through Pc-1 (Wnt signaling, AP1, and G proteins activation) and Pc-2 (p38 and AP1 activation) has been reported, and a role for polycystins in cell differentiation and proliferation has been suggested (reviewed in^{2,3}).

In ADPKD renal cysts, somatic mutations of the wild-type allele of PKD1 and PKD2 and subsequent loss of the functional polycystin complex presumably trigger a cascade of signaling and gene expression events.³ To further understand how the Pc-1/Pc-2 disruption leads to cystogenesis, we performed cDNA array experiments to identify abnormally expressed genes in ADPKD. Among genes differentially expressed between cells derived from control and ADPKD kidneys, we decided to focus on those involved in cell proliferation, cell adhesion, and cell migration, because these events presumably play a key role in cystogenesis.

We were particularly interested by the detection of a strong overexpression of β_4 integrin in ADPKD cyst-derived cells. β_4 subunit associates with α_6 to form $\alpha_6\beta_4$ integrin, which is primarily expressed at the basal surface of most epithelia, and may participate in both cell adhesion and migration. Integrin $\alpha_6\beta_4$ is a key component of hemidesmosomes⁴ that link the keratin cytoskeleton with laminins in the basement membrane.⁴ Integrin β_4 activation can also trigger intracellular signaling through its particularly long cytoplasmic tail. Epidermal growth factor (EGF) stimulation leads to β_4 cytoplasmic tail phosphorylation, disruption of hemidesmosomes, and mobilization of β_4 to actin protrusions.⁵ Integrin β_4 interaction with laminin 5 (Ln-5) may also trigger intracellular signal transduction.⁶ Epithelial cells adhere to Ln-5 via two adhesive structures, focal adhesions and hemidesmosomes,

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through binding to $\alpha_3\beta_1$ or $\alpha_6\beta_1$ and $\alpha_6\beta_4$ integrins, respectively.⁷ On processing, Ln-5 may also trigger integrin-dependent migration and integrin-independent cell scattering.^{8,9} Ln-5 ligation to $\alpha_6\beta_4$ integrin receptor, for instance, may activate PI3K signaling and subsequent stimulation of other integrins implicated in cell motility, such as $\alpha_3\beta_1$.¹⁰

In this study, we identify $\alpha_6\beta_4$ integrin and Ln-5 aberrant expression in ADPKD, and characterize the functional consequences of $\alpha_6\beta_4$ integrin-Ln-5 interactions on ADPKD cell adhesion and migration. We report that 1) Ln-5 enhances $\alpha_6\beta_4$ integrin-mediated adhesion of CC, 2) Ln-5 induces $\alpha_6\beta_4$ - and $\alpha_3\beta_1$ -dependent haptotactic migration of CC, and 3) under EGF stimulation, β_4 integrin ligation promotes migration independently of $\alpha_3\beta_1$ integrin. These results point to distinct properties of $\alpha_6\beta_4$ in Ln-5 expressing epithelia, that may contribute *in vivo* to renal cyst enlargement in ADPKD.

Experimental Procedures

Kidney Specimens

We had the unique opportunity to benefit from an ADPKD kidney harvested before the onset of renal failure. This 32-year-old woman was pronounced brain-dead after a ruptured cerebral aneurysm and underwent organ harvesting. However, both kidneys appeared enlarged and multicystic and were refused for cadaveric donation, thus becoming available for research purposes. The serum creatinine at the time of nephrectomy was subnormal (15 mg/dl). The clinical diagnosis of ADPKD was retrospectively confirmed, based on 1) bilateral enlarged polycystic kidneys with typical histological features, 2) intracerebral aneurysm, and 3) a family history of autosomal dominant polycystic kidney disease. We also handled nine other polycystic kidneys, retrieved from ADPKD patients with end-stage renal failure before renal transplantation. As control, we handled normal portions of kidneys containing localized adenocarcinoma obtained from four age-matched patients.

Primary Cell Cultures of Cystic and Non-Cystic Epithelium

ADPKD and control kidneys were used to rise primary cultures of cystic renal tubular epithelial cells (CC) and non-cystic renal tubular epithelial cells (NC). The detailed methods have been published.¹¹ Each primary culture was derived from a pool of all cysts dissected within a kidney. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) containing 1% fetal bovine serum, 5 μ g/ml insulin, 10 μ g/ml transferrin, 5 ng/ml sodium selenite, 6.5 ng/ml Triiodothyronine, 10 ng/ml EGF, 500 ng/ml hydrocortisone, and 1% HEPES (Invitrogen). Reagents were purchased from Sigma (St. Louis, MO) unless indicated. For functional studies (adhesion, migration; see below) cells were cultivated 24 hours in DMEM, 1% HEPES (referred as "starvation"), harvested with cell dissociation buffer (Invitrogen), washed and resuspended in DMEM, 1% HEPES, even-

tually supplemented with various antibodies or EGF. We elected to exclusively use these primary cultures between passages 2 and 4.

cDNA Arrays

RNA was isolated using RNeasy miniKit (QIAGEN, Hilden, Germany) according to manufacturer. Atlas Human cDNA array-1 (BD Biosciences Clontech, Palo Alto, CA) was used for expression analysis per manufacturer's instructions. Briefly, 5 μ g of DNase-treated total RNA was converted to ³²P-labeled first-strand cDNA and purified using column chromatography to remove unincorporated nucleotides. Labeled cDNA was then used to hybridize Atlas cDNA nylon array in ExpressHyb mix (BD Biosciences Clontech) at 68°C overnight. The membrane was then washed twice in 2X SSC at 65°C for 30 minutes each and exposed to autoradiography for 24 hours. The orientation grid supplied by the manufacturer was then used to identify the positions of the hybridization signals.

