ABSTRACT

Autosomal dominant polycystic kidney disease (ADPKD) is characterized by progressive renal enlargement, culminating in renal insufficiency in over one half of affected individuals (1). Although most cases of ADPKD are caused by a single mutated gene, it is a disease with a highly variable age of onset and clinical course (2-4). Thus, the progression of ADPKD may be due to factors extrinsic to the genetically defined renal cysts. In this study, cyst fluid samples from 12 nonazotemic and 18 azotemic ADPKD subjects were examined for in vitro biologic activity that promotes cellular proliferation and the secretion of fluid by renal epithelial monolayers, two pathogenetic mechanisms that have critical roles in the formation and the rate of expansion of renal cysts. In this study, cyst fluid added to culture medium (final concentrations, 1 to 20%) caused Madin-Darby canine kidney cells and human kidney cortex (HKC) cells derived from primary cultures to form cysts in Type I collagen matrix. Cyst fluid stimulated the net transepithelial secretion of fluid by polarized monolayers composed of these same cells. Absolute levels of fluid secretory activity determined by MDCK bioassay were correlated directly with the rate of fluid secretion by HKC cell monolayers and with the extent of cyst formation by MDCK and HKC cells embedded in collagen matrix. The secretory activity of urine was negligible; secretory activity was detectable in the serum of normal and ADPKD subjects, but the levels were much lower than in cyst fluid. cAMP agonists prostaglandins E1 and E2, arginine vasopressin, and 8-Br-cAMP stimulated fluid secretion by MDCK and HKC monolayers, but these substances did not cause HKC cells to form cysts in collagen matrix, whereas cyst fluid did. Among other naturally occurring growth factors and autacoids, only epidermal growth factor and transforming growth factor alpha stimulated cyst formation by HKC cells; however, the capacity of cyst fluid to stimulate fluid secretion was not affected by treatment with antiserum to epidermal growth factor. It was concluded that potent, and possibly unique, substances in the cyst fluids of individuals with ADPKD support and augment biologic processes in renal epithelial cells that may be important in the promotion of progressive cyst expansion.

Key Words: Cysts, cystic, renal, fluid secretion, hyperplasia, kidney, polycystic kidney disease, renal insufficiency
established cell lines and primary cultures of human kidney cortex can be modulated by hormones and growth factors (5–8). The in vitro studies provide a framework for evaluating mechanisms of cyst formation and expansion that may be applicable to the development and progressive enlargement of renal cysts in vivo.

The initiation and growth of in vitro cysts involve integration between cellular proliferation, which increases the surface area of the cyst wall, and fluid secretion, which fills the cavity. If common or unique extrinsic cyst growth modulators are involved in promoting the expansion of established cysts in ADPKD, traces of the pertinent substances might be sequestered in the fluid contained within the cysts. To test this hypothesis, we collected cyst fluids from nonazotemic and azotemic subjects with ADPKD and tested them for the capacity to initiate cyst formation, promote cyst expansion, and increase fluid secretion by renal epithelial cells in vitro.

The extent to which cysts develop from solitary renal epithelial cells suspended in polymerized collagen matrices in culture forms the basis of the cystogenesis assay reported here (7–10). Cystogenesis in vitro is a highly integrated process, and the assay based on the induction of cysts reflects the capacity of a candidate cystogen to promote cellular proliferation, cyst morphogenesis, and fluid secretion. The fluid secretion component of in vitro renal cystogenesis can be examined separately from the proliferative component in polarized monolayers of epithelial cells (10–12). Madin-Darby canine kidney (MDCK) cells and human renal cortex cells, when treated with adenylyl cyclase agonists, secrete fluid from cell base to apex, opposite of the usual absorptive flow of fluid in renal epithelia. The rate of fluid secretion can be measured across polarized monolayers of these epithelial cells and forms the basis of the in vitro fluid secretion bioassay reported here.

