Role of lactosylceramide and MAP kinase in the proliferation of proximal tubular cells in human polycystic kidney disease

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Abstract Polycystic kidney disease (PKD) is a common genetic disease characterized by the proliferation of epithelial cells, formation of cysts, and the progression of renal deficiency. We have investigated a possible role of glycosphingolipids in the proliferation of human kidney cells in this disease. The levels of glucosylceramide and lactosylceramide and the activity of glucosylceramide synthase (GlcT-1) and lactosylceramide synthase (GalT-2) were elevated 2-fold and 3-fold, respectively, in the PKD tissue compared to control. Lactosylceramide, but not glucosylceramide (10 μM) derived from PKD exerted a 4-fold stimulation in the proliferation of these cells. However, at a concentration of 40 μM, lactosylceramide and glucosylceramide both stimulated cell proliferation on the order of 10-fold and 2.5-fold, respectively, as compared to control. This phenomenon may be due to the enrichment of lactosylceramide containing shorter chain fatty acids (C16:0–C18:0). Lactosylceramide, but not glucosylceramide exerted a time-dependent stimulation in the phosphorylation of mitogen-activated protein kinase (p^{44} MAPK) in normal human kidney proximal tubular cells. Moreover, the kidneys and cultured cells from the PKD patients contained higher levels of the p^{44} MAPK as compared to normal human kidneys. In sum, our studies indicate that lactosylceramide present in the PKD kidney may stimulate cell proliferation via activation of the p^{44} MAPK, and contribute to the pathophysiology in this disease.—**Chatterjee, S., W. Y. Shi, P. Wilson, and A. Mazumdar.** Role of lactosylceramide and MAP kinase in the proliferation of proximal tubular cells in human polycystic kidney disease. *J. Lipid Res.* 1996. **37:** 1334–1344.

Supplementary key words lactosylceramide biosynthesis • gas chromatography–mass spectrometry • cpk mice • tyrosine kinase

Autosomal dominant polycystic kidney disease (ADPKD) is the most common autosomal genetic disease, affecting 500,000 Americans, and 7,000 new patients are recognized each year. Two major types of PKD have been recognized, autosomal dominant PKD (ADPKD) and autosomal recessive PKD (ARPKD). This disease ranks first among the inherited and congenital conditions leading to end-stage renal disease (ESRD). The ARPKD typically affects infants and children at an estimated incidence up to one in 20,000 live births (1) while ADPKD typically affects adults with a prevalence of 1:1,000. The locus responsible for more than 85% of ADPKD has been localized to human 16p 13.3 (PKD-1; European Consortium 1994) and PKD-2 to chromosome 4q (2). Recently, the gene for ARPKD has been mapped to human chromosome 6 (3).

The common feature of various kinds of PKD is the formation of cysts. The nature of the biochemical defect, which leads to cyst formation and consequent growth, and the relationship between extensive cyst formation and the development of progressive renal deficiency are not understood. There is increasing evidence that the primary abnormality is one involving abnormal cell growth whereby cyst formation and growth are controlled by the combined action of growth-promoting factors. Some evidence has suggested that epidermal growth factor may initiate cyst formation, and that cyclic AMP accelerates fluid secretion and expansion (4, 5).

Because of our recent findings that lactosylceramide mediates the proliferation of aortic smooth muscle cells (6), we rationalized that perhaps this or other glycosphingolipids may also be involved in the proliferation of the human kidney tubular epithelial cells in PKD. In this report, we present findings on the distribution of glycosphingolipids and relevant enzyme activities in normal human kidney and of patients with PKD. The effects of various glycosphingolipids on the proliferation of human kidney cells and the possible role of mitogen-activated protein kinases in the proliferative process is described. Our data indicate that there is a marked

Abbreviations: GlcCer, glucosylceramide; LacCer, lactosylceramide; ADPKD, autosomal dominant polycystic kidney disease; ARPKD, autosomal recessive polycystic kidney disease; PT, proximal tubular; MAPK, mitogen-activated protein kinase; ERK, external receptor-mediated kinase; GlcT-1, UDP glucose; ceramide β 1 → 4 glucosyltransferase; GalT-2, UDP galactose; GlcCer β 1 → 4 galactosyltransferase.

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TABLE 1. Neutral glycosphingolipid composition in normal human kidney and polycystic kidney disease

Glycosphingolipid	Normal Human Kidney	Polycystic Kidney Disease
	nmol/mg protein	
GlcCer	0.29 ± 0.01	0.69 ± 0.05
LacCer	0.24 ± 0.04	0.73 ± 0.05
$GbOse_3Cer$	0.22 ± 0.05	0.18 ± 0.05
$GbOse_4Cer$	0.02 ± 0.01	0.03 ± 0.01

About 1–2 g of kidney tissue was subjected to extraction with organic solvents. The total glycosphingolipids were purified and after perbenzoylation were subjected to HPLC analysis as described in Materials and Methods. The data represent glycosphingolipid levels obtained from three normal kidney samples and six polycystic kidney disease samples.

increase in the level of lactosylceramide in patients with PKD. Lactosylceramide added to cultured human kidney cells resulted in marked induction of p^{44} MAPK and cell proliferation. Moreover, the basal levels of p44 MAPK were markedly elevated in kidney tissue and cultured kidney cells derived from patients with PKD compared to control.

MATERIALS AND METHODS

Supplies

[^{14}C]UDP-glucose (specific activity, 9.25 Gbq/mmol), [^{14}C]UDP-galactose (specific activity, 12.2 Gbq/mmol), and methyl [^{3}H]thymidine (specific activity, 9.25 Gbq/mmol) were purchased from American Radiochemical Company (St. Louis, MO). Antibodies against p^{42} and p^{44} MAPKs were purchased from United Biotechnology Institute (Boston, MA).

Cell culture media

Click-RPMI 1640 was from Quality Biologicals (Gaithersburg, MD); collagen type I was from Becton-Dickinson (Bedford, MA); human transferrin, dexamethasone, and triiodothyronine were purchased from Sigma (St. Louis, MO); insulin from Gibco (Grand Island, NY) and fetal bovine serum (FBS) was supplied by Gemini Bioproducts (Calabasas, CA). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

Source of human kidneys

Normal human kidneys (n = 3), age ranging from 32–55 yrs, were obtained from cadaver nephrectomy specimens prepared for transplantation by in situ flushing with Collins balanced salts solutions and judged unsuitable for surgery for technical but not medical responses. ADPKD kidneys (n = 7), ranging from 32–57 yrs, were obtained at surgery from patients undergoing nephrectomy. All kidneys were flushed with balanced salts and maintained in tissue culture medium over ice for no longer than 24 h before use. End-stage ADPKD kidney showed 100% cystic involvement with multiple cysts throughout the cortex and medulla, no normal tubules, and no renal function.

All kidneys were sliced and rinsed in cold sterile Click-RPMI media or phosphate-buffered saline (PBS) and used immediately to generate primary cell cultures.