Antibodies, Extracellular Matrix Proteins, and Reagents

Monoclonal antibodies used in this study were: anti-integrin α_2 (clone P1E6), anti-integrin α_3 (clone P1B5), anti-integrin α_6 (clone NKI-GoH3), anti-integrin β_4 (clone 3E1), and anti-integrin β_1 (clone 6S6) from Chemicon (Temecula, CA); anti-laminin 5: clone BM-165 against the $\alpha 3$ chain (Dr. Patricia Rousselle, Lyon, France) and clone D4B5 against the $\gamma 2$ chain (Chemicon); anti-cytokeratin (clone AE1-AE3) from DAKO (Glostrup, Denmark). Polyclonal antibody anti- β_4 (H-101) was from Santa Cruz Biotechnology (Santa Cruz, CA). Human laminin 5 (Ln-5; $\alpha 3\beta 3\gamma 2$) was purified from SCC25 cells;¹² collagen I and collagen IV were purchased from Sigma.

Immunohistochemistry

Cryostat sections of tissues were fixed with acetone and stored at -20°C . Before use, specimens were fixed again in chloroform, blocked with 5% goat serum and 1% bovine serum albumin (BSA) in PBS, incubated with the primary antibody for 1 hour, and treated with LSAB2-system horseradish peroxidase (HRP) (DAKO) according to the manufacturer's recommendations. Peroxidase staining was examined with an Ortho LEICA microscope coupled to a CDD camera (Olympus). Cells grown on coverslips (Costar, Cambridge, MA) were fixed with acetone, incubated with 3% hydrogen peroxide (DAKO), incubated 1 hour with primary antibody or negative control reagent, and subsequently treated as above.

Immunofluorescence

Monolayers were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes at room temperature, treated with 0.25% Triton X-100 for 10 minutes, and saturated for 30 minutes with 2% BSA in PBS. Cell monolayers were successively (after intermediate washes in PBS) incubated for 1 hour with a monoclonal

Table 1. Primers and PCR Conditions

Gene	Sequence of F and R primers	PCR product (bp)	Tam (°C)	Number of cycles
β_4 Integrin	F 5'-CCG TGT GGA TAA GGA CTG CG-3' R 5'-TGT AGG TGC CCG TGG TGT CC-3'	764bp	60	35
Laminin 5 ($\gamma 2$ chain)	F 5'-AAT GGG AAG TCC AGG CAG TGT ATC-3' R 5'-ACA GCG TTC TCC AGT AAC AGC TG-3'	407bp	58	33
GAPDH	F 5'-TCT CTG CCC CCT CTG CTG AT-3' R 5'-ATG ACC TTG CCC ACA GCC TT-3'	301bp	58	23

F, forward; R, reverse.

antibody to Ln-5 $\gamma 2$ chain (D4B5, 1:100), and a fluorescein isothiocyanate goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). The coverslips were mounted with Aquapolymount antifading solution (AGAR, Stansted, Essex, UK) onto glass slides and the slides were observed under a Leica fluorescence microscope.

RT-PCR Analysis

Aliquots of total RNA extracted from cells (1 μ g, RNeasy mini Kit; QIAGEN) or tissues (20 μ g, Trizol reagent; Invitrogen) were used as a template for cDNA synthesis. Reverse transcription was carried with 200 ng/ μ l random hexamers (C1181; Promega, Madison, WI) and M-MLV reverse transcriptase (Invitrogen). Polymerase chain reaction (PCR) was performed using 50 ng (cells) or 250 ng (tissues) of cDNA samples, 2U TaqDNA polymerase (M1665; Promega) in 10 mmol/L (pH 9) TrisHCl, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μ mol/L of each deoxynucleotide, and 500 nmol/L of each primer pair. Primers and PCR conditions are listed in Table 1. PCR products were analyzed by electrophoresis using 1.5% agarose gels and photographed as ethidium bromide fluorescent bands. GAPDH was used as control, allowing quantitative estimation of gene expression by computed densitometry (NIH Imager 4.1 software).