We used the cystogenesis and fluid secretion bioassays to determine if ADPKD cyst fluids contain substances that may contribute to the expansion of cysts through stimulation of cellular proliferation and fluid secretion. The results provide evidence in support of the hypothesis that potent biologically active substances in cyst fluid may have a role in promoting the progressive enlargement of cysts in ADPKD.

METHODS

Sources of Biologic Fluids

ADPKD was verified in 30 subjects by radiologic or anatomic examination and by positive family history. In 22 subjects, clear fluid was removed by percutaneous aspiration or open surgical decompression for medical indications, usually intractable pain (13). These samples, together with serum and urine, were immediately placed on ice and shortly thereafter frozen to −70°C. The samples were shipped by overnight carrier on dry ice. In eight cases, nephrectomy was performed in preparation for renal transplantation. The kidneys were inspected for unusual pathologic features, cooled to ice temperature (4°C), and shipped by overnight carrier. Several kidneys were retrieved locally and processed on the same day. Fluid from nephrectomy specimens was aspirated from surface cysts (cortex) into plastic syringes. In some instances, fluid from several individual cysts was collected separately; in other cases, fluid from several cysts was collected in large syringes as a pooled specimen. We did not attempt to segregate cyst fluid samples on the basis of sodium concentration or pH.

MDCK Cells

The MDCK culture methods used in our laboratory have been described in detail previously (7,8,10,11). A subculture of MDCK cells (originally obtained from American Type Culture Collection) was maintained in plastic dishes in a 1:1 mixture of DME and F12 containing 5% fetal bovine serum (FBS), 10mM N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 24 mM sodium bicarbonate, 100 IU/mL of penicillin G, and 0.1 mg/mL of streptomycin.

Primary Cultures of Human Kidney Cortex

Normal portions of cortex from four human kidneys were used to make primary cultures as described previously (7,10). Briefly, renal cortex from each kidney was minced and incubated in unsupplemented DME/F12 medium containing collagenase (1 mg/mL; Cooper Biochemicals, Malvern, PA), penicillin G (100 U/mL), and streptomycin (0.1 mg/mL) for sufficient time (usually several hours) to cause dispersion of individual tubular fragments. The tubular-rich pellet was rinsed in DME/F12 and plated onto T-75 flasks in medium containing DME/F12, 5% FBS, 5 μg/mL of insulin, 5 μg/mL of transferrin, 5 ng/mL of sodium selenite, penicillin, and streptomycin. Cells were grown at 37°C in 5% CO2-air. After 24 h, loose cells and debris were decanted. Subconfluent cultures of epithelial cells were either taken directly for study after 2 to 4 days or were frozen in 10% DMSO in supplemented DME/F12 medium (see below) at −90°C for future use.

In Vitro Cyst Formation in Polymerized Collagen Matrix

We used a method reported previously from this laboratory (7,8,10). Briefly, MDCK or primary cultures of HKC cells were released from plastic dishes with trypsin and agitated to produce a suspension of single cells. After the removal of trypsin, cells were
dispersed in ice-cold Type I collagen (Vitrogen; Collagen Corp., Palo Alto, CA) at pH 7.4 and 0.4 mL (MDCK, 5,000 cells; HKC, 10,000 cells) was deposited in specific wells of a 24-well culture plate. After polymerization, 1.5 mL of defined incubation medium (see Media and Chemicals) was added above the gelled matrix. The plates were kept in a Queue CO2 incubator (Queue, Parkersburg, WV) in an atmosphere of 5% CO2 in room air at 37°C. Medium was replaced three times each week, and the cells were inspected by light microscopy at periodic intervals. Experience showed that cyst initiation and growth of sufficient magnitude for quantitative study were adequate after 13 days of incubation (10).