Cell culture

The techniques used for primary culture of normal human defined renal tubule and ADPKD cyst epithelia have been described in detail (7–9). Briefly, individual normal renal proximal tubules or ADPKD cysts were micro-dissected or finely minced from cortical slices and plated onto collagen-coated tissue culture plates. Epithelial cells were grown to confluence in defined Click-RPMI media buffered to pH 7.4 with 10 μM HEPES and 200 mg% sodium bicarbonate and the addition of 5 μg/ml human transferin, 200 U/ml penicillin, and 0.1 mg/ml streptomycin. Normal and ADPKD epithelia were grown in media supplemented with dexamethasone (5×10^{-8}M), insulin (5 μg/ml), and triiodothyronine (10^{-12}M). Fetal bovine serum (1%) was added to the media to initiate growth after which it was removed.

Characteristics of normal and ADPKD epithelia

Primary cultures of normal human renal tubule and ADPKD cyst were shown to retain structural, biochemical, and functional characteristics of the epithelia in vivo from which they were derived (7, 8). Several important differences have been identified between normal and ADPKD epithelia: notably, abnormal basement membrane assembly and mispolarization to the apical plasma membrane of the membrane proteins NaK-ATPase and EGF receptor in ADPKD epithelia (5). These abnormalities have functional consequences of abnormally high proliferative potential and basal to apical ion transport seen in ADPKD epithelia (4, 5).

Measurement of GSL, glycosyltransferase and glycosidase activity in cultured cells from normal and PKD patients

Confluent cultures of cells were incubated for 24 h in medium containing lipoprotein deficient serum (1 mg/protein per ml). Cells were harvested and stored in cacodylate buffer as described (9). The activity of UDP-Glc: ceramide, β 1 → 4 glucosyltransferase (GlcT-1) and UDP-Gal:GlcCer, β 1 → 4 galactosyltransferase (GalT-2)

was measured using [^{14}C]UDP-glucose and [^{14}C]UDP-galactose as nucleotide sugar donors, respectively, as described previously (10). The activity of LacCer β-galactosidase was measured using [^{3}H]LacCer as substrate as described previously (11).

Incubation of cells with various glycosphingolipids

Normal human kidney proximal tubular cells ($\times 10^4$) were seeded in 24-well microtiter plates. On the day of the experiment, medium was removed, cells were washed with sterile phosphate-buffered saline (PBS), and incubated with Ham's F-10 medium containing glycosphingolipids for various time periods (6). After incubation, cell proliferation assays were pursued. All assays were performed in duplicate or triplicate from three separate dishes and results from a typical experiment are presented. Another set of dishes incubated ± GSL was harvested in buffer (PBS containing 1 mM sodium vanadate), and subjected to immunoblot assays for mitogen-activated protein kinase (MAPK) (see below).

Measurement of cell proliferation

Cell proliferation was assessed using [^{3}H]thymidine incorporation as described previously (12, 13).

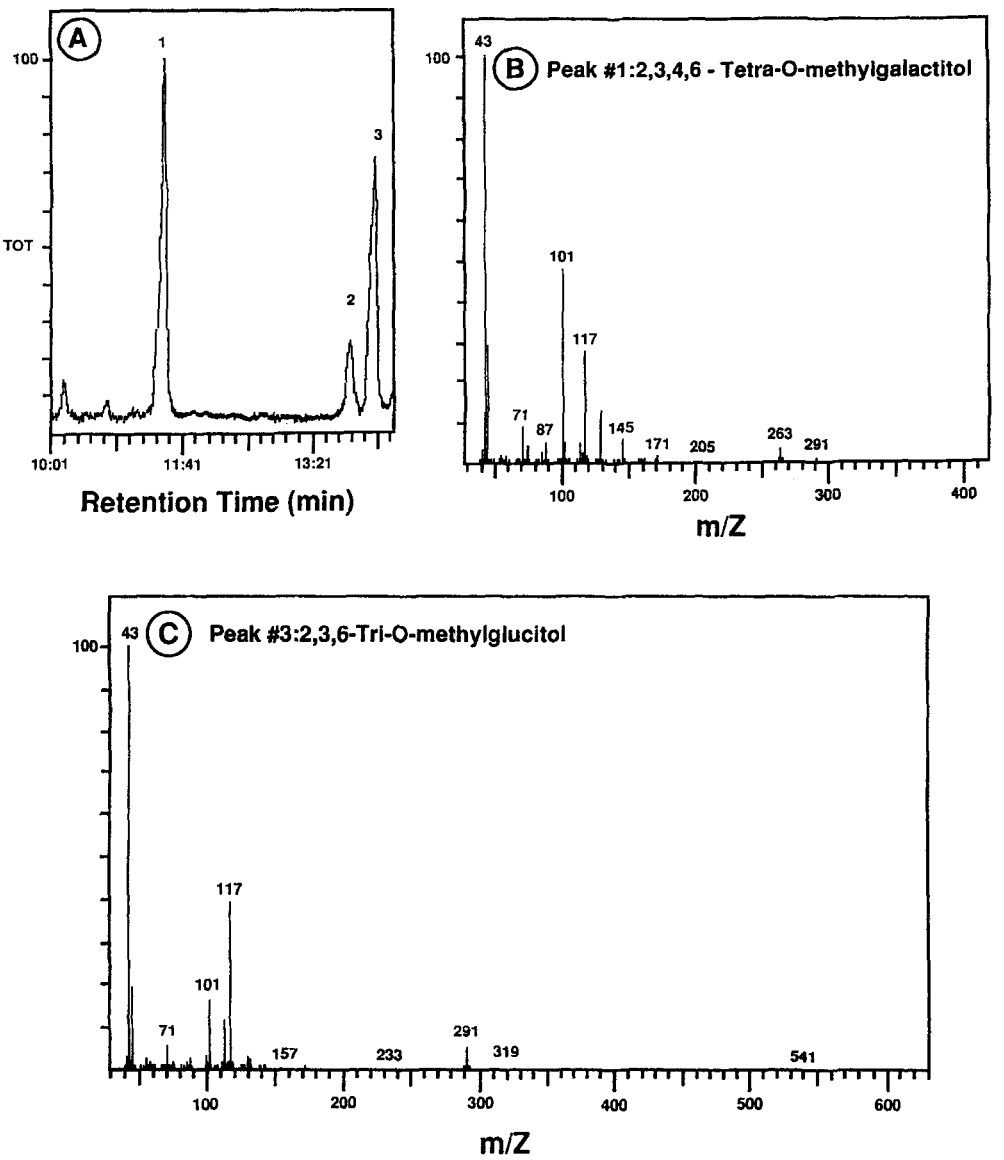

Fig. 1. GC–MS analysis of permethylated alditol acetate derived from sugars from LacCer. A: Capillary gas chromatograph of permethylated alditol acetate sugars from LacCer derived from polycystic kidney tissue; B: mass spectrum of peak #1 shown in Fig. 1A; C: mass spectrum of peak #3 shown in Fig. 1A.