Western Blotting

Cells were harvested with cell dissociation buffer (Invitrogen-BRL), lysed and centrifuged; proteins (20 μ g) were boiled under denaturing condition, separated on 7.5% SDS-PAGE, and blotted to PVDF membranes (Millipore Corp., Bedford, MA). Blots were saturated 1 hour at 37°C with TBST (20 mmol/L/L Tris-HCl, pH 8, 137 mmol/L/L NaCl, 0.1% Tween-20) containing 2% BSA and incubated 1 hour at room temperature with 1:200 anti- β_4 antibody sc-9090 (Santa Cruz); β_4 integrin was visualized using an enhanced chemiluminescence system (ECL plus; Amersham Biosciences Inc., Buckinghamshire, UK) and Biomax films (Kodak). Membranes were rehybridized with anti-ERK1/2 (Sigma) to ensure equal loading.

Cell Adhesion and Motility

Adhesion

Adhesion assays were performed in 96-well plates. Wells were coated 3 hours at 37°C with 10 μ g/ml Ln-5,

collagen I or collagen IV, washed in PBS, and blocked with 1% BSA (Sigma) at 37°C for 2 hours. 1×10^4 cells were then plated and allowed to attach for 1 hour. Non-adherent cells were washed away. Adherent cells were fixed with absolute ethanol, colored (crystal violet 0.2% in 2% ethanol), visualized using a $\times 20$ objective, and counted under a light microscope with an eyepiece grid. At least three optic fields in triplicate wells were counted for each condition.

Modified Boyden Chamber Motility Assays

Haptotactic migration (ie, migration toward ECM components) was studied using modified Boyden chambers (Transwell 24-well plates, 8.0- μ m pore size; Costar). The lower chambers of the plate were loaded with DMEM and overlaid by a 8.0 μ m polycarbonate filter eventually coated with Ln-5, collagen I, or collagen IV (as above). CC or NC starved cells (2×10^4 in 100 μ l of DMEM) were then added to the upper chamber of the filters and allowed to migrate through the filters for 12 hours at 37°C in 5% CO_2 . On completion filters were removed and fixed with absolute ethanol. Cells that had not migrated were removed from the top of the filter with a cotton swab; cells that had migrated (remaining on the bottom side of the filter) were stained (crystal violet 0.2% in 2% ethanol) and enumerated as above (cf adhesion assays).

The same modified Boyden chambers were used to quantify EGF-stimulated migration. For these assays, starved CC or NC cells (4×10^4 in 100 μ l) were stimulated by EGF at the time of cell plating and allowed to migrate through uncoated filters for 18 hours. Cells that migrated through the filters were identified and counted as outlined above.

Wound Healing

When reaching 90% to 100% confluence, NC or CC cells grown on plastic coverslips (Costar) were starved and the following day, a straight and uniform scratch was made on the monolayer with a 200- μ l plastic pipette tip. Monolayers were washed gently and incubated 20 hours at 37°C in DMEM $\pm$ EGF. For each experimental condition, the area of the wound was assessed at various time points in 10 fields initially marked for reference, using a 100-unit eyepiece optic grid under a light microscope. The difference in number of optic units filled by cells before and after wound repair is expressed as mean $\pm$ SD.

Figure 1. β_4 integrin overexpression in primary cultures derived from ADPKD kidneys. **A:** RT-PCR products for β_4 and GAPDH genes. Each bar represents the β_4 /GAPDH ratio for primary tubular epithelial cells derived from normal (white bars, NC 1 to 4) or ADPKD kidneys (black bars, 1 to 10). **B:** Immunoblot analysis of β_4 integrin in control (NC) and cystic cells (CC); anti-ERK1/2 was used as a control for loading. **C:** Immunocytochemical peroxidase staining on subconfluent CC and NC with the following mAbs: anti- β_4 3E-1; anti- α_3 P1B5; anti- α_6 GoH3; anti- β_1 6S6; anti-cytokeratin positivity assesses the epithelial nature of the cells. Negative isotypic controls are not shown.

Inhibition Experiments

For adhesion and Transwell migration inhibition assays, cells were first pre-incubated for 30' at 4°C with anti-integrin antibodies. For wound healing inhibition assays, antibodies (and EGF) were added to the medium later, at the time of stimulation. The concentrations of the antibodies used for inhibition experiments (10 μ g/ml) were equal or up to two times the concentration giving the maximal inhibition in preliminary dose-response experiments.

Statistical Analyses

Experiments were performed in triplicate at least three times each, with primary cultures of different origin. Data are expressed as means $\pm$ SD. Statistical significance was determined using Student's *t*-test. *P* < 0.05 was considered to be significant.

Results

Cystic Epithelial Cells of ADPKD Patients Aberrantly Express β_4 Integrin and Ln-5

mRNA isolated from a set of ADPKD cyst-derived cells (CC, *n* = 3) and control kidney tubular cells (NC, *n* = 3) were used to perform cDNA arrays. Comparison of hybridization signals by optic density scanning suggested that the gene encoding β_4 integrin was overexpressed in ADPKD cyst-derived cells (not shown). These results were confirmed by semiquantitative RT-PCR: the β_4 /GAPDH ratio was consistently higher (two to four-fold) when tested in 10 CC when compared to four NC primary cultures (Figure 1A). To confirm that this difference holds true at the protein level, we performed Western blot analysis of cell lysates using H-101 polyclonal antibody. Indeed, a much stronger β_4 protein expression was detected in CC than in NC (Figure 1B). To test whether β_4 integrin overexpression was specific, or a more general