The experiment was terminated by the addition to each well of 1.5 mL of 2% formaldehyde or glutaraldehyde in isosmotic sodium phosphate buffer, which fixed the cysts for an indefinite period without anatomic distortion or change in size. The diameter of each well was 1.55 cm (area, 1.89 cm²). To quantify cyst initiation, each well was divided into five contiguous microscope fields at low power (magnification, ×50), each covering ~0.126 cm² (total area/well, ~0.63 cm²). A total of 10 fields in two wells (33.3% of total area of the two wells) were examined at a magnification of ×100 for each experimental condition. Cysts were defined as spheroids (a geometrical shape readily determined by varying the depth of focus) with transparent cavities, distinct cellular linings, and outer diameters exceeding 80 μm. The size limit ensured that small cellular clumps or loose aggregates were not counted as cysts during quantitation at low-power magnification (×100). Inspection of the gels at high magnification (×400) confirmed that the number of cysts per field, determined with the low-power objective, was a valid reflection of the total number of cysts (larger and smaller than the 80-μm-diameter limit) that had formed in each experimental condition. The outer diameter of each cyst was determined with an eyepiece micrometer from the average of two measurements at right angles. Average surface area (πD²) was determined from the combined surface area of cysts in each of 10 fields.

Measurement of Fluid Secretion Rate

The method for measuring fluid secretion rate across polarized epithelial monolayers has been described in detail (11). Cells (MDCK and human kidney cortex [HKC] cells) were grown in plastic dishes in respective medium supplemented with 5% FBS. One million cells, seeded onto the upper surface of 24.5-mm-diameter Transwell-Col (Costar Corp., Cambridge, MA) culture chamber inserts coated with Type I and Type III collagen, developed confluent monolayers in 1 to 2 days and were adapted another 3 to 4 days before study. Experience showed that 5% FBS in the basolateral medium was necessary for the cellular monolayers to remain attached to the supporting membrane. To begin the study, the upper chamber fluid was completely replaced with water-saturated mineral oil (1.5 mL); the outer bath contained 2.5 mL of culture medium, a volume that assured that the level of fluid in the inner chamber above the cell layer was always higher than that of the bath. With this arrangement, fluid could move passively in response to hydrostatic gradients only from apical to basolateral media; movement of fluid from the outer to inner chamber compartment required active transport by the cellular layer.

Basal medium (see Media and Chemicals) was placed in the outer chamber, and cyst fluid or other agents were added individually to this medium. Every 24 h, the oil/fluid layer in the apical [inner] compartment was collected by aspiration and the volume of fluid was determined with calibrated capillary tubes (11). Net fluid secretion rate (in microliters per square centimeter per hour) was equal to the volume of fluid collected divided by the surface area of the chamber (4.71 cm²) and the duration of the experimental period (24 h).

Media and Chemicals

Media and chemicals used are as follows: DME/F12 (Hazeltone Biologicals, Lenexa, KS); from Sigma (St. Louis, MO), epidermal growth factor (EGF) (receptor grade), EGF antisera, nerve growth factor (NGF), platelet-derived growth factor (PDGF), atrial natriuretic peptide (ANP)[3-28], arginine vasopressin (AVP), parathyroid hormone (PTH), vasoactive intestinal peptide (VIP), prostaglandin (PG)E1, PGE2, forskolin, chola toxin, 3',5'-cAMP, 8-Br-cAMP, angiotensin II, renin, insulin-like growth factor (IGF)-1, IGF-2, Escherichia coli heat-stable endotoxin, leukotrienes (LT)B4, C4, and D4, glucosylceramide III and IV, bradykinin, urodilatin, and histamine. Smooth and rough E. coli endotoxin was a gift of Dr. David Morrison. Normal human serum and urine were obtained from the authors, and random ADPKD serum and urine were obtained after informed consent.

Basal medium, as defined in this study, was DME/F12 to which we added 5% FBS, 5 μg/mL of insulin, 5 μg/mL of transferrin, 5 ng/mL of sodium selenite, 100 U/mL of penicillin, and 0.1 mg/mL of streptomycin. Defined medium was DME/F12 medium to which we added 5 μg/mL of insulin, 5 μg/mL of transferrin, 5 ng/mL of sodium selenite, 5 × 10⁻⁸ M hydrocortisone, and 5 × 10⁻¹³ M triiodothyronine.