TABLE 2. Fatty acid composition of LacCer derived from normal human kidney and polycystic kidney disease

Fatty Acid	Normal Human Kidney	Polycystic Kidney Disease
	% distribution	
C16:0	6.8	30.43
C18:0	4.1	17.72
C18:1 (6-ene)	ND	34.04
C19:0	17.7	ND
C20:0	5.0	ND
C24:0	16.6	9.53
C24:1	39.1	8.29
Others[a]	10.3	

In this experiment, lactosylceramide from PKD tissue was purified and then subjected to acid-catalyzed methanolysis. The methyl fatty acids were isolated and identified by GC–MS; ND, not determined.

[a]Include C14, C15, C16:1, C18:2, C22 and (22:2) (taken from Rapport, M. M. et al. 1964. *Arch. Biochem. Biophys.* **105:** 431–438).

Western immunoblot assay of MAPK in normal and PKD tissue and cultured proximal tubular cells derived from normal human kidney and individuals afflicted with PKD

Five hundred micrograms of kidney tissue from normal (n = 6) and ADPKD patients (n = 6) was homogenized in 1 ml of lysis buffer (150 nM NaCl, 5 mM EGTA, 5 nM EDTA, 10 mM NaF, 1 mM Na_3VO_4, 1 nM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM pepstatin, 25 mM Tris-HCl, pH 7.4, 1% Triton X-100, and 0.5% Nonidet P-40. The homogenate was centrifuged (10,000 rpm for 10 min at 4°C) in a microcentrifuge. The supernatant was saved and protein content was measured. In one experiment, the cell supernatants (40 μg/protein per well) were subjected to a 12.5% SDS-polyacrylamide gel electrophoresis under denaturing and reducing conditions along with low molecular weight standard markers (14). The sample was then transferred onto polyvinylidine fluoride (PVDF) membrane for 1 h at 50 V and 52 mA per gel at room temperature. Next, the membrane was incubated for 1 h with 3% non-fat dry milk in TBS-T (10 mM Tris-HCl and 50 mM NaCl with 0.05% Tween 20, pH 8.0), and then incubated overnight at 4°C with the primary antibody (rabbit anti-rat MAPK against 44 MAPK and 42 MAPK, diluted 1/1000 in 1% non-fat dry milk TBS-T). After washing 4 times with TBS-T, the membrane was incubated for 2 h with the secondary antibody (alkaline phosphatase-conjugated anti-rabbit IgG). Next, the membrane was washed and incubated in the dark for 3–6 min with the substrate (5-bromo-4-chloro-4-indolyl-phosphate) AP buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5), and nitroblue tetrazolium. The reaction was terminated by rinsing the membrane with distilled water.

In another experiment, 1 mg of supernatant protein was immunoprecipitated overnight at 4°C with antiphosphotyrosine antibody conjugated with protein A/agarose (Transduction Laboratories, Lexington, KY). The immunoprecipitates were washed twice with 25 mM Tris buffer, pH 7.4, containing 2 mM EDTA and 150 mM NaCl. The immunoprecipitates were solubilized in Laemmli sample buffer, rapidly centrifuged, and supernatant was subjected to Western immunoblot assays as above using antibody specific for p^{44} MAPK.

Isolation and quantitation of glycosphingolipids from normal human kidney and kidney from patients with polycystic kidney disease

Normal human kidney was obtained at autopsy and stored frozen. Surgical specimens of kidney tissue from patients with ADPKD were obtained at nephrectomy from National Disease Repository Institute (NDRI; Philadelphia, PA). Tissue sections from the affected kidneys were sliced and stored frozen until further assays were pursued. The tissue was weighed in an analytical balance and then subjected to extraction with various combinations of organic solvents such as chloroform–methanol and water (15). After extraction, the organic solvent was subjected to partition. The total lower phase was dried in nitrogen atmosphere and then subjected to silicic acid column (2.5 cm × 100 cm) chromatography. The total glycosphingolipid fraction was eluted from the column with 300 ml of acetone–methanol 9:1 (v/v). Next, the individual GSL were separated by silicic acid column chromatography and various fractions were then subjected to thin-layer chromatography. Suitable aliquots of total and individual glycosphingolipids were also subjected to quantitative analysis by HPLC after perbenzoylation (15, 16).

TABLE 3. Activity of UDP-g:Cer, β 1 → 4 glucosyltransferase (GlcT-1) and UDP-Gal: GlcCer, β 1 → 4 galactosyltransferase (GalT-2) and LacCer β-galactosidase in cultured proximal tubular cells from normal kidneys and individuals afflicted with polycystic kidney disease

Kidney	GlcT-1	GalT-2	LacCer β-Galactosidase
		nmol/2 h/mg protein	
Normal (n = 3)	0.31 ± 0.05	1.70 ± 0.3	0.55 ± 0.05
PKD (n = 4)	0.68 ± 0.31	5.10 ± 2.0	0.60 ± 0.05

In this experiment, proximal tubular cells from normal kidney and those from polycystic kidney were grown to confluence. Then the cells were harvested and the activities of GlcT-1 and GalT-2 were measured as described previously (10, 11).

TABLE 4. Activity of UDP-g, β 1 → 4 glucosyltransferase (GlcT-1) and UDP-Gal, β 1 → 4 galactosyltransferase (GalT-2) in normal mouse kidney and polycystic mouse kidney

Kidney	GlcT-1	GalT-2
	nmol/2 h/mg protein	
Normal	.5 ± 0.5	.5 ± 0.4
PKD	2.0 ± 0.05	3.0 ± 0.4

Kidneys from normal and PKD mice were homogenized in cacodylate buffer. The protein content was measured and the activities of GlcT-1 and GalT-2 were measured as described (10, 11).

Gas-chromatography–mass spectrometric characterization of LacCer derived from ADPKD tissue

First LacCer was subjected to acid-catalyzed methanolysis (17). The fatty acid methyl esters, methyl sphingosines, and methyl sugars were analyzed by gas chromatography–mass spectrometry after derivatization by using trimethylchlorosilane reagent. The fatty acid methyl esters suspended in hexane were injected into a Varian-3400 gas chromatograph (DB-wax capillary column) 30 meters; J&W Scientific Company, Rancho Cordova, CA) that was attached to a mass spectrometer ITD-850, Finnigan ion trap detector. Helium was used as a carrier gas. Temperature programming (60°C–250°C) at 2°C per min was used to separate the various fatty acid methyl esters and methylsphingosines. The TMSi sugars were separated on a DB-5 column using temperature programming from 160°C–250°C at 1°C per min. Data analysis of TMSi sugars was pursued by the use of a Compaq Deskpro-2862 computer.