Figure 2. β_4 integrin is overexpressed in ADPKD kidneys. **A:** Integrin β_4 mRNA levels in control and ADPKD kidneys. **Bottom**, ethidium bromide stained RT-PCR products. **Top**, graphic representation of the β_4 /GAPDH ratio in kidneys retrieved from three control subjects (open bars, normal tissue, NT 1 to 3) and 2 ADPKD patients (filled bars, cystic tissue, CT 1 and 2). **B, a to d:** β_4 immunohistochemical peroxidase staining on kidney sections with 3E1 antibody. **a:** Normal adult kidney ($\times 10$); **b**, ADPKD patient ($\times 10$, * denotes large cysts); **c**, undilated tubules in a ADPKD patient with normal renal function ($\times 40$); **d**, normal fetal kidney at 33 weeks of gestation ($\times 40$). **e** and **f:** Serial sections of an ADPKD kidney, stained with anti- β_4 3E1 (**c**, $\times 5$) and anti- α_6 GoH3 (**d**, $\times 5$).

feature of integrin expression in ADPKD, we performed immunocytochemical experiments. β_4 integrin was undetectable in NC and positive in CC with two different anti- β_4 antibodies, 3E1 (Figure 1C) and ASC-9 (not shown); by contrast, α_3 , α_6 , and β_1 integrins were strongly expressed in both types of cells (Figure 1C).

We next wanted to test whether β_4 overexpression was also a feature of *in vivo* kidneys. With a set of primers designed to amplify a band of 764 bp, which corresponds to all four known β_4 isoforms, RT-PCR amplification showed that the β_4 /GAPDH transcript ratio was much higher (three to fourfold) in two ADPKD kidneys compared to three control kidneys (Figure 2A). Immunohistochemistry with the 3E1 antibody, directed against the extracellular domain of β_4 integrin, showed no detectable β_4 integrin expression in normal adult kidney [Figure 2B(a)]. By contrast, in five different adult ADPKD patients, 70% to 80% of the kidney cyst-lining epithelia exhibited a strong β_4 basolateral expression [Figure 2B(b)]. Of note, aberrant β_4 expression was also observed on undilated tubules in a patient with ADPKD and normal renal function [Figure 2B(c)], suggesting that it is an early event. Since ADPKD epithelial cells have been reported to express fetal markers, we also studied β_4 immunostaining

in two fetal kidneys (33 weeks of gestation), which was found positive in the medullar collecting ducts [Figure 2B(d)]. Additional stainings performed on serial sections showed a perfect match between β_4 and α_6 integrin stainings, as expected [Figure 2B(e, f)]. The proportion of cysts of proximal origin (stained by lotus tetragonolobus lectin), of distal origin (stained by $\alpha 3$ chain of collagen IV and epithelial membrane antigen antibodies) and originating from the collecting ducts (stained by dolichos biflorus agglutinin lectin) or showing no nephron segment specificity were the same, whether the cysts were positive or negative for β_4 staining (not shown). Positive β_4 staining was also independent of the size of the cysts.

We then asked whether Ln-5, an $\alpha_6\beta_4$ integrin ligand, was expressed in ADPKD kidneys. RT-PCR was performed using primers designed to amplify the LAM γ 2 gene, encoding for the Ln-5-specific $\gamma 2$ chain. LAM γ 2/GAPDH transcript ratio was two- to 3.5-fold higher in ADPKD than in normal renal tissues (Figure 3A). Additionally, immunohistochemical staining for Ln-5-specific $\gamma 2$ chain (D4B5 Ab) and Ln-5 $\alpha 3$ chain (BM 165 Ab) showed strong positivity in BMs adjacent to cystic epithelia in ADPKD patients [Figure 3B(a and b)]. By contrast, no significant Ln-5 staining was observed in control

Figure 3. Ln-5 is overexpressed in ADPKD kidneys. **A:** Overexpression of Ln-5 γ 2 chain (LAM γ 2) mRNA in ADPKD kidneys. Results are presented as in Figure 2A. **B:** Immunohistochemical peroxidase staining of ADPKD kidneys using anti-Ln-5 γ 2 chain (D4B5) antibody on an end-stage renal disease ADPKA patient (**a**) and anti-Ln-5 α 3 chain (BM 165) antibody on the early ADPKD kidney with preserved renal function (**b**). **c:** Immunohistochemical staining of control kidney with D4B5 Ab. **d:** Fetal kidney at 12 weeks of gestation, stained with BM 165 Ab. All magnifications, $\times 10$.

adult kidneys [Figure 3B(c)]. Overall, Ln-5 positivity was found in 70% of ADPKD cysts. Importantly, Ln-5 pericyclic aberrant expression was already observed in a patient with preserved renal function [Figure 3B(b)]. Interestingly, Ln-5 and β 4 integrin aberrant expression correlated nicely in individual cysts (not shown). When fetal kidneys were studied, Ln-5 expression was found early in development, at 12 weeks, in the ureteric bud [Figure 3B(d)]. As for β 4 integrin, this suggested aberrant reexpression of a fetal marker in ADPKD. To test the specificity of this aberrant laminin expression, we studied the expression of laminin 10/11 α 5 chain; immunohistochemistry showed a strong positivity in ADPKD but similar to control kidneys (not shown).