Statistics

Mean, standard deviation, and standard error were calculated in the usual fashion. Statistical differences between means were determined by grouped and ungrouped t test. Differences between more than two measurements were assessed by one-way analy-
sis of variance and Fisher's test; \( P < 0.05 \) was accepted as significant.

RESULTS

Fluid Secretion: MDCK

The polarized MDCK preparation was used to screen samples of cyst fluid for potential secretory activity. Cyst fluid was added to the medium bathing the basolateral surfaces of confluent polarized MDCK monolayers. We made no attempt in these studies to determine the sidedness of the cyst fluid effect, because there was no a priori reason to suppose that receptors for biologically active cyst fluid components would be preferentially located in the apical as opposed to the basolateral membranes of MDCK and human kidney cortex (HKC) cells. Samples with detectable secretory activity caused a concentration-dependent increase in fluid secretion. Figure 1a illustrates an expanded concentration-dependence study for a sample of cyst fluid in which the maximally effective concentration was 10%. In contrast to Figure 1a, maximal effects of cyst fluid were usually observed when diluted 5% with medium. The effect of cyst fluid was maintained above the baseline fluid secretion rate for at least 5 days (Figure 1b). The rate of fluid secretion returned to the baseline within 48 h of the removal of the cyst fluid.

In each assay, a maximally effective concentration of forskolin (10^{-5} M) was added to standardize the assay preparation. Maximally effective cyst fluid concentrations rarely caused fluid secretion rates by MDCK monolayers to exceed that caused by forskolin. Dilution of medium with phosphate-buffered saline up to 30% did not affect the secretory response to 10^{-5} M forskolin (data not shown). In 17 separate assays, the mean response to forskolin was 0.309 ± 0.078 (SD) \( \mu \)L/cm²/h. The interassay coefficient of variations was 25%, and the intra-assay coefficient of variation was 5.1%. Ouabain (10^{-7} M) completely inhibited the secretory response to cyst fluid and forskolin (data not shown).

Secretory activity induced by cyst fluid was expressed in absolute dimensions (microliters per square centimeter per hour) and was relative to the maximally effective rate of fluid secretion caused by forskolin in the respective assays

\[
\frac{V_{\text{cf}} - \text{basal}}{V_{\text{forskolin}} - \text{basal}} \times 100 = \text{relative secretion rate}
\]

where \( V_{\text{cf}} \) is the rate of fluid secretion caused by cyst fluid, basal is the rate of fluid secretion in basal medium, and \( V_{\text{forskolin}} \) is the rate of fluid secretion caused by forskolin. A cyst fluid that stimulated fluid secretion to the same extent as forskolin would have 100 U of secretory activity. Fluid from a single cyst was assayed on five separate occasions. The mean secretory activity was 31.3 ± 5.9 (SD) U; the inter-assay coefficient of variation was 18.7%.

Twenty-two of 30 subjects (9 nonazotemic [serum creatinine <2.0 mg/dL], 13 azotemic) had one or more cyst fluids that stimulated fluid secretion by MDCK cells. Some individuals had both stimulatory and nonstimulatory fluids. Seventy-two (72.7%) of a total of 99 cyst fluid samples (both single and pooled collections) exhibited secretory activity. In 82 samples from azotemic subjects, the mean secretory activity was 18.9 ± 2.6 (SE) (range, 0 to 82) U. In 17 samples
from nonazotemic subjects, the mean secretory activity was 21.8 ± 6.2 U (range, 0 to 64). The difference between nonazotemic and azotemic subjects was not significant. Secretory activity was detected in fluids from both males and females.

Serum from four normal and six ADPKD subjects had mean secretory activities of 7.4 ± 3.2 (SE) and 4.3 ± 1.2 Units, respectively. The difference between means was not significant. Urine from three normal and four ADPKD subjects had mean secretory activities of 0.3 ± 0.2 and 3.5 ± 2.0 U, respectively, that were not different from zero. The secretory activity values for serum and urine were significantly less than those of cyst fluid.