Second, LacCer was subjected to further structural analysis by GC–mass spectrometry after microscale permethylation according to Levery and Hakomori (18). The gas chromatography column (DB-5 capillary column 0.25 mm × 30 m) was calibrated with authentic standard mixtures of partially methylated alditol acetates (Biocarb. Chemicals, Sweden). Temperature programming was from 160°C–250°C at 1°C per min and from 250°C–350°C at 2°C min.

Anomeric linkage of the purified LacCer was determined by the method of Hakomori (19) using β-galactoside, α-galactosidase, and β-glucosidase. An aliquot of the purified GSL was incubated with or without β-galactosidase, α-galactosidase, and β-glucosidase in 0.05 M citrate buffer (pH 5.4) containing taurodeoxycholate for 18 h at 37°C. The reaction was terminated with cholorform–methanol 2:1 (v/v) and the lower choloroform layer was subjected to HPTLC. The plate was developed with aniline diphenylanine (DPA) reagent (15).

RESULTS

Glycosphingolipid composition of normal human kidney and patients with polycystic kidney disease

The major glycosphingolipids present in normal human kidney and ADPKD are: glucosylceramide (GlcCer), lactosylceramide (LacCer), globotriosylceramide ($GbOse_3Cer$), and ($GbOse_4Cer$) (**Table 1**). We found that the levels of glucosylceramide, and lactosylceramide were elevated about 2-fold and 3-fold, respectively, in patients with ADPKD as compared to normal human kidney. The levels of globotetraosylceramide and globotriosylceramide in ADPKD were within normal range.

GC–mass spectrometry of acid methanolyzed product of LacCer derived from PKD tissue after trimethysilyl derivatization

GC–mass spectrometry revealed that LacCer in ADPKD tissue is composed of galactose and glucose

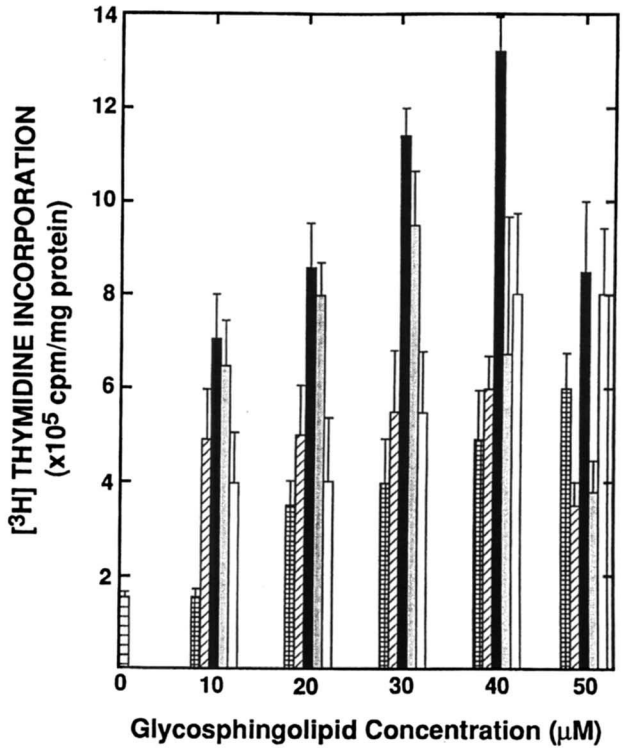

Fig. 2. Effects of various glycosphingolipids on the incorporation of [^{3}H]thymidine in normal human kidney proximal tubular cells. Glucosylceramide and lactosylceramide from polycystic kidney were purified and characterized, and cells were incubated with these glycosphingolipids along with steroyl lactercerobroside, porcine lactosylceramide, and bovine lactosylceramide. After incubation for 24 h at 37°C, [^{3}H]thymidine (5 μCi/ml) was added to each well. After incubation for 2 additional h, the medium was removed, and the incorporation of [^{3}H]thymidine was measured as described previously (11); ⊟, control; ◪, steroyl LacCer; ■, PKD LacCer; ▨, porcine LacCer; □, bovine LacCer; and ⊞, PKD GlcCer.

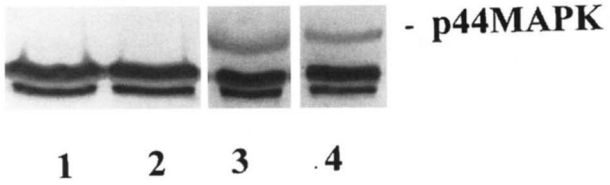

Fig. 3. Western immunoblot analysis of mitogen-activated protein kinase (MAPK) in normal and polycystic human kidney. Approximately 40 μg of solubilized protein (see Materials and Methods for details) from normal and polycystic kidney disease was subjected to Western immunoblot assay using antibody against mitogen-activated protein kinases (42 MAPK and 44 MAPK).

having a molar ratio of 1.0:0.94. Galactose resolved into four peaks; glucose resolved into three peaks and their retention times agreed well with TMSi-galactose and TMSi-glucose (data not shown).

Gas chromatography–mass spectrometry of permethylated-alditol acetates of LacCer

In order to determine the linkage between the sugar components present in LacCer derived from PKD tissue, we pursued GC–MS analysis of permethylated alditol acetates (**Fig. 1A–C**). As shown in Fig. 1A, the gas chromatograph revealed three major peaks: the first, peak #1 had a retention time of 11.2 min and co-chromatographed with 2, 3, 4, 6 tetra-O-methyl-galactitol; peak #2 had a retention time of 13.4 min and co-chromatographed with 2, 3, 6 triomethylgalactitol. Peak #3 had a retention time of 14 min and co-chromatographed with 2, 3, 6-tri-O-methylglucitol. The molar ratio of the galactitols and glucitol was 1.0:0.88. In Figs. 1B and 1C, mass chromatograms for peak #1 and peak #3 are shown. Based upon these analyses, the molecular weight of peak #1 (2, 3, 4, 6 tetra-O-methyl alditol acetate) is 235 and the molecular weight of peak #2 is 222. Taken together, these studies reveal that the linkage between the two sugar residues in LacCer derived from ADPKD tissue is Gal 1 → 4 Glc.

Determination of anomeric linkage in LacCer from PKD tissue

Suitable aliquots of the LacCer were subjected to digestion in buffer alone containing β-galactosidase and α-galactosidase. Only β-galactosidase hydrolyzed the LacCer derived from PKD kidney to glucosylceramide (data not shown). Thus, the LacCer in PKD tissue most probably has the following structure: Cer Glc 4 → 1 β-Gal.

Fatty acid composition of lactosylceramide in normal and ADPKD kidneys

The LacCer derived from PKD tissue was subjected to acid-catalyzed methanolysis. The GSL methyl esters were subjected to TMSi derivatization and capillary GC–MS analyses (**Table 2**). Major fatty acids present in lactosylceramide in normal human kidney (reported previously) include C16:0, C18:0, C19:0, C19:1, C20:0, C24:0, and C24:1 (Table 2). The absence of C:18:1 in LacCer in normal kidney has been reported previously. In comparison, lactosylceramide derived from ADPKD has a highly enriched level of C16:0, C18:0, and C18:1 fatty acids. LacCer from ADPKD was devoid of C19:0 and C20:0 fatty acids. Finally, the levels of C24:0 fatty acids as well as C24:1 fatty acid were decreased on the order of about 2- to 4-fold compared to normal human kidney.