α 6 β 4 Integrin Mediates Enhanced Cystic Cells Adhesion to Ln-5

We first assessed the binding of CC and NC on plastic support coated with increasing concentrations of Ln-5 (1 to 50 μ g/ml). As shown on Figure 4A, Ln-5 is a preferential adhesion substrate for CC, in a dose-dependent manner, with an optimal differential adhesion seen at 50 μ g/ml. The 10 μ g/ml Ln-5 concentration was chosen for subsequent adhesion assays. The Ln-5 specificity of this adhesion preference was demonstrated by comparing adherence of CC and NC to other ECM components, ie, collagen I and IV. No significant differences were seen on plastic, collagen I- or collagen IV- coated dishes, whereas adhesion to Ln-5 was more than twofold higher for CC (Figure 4B). A role for α 6 β 4 integrin in preferential CC adhesion to Ln-5 was studied using monoclonal blocking antibodies (Figure 4C). On a Ln-5 support, anti-

Ln-5 mAb (BM 165) exerted a 70% inhibition of CC attachment; mAbs against integrins α 6 and β 4 similarly decreased to $\sim 40\%$ CC adhesion. By contrast, anti- α 2, α 3, and β 1 mAbs had little effect on cell binding, suggesting that α 6 β 4 integrin was the major mediator of CC adhesion to Ln-5 (Figure 4C). NC adhesion to Ln-5 was not blocked by anti- β 4 mAb, and was partially blocked by anti- α 6, α 3, and β 1 mAb, suggesting that the two other integrin receptors for Ln-5, ie, integrins α 3 β 1 and α 6 β 1, were responsible for NC adherence to Ln-5 (not shown). On a collagen I support, a preferential ligand for α 2 β 1 integrin, CC adhesion was not influenced by anti-Ln-5 and anti-integrins α 6 or β 4 mAbs, showing the specificity of the effects observed in Figure 4C, while anti- α 2 and anti- β 1 blocking mAbs exerted a strong inhibitory effect, as expected (Figure 4D).

Laminin 5 Is a Strong Haptoattractant for CC and Is Required for EGF-Stimulated Chemotactic Migration

To study the role of Ln-5 in CC motility, we first performed haptotactic migration assays using Ln-5-coated Transwell filters. Quiescent CC displayed a two-fold higher spontaneous migration toward Ln-5 than NC (Figure 5A). The Ln-5 specificity of this migration preference was demonstrated by comparing haptotaxis of CC and NC toward other ECM components; both cell types showed weak and comparable spontaneous migration toward collagen I or IV, and virtually no migration through uncoated filters (Figure 5A). Ln-5 displayed a dose-dependent haptotactic effect on CC (Figure 5B), and a 10 μ g/ml

Figure 4. Enhanced adhesion of CC to Ln-5 is $\alpha_6\beta_4$ -integrin-dependent. **A:** CC (triangles) and NC (squares) were plated on Ln-5 (0, 1, 10, and 50 $\mu\text{g/ml}$) in triplicate. Adherent cells were stained and counted as described in Materials and Methods. Results are expressed as mean $\pm$ SD ($n = 3$) of relative cell adhesion, with cell adherence to uncoated support set as 1. **B:** CC (filled bars) and NC (open bars) were plated on 10 $\mu\text{g/ml}$ collagen I, collagen IV, or laminin 5. Results are expressed as mean $\pm$ SD of the absolute number of cells counted in three optic fields per well. One representative experiment out of three is shown. **C:** CC were preincubated at 4°C for 30 minutes with the indicated antibodies: none, P1B5 (anti- α_6), GoH3 (anti- α_3), 6-S6 (anti- β_1), 3 E1 (anti- β_4), or BM 165 (anti-Ln-5), all at 10 $\mu\text{g/ml}$, plated on plastic precoated with 10 $\mu\text{g/ml}$ Ln-5 (filled bars). Open bar represents cell adhesion on uncoated support without blocking antibodies. Results are expressed as mean $\pm$ SD ($n = 3$) of relative cell adhesion, with cell adherence to Ln-5 set as 100%. **D:** CC were preincubated with the indicated antibodies before plating on collagen I (10 $\mu\text{g/ml}$). Results are expressed as mean $\pm$ SD ($n = 3$) of relative cell adhesion, with cell adherence to collagen I set as 100% (* $P < 0.05$; ** $P < 0.01$).

coating concentration was chosen for subsequent experiments. To further confirm the specificity of Ln-5-mediated CC haptotaxis, we used an anti-Ln-5 blocking mAb (BM 165), which almost completely blocked CC migration toward Ln-5 (Figure 6A, column 1).