**Fluid Secretion: Human Kidney Cortex Cells**

Basal rates of fluid secretion were generally higher in monolayers of normal kidney cortex cells than in MDCK monolayers (compare Figures 1 and 2). Forskolin also stimulated fluid secretion in these cells and was used as a reference marker as in the MDCK assays. Cyst fluid caused a sustained increase in fluid secretion that was reversible on removal of the cyst fluid from the medium (data not shown). Ouabain inhibited the stimulatory effect of cyst fluid as well as basal fluid secretion (Figure 2). the IC50 of ouabain was approximately 4 x 10^-7 M.

**Cystogenesis: MDCK**

The formation of intact cysts from MDCK cells in polymerized collagen gel provides an assay for highly differentiated functions that act in concert because each cyst derives from a solitary cell that proliferates to become a continuous polarized monolayer surrounding a cavity filled with fluid (14). The outer diameter of individual spherical cysts provides an index of cell number, because the individual cells remain of uniform size as the cysts expand (15). Fluid is pumped into the cysts filling the cavity created by the proliferating cells. Thus, changes in cyst volume and outer surface area provide an index of net fluid secretion rate as well as cell proliferation rate.

In defined medium, MDCK cells remained stationary in the matrix and did not proliferate or form cysts (Figure 3). The addition of EGF, a substance that increases the rate of cell proliferation and inhibits net fluid secretion (data infra), led to the formation of dense cell masses in which the cells of the original cyst wall appeared to have hypertrophied, migrated into the adjacent matrix, and desquamated into the cyst lumen (Figure 3). By contrast, the addition of a cystogen that stimulated both cell proliferation and fluid secretion (e.g., PGE1, AVP, forskolin) caused the formation of cysts with relatively thin walls. Because examination at high magnification (x400) showed that cysts did not develop in defined medium, the capacity of a substance to initiate cyst formation (cystogenesis) was adduced from the average number of cysts observed in each microscope field at relatively low power; the average total surface area of cysts per microscope field was interpreted to indicate the capacity of a substance to promote fluid secretion and cellular proliferation.

Cyst fluid caused a concentration-dependent increase in the number of cysts per microscope field and in the total surface area of cyst wall epithelium per microscope field (Figures 3 and 4). Cyst fluid concentrations of 5% elicited maximal cyst forma-
a. S.,

Figure 3. Appearance of MDCK and HKC cells in collagen matrix in response to treatment with forskolin, EGF, and cyst fluid. (a–d) MDCK cells after 13 days of treatment with (a) defined medium, (b) 10 μM forskolin, (c) 25 ng/mL of EGF, and (d) 5% cyst fluid. Cysts with clear cavities and relatively thin walls formed in response to forskolin and cyst fluid. EGF caused dark masses to develop instead of clear-cut cysts, which on fixed/stained sections proved to be dense collections of hypertrophied MDCK cells with an attenuated fluid cavity. EGF also caused MDCK cells to migrate into the adjacent matrix and desquamate into the cyst cavity. (e–h) HKC cells after 13 days of treatment with (e) defined medium, (f) 10 μM forskolin, (g) 25 ng/mL of EGF, and (h) 5% cyst fluid. Cysts with clear cavities and relatively thin walls formed in response to EGF or cyst fluid. Original magnification, ×50. Marker in panel a indicates 100 μm.

tion; in some instances, concentrations higher than 5% inhibited cystogenesis (data not shown). Forskolin, used as a reference cystogen, produced a concentration-dependent increase in cyst formation and size (Figure 4). Urine and serum were not tested for cystogenic activity.