Sphingosine composition of LacCer derived from ADPKD

Using GC–mass spectrometry, we found that C18-sphingosine (4,8-enine) and C22-sphingosine (4,8-enine) represented 97.4% and 2.54%, respectively, of the sphingosines present in LacCer in ADPKD. A very small amount of C18:2 sphingosine was also found.

Activity of glycosphingolipid glycosyltransferases and glycohydrolases in cultured cells from normal and ADPKD kidney tissue and normal and cpk mouse tissue

We found that the activity of UDP-glucose: ceramide β 1 → 4 glucosyltransferase and UDP-galactose GlcCer β 1 → 4 galactosyltransferase in cultured cells from ADPKD kidney tissue were about 2-fold and 3-fold higher, respectively, than normal, unaffected kidney cells (**Table 3**). In contrast, the activity of LacCer β-galactosidase in ADPKD kidney cells was similar to control.

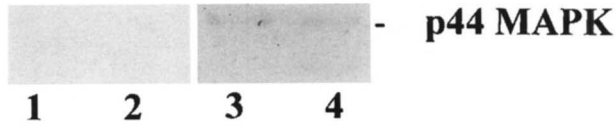

Fig. 4. Western immunoblot analysis of the phosphorylated form of mitogen-activated protein kinase (p^{44} MAPK) in polycystic human kidney and normal human kidney. Approximately 1 mg of solubilized protein (see Materials and Methods for details) from polycystic tissue or normal human kidney tissue was subjected to immunoprecipitation with antiphosphotyrosine antibodies conjugated to protein A/agarose. The immunoprecipitates were subjected to polyacrylamide gel electrophoresis. Subsequently, the protein was transferred on a PVDF membrane and then subjected to Western immunoblot assays using antibodies against p^{44} MAPK (ERK_1) as described previously (12). Six normal and six ADPKD tissues were analyzed in this study.

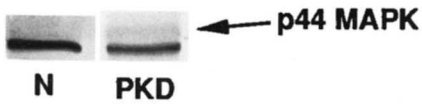

Fig. 5. Western immunoblot analysis of mitogen-activated protein kinase in cultured cells derived from ADPKD patients and normal kidney. In this experiment, both cultured normal human kidney proximal tubular cells and cells from polycystic human kidney tissues were cultured in defined medium as described in Materials and Methods. Subsequently, the cells were lysed in lysis buffer, and 40 μg protein of supernatant was subjected to Western immunoblot analysis for various 44 MAPK and 42 MAPK. Cell pellets from three normal individuals and four patients with ADPKD were analyzed in this study.

Similarly, the activities of GlcT-1 and GalT-2 in cpk mouse kidney were elevated on the order of 4-fold and 6-fold, respectively, compared to normal kidney tissue (**Table 4**).

Effects of glycosphingolipids on the proliferation of normal human kidney cells

In this study, we compared the effects of glucosylceramide and lactosylceramide obtained from ADPKD kidney, bovine brain, porcine brain, and stearoyl lactosylceramide on the proliferation of normal human kidney PT cells. We found that glucosylceramide (10 μM) derived from ADPKD kidney (**Fig. 2**) did not stimulate the incorporation of [^{3}H]thymidine incorporation into human kidney cells as compared to control. In contrast, lactosylceramide (obtained from various sources) exerted a concentration-dependent stimulation of the incorporation of [^{3}H]thymidine into normal human kidney cells. Most interestingly, lactosylceramide derived from patients with ADPKD exerted a marked concentration-dependent increase in the stimulation of [^{3}H]thymidine incorporation in normal cultured proximal tubular cells. At 10 μM, LacCer from ADPKD exerted a 4-fold increase in cell proliferation compared to GlcCer and control. At 40 μM, LacCer from ADPKD exerted a 10-fold increase in the proliferation of cells. Moreover, at this concentration, LacCer from ADPKD was at least 2-fold more effective in stimulating cell proliferation than bovine or porcine LacCer (Fig. 2). Glucosylceramide (40 μM) also stimulated cell proliferation on the order of 2.5-fold compared to control.

Western immunoblot assays of mitogen-activated protein kinase in normal human kidney tissue, ADPKD tissue, and cultured cells derived from these tissues

We found that normal human kidney contained 42 and 44 kDa MAPKs (**Fig. 3**). In contrast, tissues obtained from two representative (six normal and six PKD tissues were analyzed all together) ADPKD patients revealed that the basal level of the phosphorylated form of 44 MAPK (p^{44} MAPK) was high (Fig. 3, lanes 3, 4) as compared to normal kidney (Fig. 3, lanes 1, 2) that did not reveal the p^{44} MAPK. The level of the unphosphorylated form of 44 kDa MAPK (44 MAPK) and 42 kDa MAPK (42 MAPK) was similar in normal and ADPKD kidney tissues (data not shown).

Western immunoblot assay of phosphotyrosine antibody immunoprecipitate

Normal human kidney and PKD tissue supernatants were immunoprecipitated with antiphosphotyrosine antibodies, and next subjected to Western immunoblot assay against p^{44} MAPK. As shown in **Fig. 4,** only PKD tissue was found to have p^{44} MAPK (Fig. 4, lanes 3, 4), but not the normal kidney tissue (Fig. 4, lanes 1, 2).

Western immunoblot assay of mitogen-activated protein kinase in cells from normal human kidney and ADPKD kidney

p^{44} MAPK was undetectable in cultured proximal tubular cells from normal human kidney samples, but was present in cells derived from ADPKD tissue (**Fig. 5**). The major MAPK in these cells was 44 MAPK. Densitometric analysis revealed that the level of this protein kinase was similar in normal and ADPKD cells (data not shown).

Effects of glycosphingolipids on MAPKs in cultured normal human kidney cells

Incubation of cells with glucosylceramide exerted a rapid increase in the levels of the unphosphorylated form of 42 MAPK and 44 MAPK. Within 5 min, the level of 44 MAPK (**Fig. 6A**) and 42 MAPK (Fig. 6B) increased

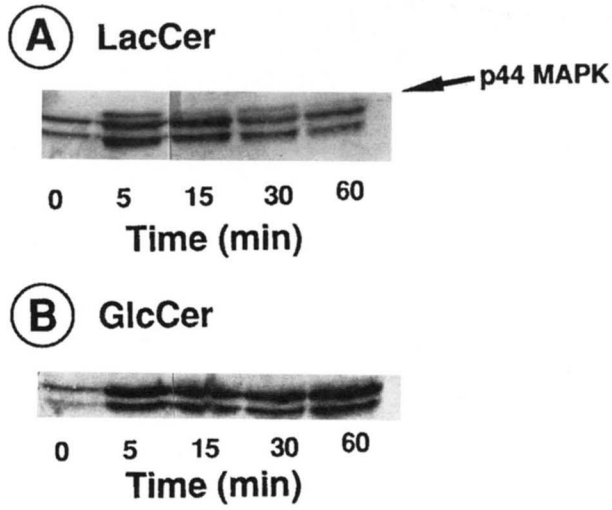

Fig. 6. Densitometric scan of MAPKs in cells incubated with and without glucosylceramide. A: 44 MAPK; B: 42 MAPK.