As cystic cells *in vivo* are in contact with cyst fluid containing relatively high concentrations of EGF,¹³ we next analyzed renal epithelial cell EGF-stimulated motility. EGF stimulation of cells before migration through uncoated Transwell filters was dose-dependent, with a maximal stimulation observed at 10 ng/ml; CC exhibited a two- to three-fold stronger chemotactic migration than NC with each EGF concentration tested (Figure 5C). We further tested EGF-stimulated motility in a scratch assay, an *in vitro* model for wound healing in various epithelial systems. After 20 hours of EGF stimulation (10 ng/ml) CC displayed a 50% increase in wound healing compared to NC (Figure 5D). To test the role of Ln-5 in EGF-induced CC migration, we used BM 165 blocking mAb. Surprisingly, BM 165 was able to almost completely block EGF-induced CC migration through Transwell and during wound healing (Figure 6A, columns 2 and 3). A control isotypic mAb did not modify CC migration in both assays (not shown). To confirm the relevance of these findings, we further tested whether CC were actually synthesizing Ln-5 in our system. Indeed, immunofluorescence staining with anti- γ_2 chain mAb D4B5 revealed a strong Ln-5 network surrounding CC, while staining with control isotypic Ab was negative (Figure 6B). Altogether, these data

Figure 5. Enhanced cystic cell migration in three different models. **A:** Haptotactic migration of CC (filled bars) and NC (open bars) through Transwell filters either uncoated or coated with 10 $\mu\text{g/ml}$ collagen I, collagen IV, or Ln-5. Migrated cell count is expressed as mean $\pm$ SD ($n = 6$ microscopic fields) on two filters per condition. **B:** Effect of Ln-5 coating concentration (0 to 10 $\mu\text{g/ml}$) on CC haptotaxis through Transwell filters. **C:** Migration of CC (filled bars) and NC (open bars) through uncoated Transwell filters after EGF stimulation (0, 1, 10, or 100 ng/ml). **D:** Cell migration during EGF-stimulated wound healing. On micrographs (magnification, $\times 10$), arrows indicate the wound edges. Bar graph depicts quantification (see Materials and Methods) of cell migration after 9 and 20 hours (triangles, CC; squares, NC). Significant increase in CC wound healing was observed at 20 hours. (* $P < 0.05$; ** $P < 0.01$).

suggest that Ln-5 is a strong haptotactant for CC, and that EGF-induced CC motility requires Ln-5 production.

β_4 Integrin Ligation to 3E1 Antibody Specifically Stimulates CC Migration

The possibility that $\alpha_6\beta_4$ integrin expression influenced migration of CC was assessed *in vitro* by using the three migration models described above. Although anti- β_4 integrin antibody 3E1 exerted a blocking effect on adhesion, it consistently stimulated CC migration, either haptotaxis toward Ln-5 (+ 75%) [Figure 7A(a)], EGF-induced Transwell migration (+ 120%) [Figure 7A(b)], or EGF-stimulated wound healing (+ 50%) [Figure 7A(c)]. While increasing 3E1 concentrations had no effect on NC, they exerted a dose-dependent effect on CC haptotaxis toward Ln-5 (Figure 7B), consistent with the β_4 integrin overexpression in CC. Another adhesion blocking anti- β_4 mAb (ASC-9) resulted in a similar albeit milder stimulation of CC migration (not shown). The promigratory effect of these anti-integrin β_4 mAbs raised two non-exclusive hypothesis: 1) an effect of β_4 ligation on cell machinery that would directly promote migration, and 2) an indirect effect mediated by increased $\alpha_3\beta_1$ and/or $\alpha_6\beta_1$ -dependent migration.

Figure 6. Inhibitory effect of 10 $\mu\text{g/ml}$ BM165 anti-Ln-5 antibody on three CC migration models. **A**, panel **a**: inhibition of CC haptotaxis toward laminin-5 coated filters (filled bars, 10 $\mu\text{g/ml}$; first lane, uncoated support). Results are expressed as mean $\pm$ SD ($n = 3$) of relative cell migration, with migration toward Ln-5-coated filters set as 100%. **b**: Inhibition of CC chemotaxis. Where indicated, CC were incubated with 10 ng/ml EGF (filled bars) $\pm$ BM165 before plating. Results are expressed as mean $\pm$ SD ($n = 3$) of relative cell migration, with EGF-stimulated migration set as 100%. **c**: Inhibition of wound healing. Serum-starved CC were allowed to migrate from the edges to re-epithelialize the wounded surface for 20 hours in DMEM $\pm$ EGF (10 ng/ml, filled bars) $\pm$ BM165. Results are expressed as mean $\pm$ SD ($n = 3$) of relative cell migration, with EGF-stimulated wound healing set as 100%. **B**: Endogenous Ln-5 deposition by CC in cultures (**a**, $\times 40$) and negative isotypic control (**b**).