Cystogenesis: HKC Cells

Cyst fluid caused a concentration-dependent increase in cyst formation and total surface area that was maximal at a 5% dilution (Figure 5). EGF (2.5 ng/mL) caused cysts to form, but cAMP agonists had no effect unless EGF was also present in the medium (10). As reported previously for MDCK cysts (15), we determined the average size of cells in 10 cysts ranging in diameter from 148 to 1,184 μm. There was no correlation between cyst diameter and mean cell surface area ($r^2 = 0.006$). Mean cell surface area ranged from 720 to 970 μm$^2$. Thus, changes in the surface area may be used as a measure of cellular proliferation in intact cysts.

In a separate study, we determined the mitogenic effect of cyst fluid on HKC cells grown in the plastic wells of a Transwell plate. A suspension of single HKC (50,000 cells per dish) was allowed to attach to the plastic overnight in basal medium after which defined medium or defined medium containing 5% cyst fluid (60 forskolin units of secretory activity in a 5% solution) was added for 48 h. The mean number of cells in six wells was significantly increased from 75,650 ± 2,970 to 112,550 ± 10,740 in defined medium and defined medium plus 5% cyst fluid, respectively. In a study of similar design with MDCK cells,
Figure 4. Effect of cyst fluid and forskolin on MDCK cyst formation in collagen matrix (cystogenesis assay). MDCK cells were embedded and treated for 13 days with medium containing different concentrations of cyst fluid or forskolin. Medium was replaced three times a week. (A) Effect of forskolin on cystogenesis as reflected by increase in the number of cysts per microscope field. Symbols represent mean ± SE of 10 determinations (symbols encompass the SE bars). Cystogenesis was maximal at a 10 μM forskolin concentration; higher concentrations than this are not shown. (B) Concentration-dependent effect of 1 and 5% cyst fluid on cystogenesis. (C) The mean diameters of the same cysts examined in panel B were used to calculate the total surface area of cysts per field (C/F), an index of cellular proliferation. The size and surface area of individual cysts (surface area reflects the number of cells) were also increased by cyst fluid in a concentration-dependent manner.

Figure 5. Effect of cyst fluid on HKC cyst formation in collagen matrix (cystogenesis assay). HKC cells were embedded and treated for 13 days with medium containing different concentrations of cyst fluid. Medium was replaced three times a week. (A) Concentration-dependent effect of cyst fluid on cystogenesis as reflected by increase in the number of cysts per microscope field. Symbols represent mean ± SE of 10 determinations. Cystogenesis was maximal at a 5% concentration of cyst fluid. (B) The mean diameters of the same cysts examined in panel A were used to calculate the total surface area of cysts per field, an index of cellular proliferation. The size and surface area of individual cysts (surface area reflects the number of cells) were also increased by cyst fluid in a concentration-dependent manner.
the mean number of cells in five wells was significantly increased over 72 h from 32,000 ± 1,000 to 95,000 ± 3,000 in defined medium and defined medium plus 5% cyst fluid, respectively.

**Interrelation Among Secretory and Cystogenesis Bioassays**

To determine the extent to which the biologic assays developed for this study might be interrelated, we selected from the polycystic kidney cyst fluid library 9 samples with MDCK secretory activity levels ranging from 0 to 64.4 activity units. We determined the secretory activity of each fluid by the HKC monolayer system and the cystogenesis activity of each fluid by the MDCK and HKC methods (see legend to Figure 6 for further details). We arbitrarily chose the MDCK secretory assay as the independent variable. There was a direct relationship and good-to-excellent correlation between the secretory activity of cyst fluid determined by MDCK bioassay and the secretory activity and cystogenesis activities of the same fluids determined by MDCK and HKC cell bioassays (Figure 6).