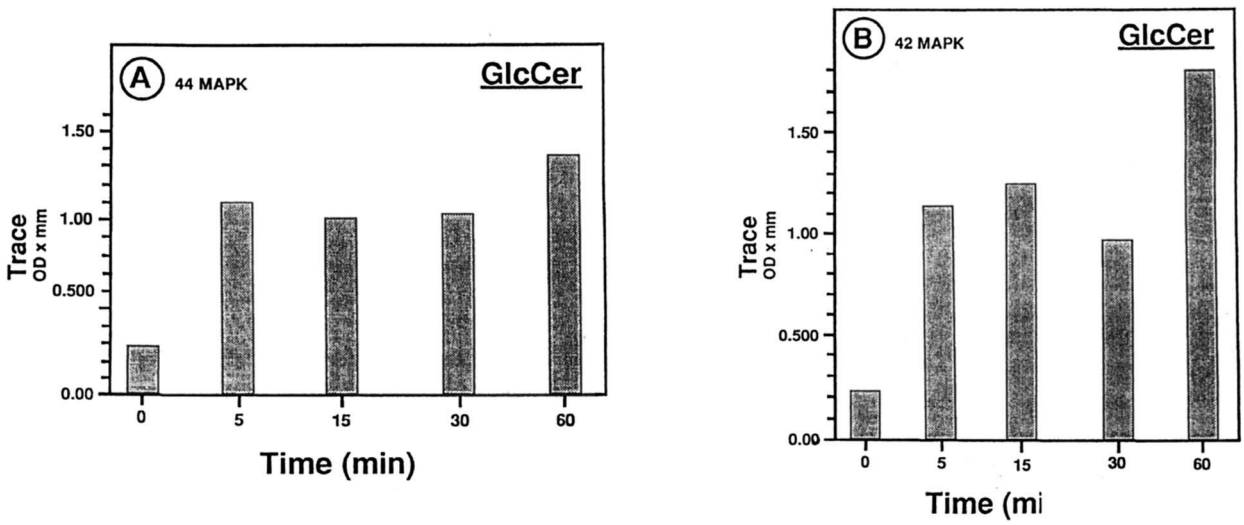

Fig. 7. Effects of time of incubation with glycosphingolipids on mitogen-activated protein kinases in normal human kidney proximal tubular cells. In this experiment, normal human kidney proximal tubular cells were grown in P-100 dishes to confluence. Next, the medium was changed and lactosylceramide or glucosylceramide (10 μM) was added. At various time points indicated, cells were harvested and solubilized, protein was measured and subjected to Western immunoblot assays as described above. Panel A, cells incubated with lactosylceramide. Panel B, cells incubated with glucosylceramide.

4- to 5-fold compared to control as determined by densitometric scanning of the gels. This phenomenon was sustained 60 min after incubation of cells with glucosylceramide. However, GlcCer did not stimulate the phosphorylation of 44 MAPK. In contrast, incubation of cells with LacCer increased the levels of p^{44} MAPK (Fig. 6 and **Fig. 8A**) as a function of time (0–60 min). Normal human kidney cells did not contain any detectable levels of the p^{44} MAPK (**Figs. 7** and 8A). Maximum increase in the level of the p^{44} MAPK occurred 5–15 min subsequent to incubation with LacCer (Figs. 7 and 8A) and decreased rapidly thereafter. Like GlcCer, LacCer also stimulated the level of the unphosphorylated form of 44 MAPK (Fig. 7), in that after 15 min of incubation of cells with LacCer, we found a 2- to 3-fold increase in the levels of the unphosphorylated form of 44 MAPK and 42 MAPK, respectively (Fig. 8B, 8C). The levels of these protein kinases subsided to control levels 30–60 min after incubation of cells with LacCer (Fig. 8B, 8C).

DISCUSSION

The current investigation led to several important findings. First, we found increased levels of glucosylceramide and lactosylceramide in the kidney of patients with ADPKD. Second, the fatty acid composition of lactosylceramide derived from patients with ADPKD was uniquely different in composition when compared with normal human kidney. Third, when normal human kidney was incubated with lactosylceramide derived from patients with ADPKD there was a remarkable increase in cell proliferation as evidenced by an increase in [^{3}H]thymidine incorporation. Fourth, the basal level of the phosphorylated form of 44 MAPK (p^{44} MAPK) was markedly elevated in ADPKD kidney tissue and cultured cyst epithelial cells derived from the same tissue, but not in normal human kidney tissue and cultured normal proximal tubular cells. Finally, when normal human kidney proximal tubular cells were incubated with glucosylceramide and lactosylceramide, only lactosylceramide was able to stimulate the level of p^{44} MAPK.

We found an increase in the levels of glucosylceramide and lactosylceramide in the kidneys of patients afflicted with ADPKD. The relative increase in the levels of glucosylceramide and lactosylceramide (2-fold and 3-fold, respectively) in human PKD tissue in our study was considerably higher compared to the reported 1.5-fold to 2-fold increase in glucosylceramide and lactosylceramide in autosomal recessive C57-BL6J-cpk mouse as compared to control in another study (20). These investigators found that the level of GM_3 was moderately elevated in cystic kidney whereas, the level of sulfatide and ceramide were significantly lower (20). The substantial increases in the levels of glucosylceramide and lactosylceramide in our study in human ADPKD were substantiated by a marked increase (3-fold) in the level of enzyme, GalT-2 involved in the synthesis of lactosylceramide compared to control. We made a similar observation with GalT-2 activity in cpk mouse kidney compared to control mice. Moreover, we found that the activity of GlcT-1 synthase was only 2-fold higher in the