β_4 Ligation to 3E1 Stimulates $\alpha_3\beta_1$ -Dependent Haptotaxis and $\alpha_3\beta_1$ -Independent Chemotaxis

To further address these hypotheses, we performed migration assays with anti-integrins blocking antibodies. In the Ln-5 haptotaxis model, migration was dramatically reduced by preincubating cells with anti-integrin α_3 (-80%), anti- α_6 (-75%) and anti- β_1 (-70%) mAbs, but not by anti- α_2 mAb (Figure 8A). The stimulating effect of 3E1 mAb was almost completely blocked by antibodies

Figure 7. Anti- β_4 integrin 3E1 mAb enhances CC haptotactic and EGF-induced chemotactic migration. **A**: Stimulating effect of 3E1 mAb on three cell migration assays (performed as in Figure 6). Anti- β_4 integrin mAb 3E1 stimulates haptotactic migration toward Ln-5 (**a**), EGF-stimulated chemotactic migration through Transwell filters (**b**), and EGF-stimulated wound healing (**c**). **B**: 3E1 stimulates CC but not NC haptotaxis toward Ln-5. CC (triangles) and NC (squares) were preincubated with or without 3E1 (1, 5, or 10 $\mu\text{g/ml}$). Spontaneous haptotactic migration toward Ln-5 was set as 100%.

Figure 8. Haptotactic migration of CC on Ln-5 is $\alpha_3\beta_1$ integrin-dependent, while chemotactic CC migration becomes $\alpha_3\beta_1$ independent after stimulation with β_4 antibody 3E1. **A**: Haptotactic CC migration toward Ln-5 coated filters was performed and quantified as described in Figure 6. Cells were preincubated with 10 $\mu\text{g/ml}$ P1E6 (anti- α_2), P1B5 (anti- α_3), GoH3 (anti- α_6), 6-S6 (anti- β_1), or 3E1 (anti- β_4). **B**: EGF-stimulated CC wound repair was performed and quantified as described in Figure 6. Cells were incubated during wound healing with 10 $\mu\text{g/ml}$ P1E6 (anti- α_2), P1B5 (anti- α_3), GoH3 (anti- α_6), 6-S6 (anti- β_1), or 3E1 (anti- β_4).

directed against $\alpha_3\beta_1$, and partially blocked by antibodies directed against $\alpha_6\beta_1$ (Figure 8A, last 3 columns), suggesting that the stimulating effects of β_4 ligation operate mainly through an $\alpha_3\beta_1$ -dependent pathway. In the EGF-stimulated wound repair assay, anti- α_3 mAb strongly inhibited CC migration (-70%), while anti- α_2 , anti- α_6 , and anti- β_1 mAb exerted a moderate inhibition (-27% , -5% and -33% , respectively; Figure 8B), suggesting that in this assay, CC migration is also mainly $\alpha_3\beta_1$ -dependent. In this assay however, the promigratory effect of 3E1 was not blocked by the combination of anti- β_1 and either anti- α_3 or anti- α_6 , suggesting that β_4 integrin ligation is able to promote migration independently of these integrins.

Discussion

Normal renal tubular epithelia synthesize and interact with the adjacent basement membrane, a specialized region of the extracellular matrix (ECM) containing type IV collagen, laminins, fibronectin, entactin, and proteoglycans. In ADPKD, it has long been stated that abnormal interactions between cyst-lining epithelia and surrounding basement membrane may play an important role in cystogenesis.³

Through cDNA arrays screening of differentially expressed genes, we identified a strong overexpression of β_4 integrin in ADPKD cystic cells. RT-PCR analysis confirmed that β_4 integrin transcripts were overexpressed in ADPKD compared to normal kidneys. Immunohistochemistry further demonstrated a strong β_4 integrin expression in most ADPKD cyst-lining epithelia, whereas no expression was detected in normal adult tubules. Our observation of β_4 integrin overexpression in slightly dilated ADPKD tubules in a patient with preserved renal function [Figure 2B(c)] suggests it is an early event in the cyst formation process and that this finding is not related to end-stage renal disease. Strong β_4 integrin expression during normal fetal kidney development suggests that β_4 integrin may be either persistently expressed after birth in ADPKD tubules, or reexpressed later during cystogenesis. Costaining with specific markers ruled out β_4 integrin aberrant expression as a nephron segment-specific event. Expression of other integrins (α_2 , α_3 , α_6 , and β_1) by immunohistochemistry was similar in ADPKD and control

kidneys (not shown), highlighting the specificity of β_4 integrin overexpression in ADPKD. The reason why a few cysts did not express β_4 integrin remains obscure.

Ln-5, the main ligand for $\alpha_6\beta_4$, was also aberrantly expressed in most of the cyst-surrounding ECM. Other authors have reported the strong expression of unspecified laminin in the Cy rat model of ADPKD,¹⁴ and the presence of uncharacterized laminin breakdown products in the cyst fluid of ADPKD patients.¹⁵ As expected, we observed a good correlation between Ln-5 and β_4 integrin-positive staining among individual cysts. In accordance with previous data, no expression of Ln-5 was detected in normal adult tubules.¹⁶ By contrast, we detected Ln-5 expression in fetal kidney ureteric bud, as reported by others [Figure 3B(d)].¹⁷