**Survey of Candidate Cystogens**

We have shown that cells derived from primary cultures of normal human kidney cortex form cysts in Type I collagen matrix only when EGF or transforming growth factor alpha (TGF-α) are present in the nutrient medium (10). Because cyst fluid caused normal human renal cortex cells to form cysts in collagen matrix, we characterized further the *in vitro* effects of EGF. EGF inhibited forskolin-stimulated fluid secretion by polarized MDCK monolayers (Figure 7a) and diminished the formation of cysts in collagen matrix caused by forskolin (data not shown). The inhibition of fluid secretion caused by EGF was relieved by including antiserum to EGF in the medium (Figure 7b); however, the antiserum had no effect on the stimulation of fluid secretion by one of the well-characterized pools of cyst fluid. On the basis of these findings, it is unlikely that the secretory activity in cyst fluid is due to the presence of EGF.

Several candidate cystogens have been examined in this or previous reports from this laboratory (7,8,10). All compounds were tested by the MDCK fluid secretion assay. cAMP and 8-Br-cAMP stimulated fluid secretion. The IC₅₀ for MDCK fluid secretion was approximately 10⁻⁵ M for 3',5'-cAMP (data not shown). Mean cAMP levels measured in five cyst fluids by RIA (method described in reference 7) were 5 x 10⁻⁶ M. The cAMP agonists AVP (10 mU/mL), VIP (3 nM), PGE₁ (25 ng/mL), PGE₂ (25 ng/mL), and cholera toxin (1 μg/mL) stimulated fluid secretion and cystogenesis by MDCK cells. PGE₁, PGE₂, AVP, cholera toxin, cAMP, 8-Br-cAMP, and PTH (100 nM) stimulated fluid secretion by HKC monolayers, but none of these agents caused cyst formation by HKC cells in collagen matrix. Substances that did not stimulate fluid secretion by MDCK of HKC cells include NGF (25 ng/mL), PDGF (5 ng/mL), IGF-1 (1 μg/mL), IGF-2 (1 μg/mL), E. coli heat-stable enterotoxin (50 U/mL), E. coli endotoxin (smooth and rough, 10⁻³ M), interleukin (IL)-1 (10 U/mL), IL-2 (10 U/mL), LTD₄, LTC₄, glucosylceramides III and IV (50 x 10⁻⁶ to 150 x 10⁻⁶ M), bradykinin (10⁻³ M), angiotensin II (10⁻⁷ M), renin (500 mU/mL), urodilatin (100 ng/mL), ANP (100 ng/mL), and histamine (50 μg/mL).

EGF, TGF-α, and cyst fluid were the only substances that caused HKC cells to form cysts in collagen matrix. The following substances were tested in the HKC system and found to have no effect (see concentrations above): PDGF, NGF, cAMP, AVP, VIP,
Figure 7. Effect of EGF on forskolin and cyst fluid induced fluid secretion by MDCK monolayers. MDCK monolayers were adapted in control medium for 5 days. Additions of forskolln or EGF were made to basolateral medium daily. (A) Forskolln increased net fluid secretion for 3 days; EGF (2.5 ng/mL) significantly diminished the forskolln response and baseline fluid secretion. The effects of forskolln and EGF were reversible. (B) Sufficient EGF antiserum was added to completely bind EGF at a concentration of 2.5 ng/mL in diluted cyst fluid. Antiserum to EGF significantly reversed the effect of EGF to inhibit fluid secretion caused by forskolln (both days 3 and 4) but had no detectable effect on the rate of fluid secretion caused by cyst fluid. Each symbol is the mean of three determinations; vertical lines indicate SE.

PTH, PGE\textsubscript{1}, PGE\textsubscript{2}, cholera toxin, IL-1, IL-2, angiotensin II, and ANP.