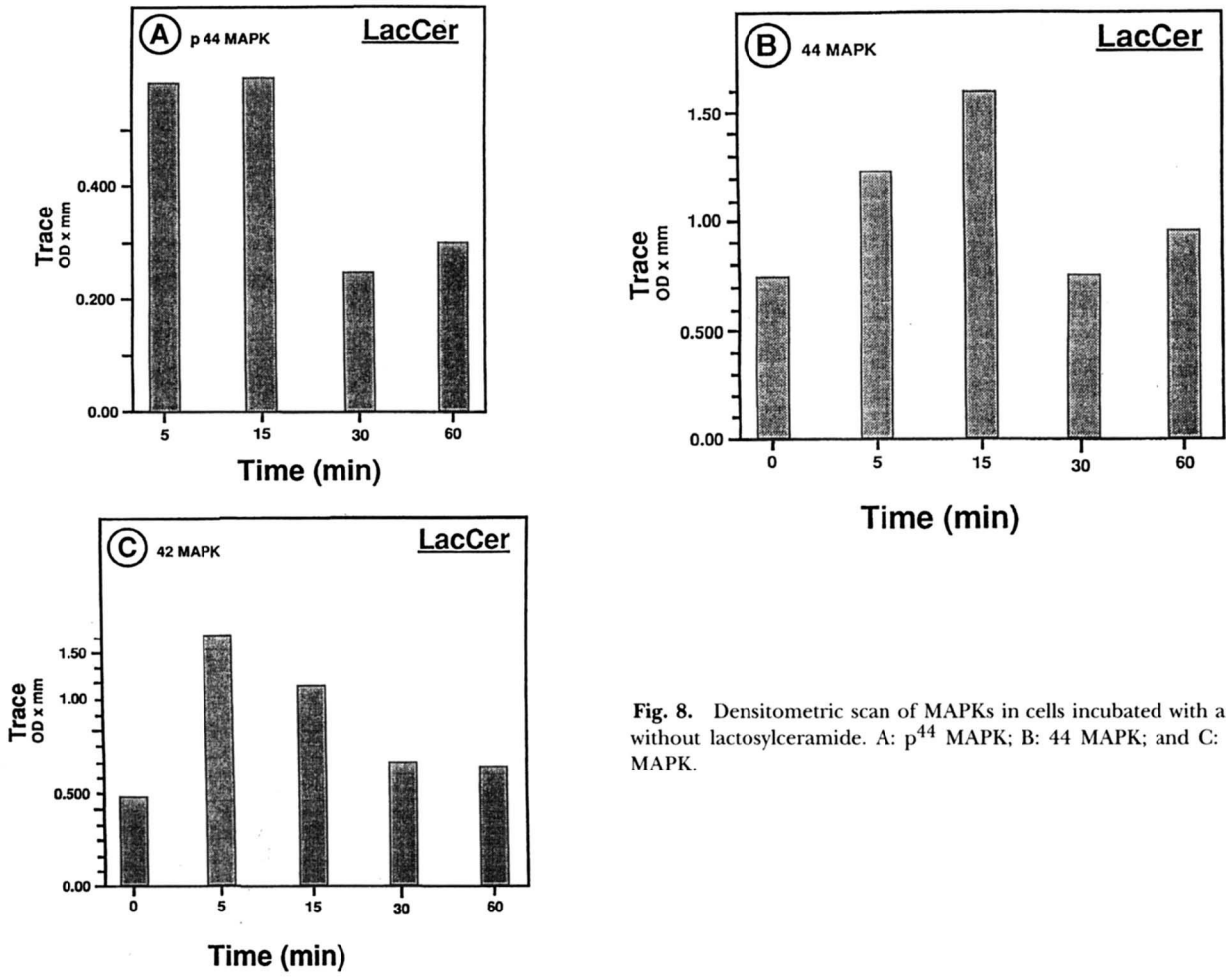

Fig. 8. Densitometric scan of MAPKs in cells incubated with and without lactosylceramide. A: p^{44} MAPK; B: 44 MAPK; and C: 42 MAPK.

kidneys of patients afflicted with ADPKD compared to control. A similar observation was made in the cpk mouse model compared to control by us as well as in the report by Desmukh et al. (20). We have taken these studies further in that we found that the activity of β-galactosidase (lactosylceramide hydrolase) was similar in human ADPKD kidney and control.

Our studies on the structural analysis of lactosylceramide revealed marked heterogeneity in the fatty acid composition of ADPKD kidney lactosylceramide, in that the levels of short chain fatty acids (C16:0–C18:0 and C18:1) were markedly higher in LacCer from ADPKD kidney compared to normal human kidney lactosylceramide (21). Moreover, several other fatty acid molecular species (C19:0 and C20:0) of LacCer found in normal human kidney LacCer were absent in PKD LacCer. When we added lactosylceramide derived from ADPKD kidney to normal cultured human proximal tubular cells, it markedly stimulated cell proliferation as compared to lactosylceramide derived from porcine kidney or bovine brain as well as from steroyl lactosylceramide. One simple explanation for the relatively marked increase in the cell proliferation observed with ADPKD lactosylceramide might be a relative ease of the uptake and transport of LacCer with the shorter chain fatty acid compared to long chain fatty acid and the subsequent activation of p^{44} MAPK. These findings are consistent with our previous observation with cultured rabbit and human aortic smooth muscle cells (6, 22).

To understand the biochemical mechanisms involved in lactosylceramide-mediated proliferation of normal human kidney proximal tubular cells, we investigated the effects of lactosylceramide and other glycosphingolipids on the mitogen-activated protein kinase. These studies were pursued in view of the recent interest in this area implicating an important role of MAP kinases in mediating the signal transduction reactions leading to cell proliferation via activation of various transcriptional factors (23, 24). Our observations support the notion that glycosphingolipids are intimately involved in the activation of MAP kinase. First, we found that when normal human kidney proximal tubular cells were

incubated with lactosylceramide, there was a time-dependent stimulation of p^{44} MAPK. In contrast, when cells were incubated with glucosylceramide, there was no stimulation of p^{44} MAPK. However, both GlcCer and LacCer stimulated the levels of the unphosphorylated forms of 44 MAPK and 42 MAPK, but the time kinetics were different. For example, GlcCer exerted a 4- to 5-fold increase, respectively, in the levels of 44 MAPK and 42 MAPK within 5 min, and this effect was sustained for up to 60 min. In contrast, LacCer-mediated stimulation of the levels of 44 MAPK and 42 MAPK reached a maximum (2-fold increase) at 15 min followed by a sharp decline in the level of these MAPKs to basal levels in 30 min. These findings clearly warrant further studies on the mechanism of action of GlcCer and LacCer in kidney cells.

A remarkable observation made in these current studies was that the normal human kidney does not contain a significant amount of p^{44} MAPK, whereas the basal levels of p^{44} MAPK in ADPKD kidneys obtained from a number of patients were elevated.

To further characterize the p^{44} MAPK in PKD kidney we used another approach. First, we immunoprecipitated all phosphotyrosine-containing proteins with antiphosphotyrosine antibody followed by Western immunoblot assay of immunoprecipitates with antibody against the p^{44} MAPK. Such studies revealed that, indeed, the migration of the phosphotyrosine antibody immunoprecitable band (Fig. 4, lanes 3, 4) co-migrated with the p^{44} MAPK antibody reactive band (Fig. 3, lanes 3, 4). Taken together, our studies indicate the presence of a p^{44} MAPK in PKD kidney tissue and not in normal kidney tissue. However, our studies cannot explain why the endogenous level of p^{44} MAPK was elevated in PKD tissue. We can speculate that the high levels of endogenous epidermal growth factor(s) and lactosylceramide may possibly contribute to this phenomena.