To clarify the role of $\alpha_6\beta_4$ integrin-Ln-5 interactions in ADPKD, we set up *in vitro* conditions to analyze cell anchorage and cell motility. As shown in Figure 1, cells derived from ADPKD cysts in primary culture maintain high production of β_4 integrin mRNA and protein. In short-term adhesion studies, CC and NC adhere similarly to collagens I and IV, while Ln-5 induce specifically CC but not NC to adhere more strongly (two-fold) in a dose-dependent manner (Figure 4, A and B). This result contradicts the previously reported weaker adhesion of ADPKD cyst-lining cells to laminin than to other ECM components,¹⁸ but the laminin composition and experimental design were clearly different in this work. Most of CC adhesion to Ln-5 was blocked by anti- α_6 or β_4 mAbs, suggesting that the strong and specific adhesion of CC to Ln-5 is mainly $\alpha_6\beta_4$ -dependent, with $\alpha_3\beta_1$ and $\alpha_6\beta_1$ playing a minor role (Figure 4C).

We then tested in Transwell assays the ability of exogenous matrix substrates to stimulate haptotactic migration, and found that Ln-5 is a much stronger haptotactant for CC than collagen I or collagen IV (Figure 5A). Furthermore, complete inhibition of EGF-stimulated migration by anti-Ln5 mAb [Figure 6(b and c)] suggests that Ln-5 endogenous production and extracellular deposition as a substrate is necessary for CC to migrate.¹⁹ Blocking experiments further indicated that among Ln-5-binding integrins, $\alpha_3\beta_1$ was the major mediator of CC haptotaxis toward Ln-5 and EGF-stimulated wound repair (Figure 8A), in agreement with findings reported in migrating keratinocytes.^{20–22} These studies failed to demonstrate a role for $\alpha_6\beta_4$ in migration, but mAbs used to block integrin $\alpha_6\beta_4$ function were generally raised against the α_6 subunit. In this study we show that the anti- β_4 subunit mAb 3E1 stimulates CC migration in the three different models tested (Figure 7). Integrin β_4 ligation to 3E1 could indirectly enhance migration by preventing adhesion. However, a similar observation was made on a colon cancer cell line in which 3E1 had no effect on adhesion.²³ Indeed, our blocking experiments suggest that that 3E1 ligation to β_4 may transactivate $\alpha_3\beta_1$ integrin in the Ln-5-driven haptotaxis assays (Figure 8A), and even directly stimulate the promigratory cell machinery in the EGF-stimulated CC motility assays (Figure 8B). Interestingly, it was reported that both 3E1 mAb ligation to β_4 and EGF receptor binding to $\alpha_6\beta_4$ could both trigger β_4 cytoplasmic domain phosphorylation, downstream signal

transduction, and subsequent epithelial cell migration and tumor invasion.^{5,24,25}

To date, $\alpha_6\beta_4$ integrin and Ln-5 overexpression have mostly been reported in neoplastic diseases and were described separately. Expression of $\alpha_6\beta_4$ integrin was positively correlated to the progression of various carcinomas²⁶ and facilitated cell motility, tumor invasion, and metastatic potential.²⁷ Classically, $\alpha_6\beta_4$ signaling is triggered by its ligation to the tumor-produced laminins,²⁶ but it has also been described in laminin-free ECM environments.²⁸ Overexpression of Ln-5 was described in various tumors²⁹ and was shown to modulate cell adhesion,³⁰ migration, and proliferation.^{10,31} Furthermore, disruption of $\alpha_6\beta_4$ -Ln-5 interaction was shown to inhibit tumor growth.³²

A role for $\alpha_6\beta_4$ and Ln-5 in kidney tubulogenesis was recently suggested by Zent et al,³³ who reported that blocking antibodies to α_6 or Ln-5 could inhibit ureteric bud branching morphogenesis in whole embryonic kidney organ culture as well as in isolated ureteric bud culture. Of note, a role for Ln-5 acting not only through $\alpha_6\beta_4$ but also through $\alpha_3\beta_1$ was reported in this model, in accordance with the involvement of $\alpha_3\beta_1$ integrin in CC migration.

In ADPKD, the mechanism of β_4 integrin and Ln-5 cystic aberrant expression remains unknown. However, we found that β_4 integrin mRNA expression by CC was dose-dependently increased by EGF (data not shown), as described in other cell types.³⁴ The positive regulation of β_4 integrin expression by EGF may be biologically relevant in ADPKD because cysts contain high levels of EGF, which is able to interact with apical EGF receptors *in vivo*.³⁵ Altogether, our *in vitro* findings suggest a role for $\alpha_6\beta_4$ and Ln-5 in kidney cystogenesis. Ln-5- $\alpha_6\beta_4$ complex mediates both adhesion and migration of cyst-derived cells. Several factors may account for this apparent duality, including the proteolytic cleavage of Ln-5 $\gamma 2$ chain⁸ and the activation of EGF receptor pathway.^{5,25} One can speculate that stable anchorage of the epithelium is necessary to maintain the cystic architecture, while cyst growth requires cell migration. Further work is currently under way to clarify the role of Ln-5- $\alpha_6\beta_4$ interaction in dysregulated proliferation, another cardinal feature of ADPKD cystic epithelia.

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