As kidney tissue is composed of a variety of cells that may contribute to the overall composition of MAPK, we pursued another line of investigation to delineate the presence of MAPKs in polycystic kidney epithelia. We cultured cyst lining epithelial cells from the microdissected cysts of patients described above. When these cells were analyzed for MAPKs using Western immunoblot assays, we again found that without any endogenous stimulation of cells with growth factors, the basal level of p^{44} MAPK was elevated in cells derived from ADPKD cells compared to normal cells that did not have detectable levels of p^{44} MAPK. We have previously shown that the ADPKD cells and tissues have higher levels of epidermal growth factor (EGF) and EGF receptors compared to control (5). EGF directly and/or by stimulating lactosylceramide synthesis may stimulate the phosphorylation of the 44 MAPK and promote cell proliferation. In this regard, our preliminary data indicate that inhibitors that inactivate GalT-2 in normal human proximal tubular cells and aorta cells (22) can impair the proliferation of cells induced by lactosylceramide (6). Clearly, further studies will be required to delineate the possible role of lactosylceramide and the biochemical mechanisms involved in the proliferation of proximal tubular cells in ADPKD.

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REFERENCES

1. Cole, B. R. 1990. Autosomal recessive polycystic kidney disease. *In* The Cystic Kidney. K. D. Gardner and J. Bernstein, editors. Kluwer, Dordrecht, Netherlands. Chapter 13.
2. Kimberling, W. J., S. Kumar, and P. A. Gabow. 1993. Identification of chromosome site of autosomal dominant polycystic kidney disease unlinked to chromosome 16. *J. Am. Soc. Nephrol.* **4:** 816–?.
3. Zerres, K., G. Mucher, L. Bachner, G. Deschenes, T. Eggerman, H. Kaarianinen, M. Knapp, T. Lennert, J. Misselwitz, K. E. von Muhlendahl, H. P. H. Neuman, Y. Pirson, S. Rudnik-Schoneborn, V. Steinbicker, B. Wirth, and K. Scharer. 1994. Mapping of the gene for autosomal recessive polycystic kidney disease (RPKD) to chromosome 6p21-cen. *Nature Genet.* **7:** 429–432.
4. Wilson, P. D., J. Du, and N. Kuo. 1993. Tyrosine kinase receptor abnormalities in human autosomal dominant kidney disease (ADPKD): implications for growth factor signal transduction. *J. Am. Soc. Nephrol.* **4:** 505–511.
5. Wilson, P. D., J. Du, and J. T. Norman. 1993. Autocrine, endocrine and paracrine regulation of growth of human adult polycystic kidney disease epithelia. *Eur. J. Cell Biol.* **61:** 131–138.
6. Chatterjee, S. 1991. Lactosylceramide stimulates aortic smooth muscle cell proliferation. *Biochem. Biophys. Res. Commun.* **181:** 554–561.
7. Wilson, P. D., M. A. Dillingham, R. Breckon, and R. J. Anderson. 1985. Defined human renal tubular epithelia in culture: growth, characterization, and hormonal response. *Am. J. Physiol.* **248:** F436–F443.
8. Wilson, P. D., R. W. Schrier, R. D. Breckon, and P. A. Gabow. 1986. A new method for studying human polycystic kidney disease epithelia in culture. *Kidney Int.* **30:** 371–378.
9. Chatterjee, S., A. Trifillis, and A. Regec. 1987. Effects of gentamicin on cell morphology and on the binding, internalization and degradation of low density lipoproteins in cultured human proximal tubular cells. *Can. J. Biochem. Cell Biol.* **65:** 1049–1056.
10. Basu, M., T. De, K. K. Das, J. W. Kyle, H. C. Chon, R. J. Shaper, and S. Basu. 1987. Glycolipids. *Methods Enzymol.* **138:** 575–607.

11. Chatterjee, S., N. Ghosh, E. Castiglione, and P. O. Kwiterovich, Jr. 1988. Role of low density lipoprotein receptors on glycosyltransferase activity in cultured cells. *J. Biol. Chem.* **263:** 13017–13023.
12. Chatterjee, S. 1993. Effects of monensin on glycosphingolipid metabolism in cultured human proximal tubular cells. *Indian J. Biochem. Biophys.* **30:** 346–352.
13. Chatterjee, S. 1992. Role of oxidized low density lipoproteins in atherosclerosis: effects on smooth muscle cell proliferation. *Mol. Cell. Biochem.* **111:** 143–147.
14. Chatterjee, S., and N. Ghosh. 1989. Neutral sphingomyelinase from human urine: purification and preparation of monospecific antibodies. *J. Biol. Chem.* **264:** 12534–12561.
15. Chatterjee, S., C. S. Sekerke, and P. O. Kwiterovich, Jr. 1982. Increased urinary excretion of glycosphingolipids in familial hypercholesterolemia. *J. Lipid Res.* **23:** 513–522.
16. Ullman, M. T., and R. H. McCluer. 1977. Quantitative analysis of plasma neutral glycosphingolipids by high performance liquid chromatography of their perbenzoyl derivatives. *J. Lipid Res.* **18:** 371–378.
17. Essleman, W. J., R. A. Laine, and C. C. Sweeley. 1972. Isolation and characterization of glycosphingolipids. *Methods Enzymol.* **28:** 140–156.
18. Levery, S. B., and S. Hakomori. 1987. Microscale methylation analysis of glycolipids using capillary gas chromatography–chemical ionization mass fragmentography with selected ion monitoring. *Methods Enzymol.* **138:** 13–25.
19. Hakomori, S. I. 1983. Chemistry of glycosphingolipids. *In* Sphingolipid Chemistry. J. N. Kanfer and S. I. Hakomori, editors. Plenum Press, New York. 52–53.
20. Deshmukh, G., N. S. Radin, V. H. Gattone II, and J. A. Shayman. 1994. Abnormalities of glycosphingolipid, sulfatide, and ceramide in the polycystic (*cpk/cpk*) mouse. *J. Lipid Res.* **35:** 1611–1618.
21. Rapport, M. M., L. Graf, and H. Schneider. 1964. Immunochemical studies of organ and tumor lipids: isolation of cytolipin K, a glycosphingolipid hapten present in human kidney. *Arch. Biochem. Biophys.* **105:** 431–438.
22. Chatterjee, S., and N. Ghosh. 1996. Oxidized low density lipoprotein stimulates aortic smooth muscle cell proliferation. *Glycobiology.* **6:** 303–311.
23. Davis, R. J. 1994. The mitogen-activated protein kinase signal transduction pathway. *Trends Biol. Sci.* **19:** 470–473.
24. Cano, E., and L. C. Mahadevan. 1995. Parallel signal processing among mammalian MAPKs. *Trends Biol. Sci.* **20:** 117–122.