DISCUSSION

In the current study, we used two uniquely relative \textit{in vitro} bioassay methods to determine if cysts accumulated substances that might be incriminated in the promotion of cyst expansion. The observations with monolayers of MDCK and HKC cells that material in cyst fluid increases the rate of fluid secretion across cultured renal epithelia of two mammalian species support the view that secretagogue(s) could have an important role in cyst filling \textit{in situ}. Candidate secretagogues, all of them participants in the cAMP signal transduction mechanism, include AVP, VIP, isoproterenol, PGE\textsubscript{1}, PGE\textsubscript{2}, and cholera toxin, as well as 3',5'-cAMP and 8-Br-cAMP (this study; 8,10–12). In MDCK cells, the mechanism of fluid secretion involves increased active transport of solute (NaCl) coupled to the osmotic flow of water across an epithelium of relatively low hydraulic conductivity (8,10–12,16). A recent report suggests that secondary active chloride transport may be the motive force in driving solute into the MDCK cyst cavity (17). Preliminary studies in HKC monolayers are consistent with a mechanism of fluid transport similar to that of MDCK (11). Thus far, we have not identified any specific agonists in cyst fluid.

The finding that cyst fluid stimulates cyst formation and enlargement of MDCK and HKC cells imbedded in collagen matrix provides additional insight into the potential mechanisms by which cyst fluid factors may augment the growth of individual cysts in polycystic kidneys. MDCK cysts develop by clonal growth from solitary cells embedded in collagen matrix (14). Although we have not explicitly confirmed this for either MDCK or HKC cells by sequentially examining a single cell develop into a cyst, the method used in this and previous studies uses a relatively dilute preparation of solitary cells as starting material. Repeated microscopic examination at high magnification has revealed no evidence that cell aggregation accounts for the initial formation of cysts in collagen matrix, in agreement with the initial report of McAteer \textit{et al.} (14). The absence of cyst formation in defined medium (Figure 3) and the appearance of cysts after treatment with cyst fluid may be interpreted to show that cyst fluid contains a cystogen(s). That the cystogen is also mitogenic is confirmed by the fact that the walls of small and large cysts are composed of cells with a relatively constant size (15; this study); consequently, the number of cells in cyst epithelium increases in proportion to the increase in cyst diameter and plane surface area of the cyst wall in response to cyst fluid. A direct mitogenic effect on the cells is supported by the observation that cyst fluid stimulated the proliferation of MDCK and HKC cells grown on a plastic surface where net transepithelial fluid secretion and apical compartment hydrostatic pressure could not be factors provoking mitogenesis.

Cyst fluid coordinately increased \textit{in vitro} cyst formation and stimulated \textit{in vitro} fluid secretion in both types of cells used in the bioassays (Figure 6). Such a result could be due to a single substance with mitogenic and secretagogue activities or could be the product of several substances that have complimentary effects in these bioassays. In this regard, the differential effects of cyst fluid on HKC and MDCK cells may provide a clue to the identity of the cystogen/mitogen. MDCK cells form cysts in a collagen matrix and secrete fluid across polarized monolayers...
in response to cAMP agonists (7,8,11,12). Thus, a variety of cAMP agonists could account for the behavior of MDCK cells in these bioassays. On the other hand, HKC cysts do not form in collagen matrix in response to cAMP agonists (Figure 3: 10) and either EGF or TGF-α is required. It is unlikely, however, that EGF is responsible for the cystogenic activity of cyst fluid, because treatment with EGF antibodies did not alter the effect of the growth factor on fluid secretion by MDCK cells. An examination of several known growth factors has not revealed a suitable candidate for the hypothetical cystogen (10; this study). Although cAMP agonists will not cause HKC to form cysts, these agents will promote an increased rate of enlargement of cysts initiated with EGF (10). The complementary effect of cAMP agonists on the growth of HKC cysts initiated with EGF suggests an economical explanation to account for the correlations between the four bioassays illustrated in Figure 6. Cyst fluid may contain an as yet unidentified growth factor that stimulates cyst formation and cellular proliferation in both HKC and MDCK cells as well as adenyl cyclase agonists that promote net fluid secretion. Combinations of the two putative components could produce an effect on fluid secretion and cyst formation greater than either secretagogue or cystogen alone. Additional studies are needed to examine this hypothesis.

The majority of kidneys and cysts available to us contained "bioassayable" material that increased in vitro cyst initiation and expansion in cells of distal phenotype (10) derived from two species: dog (MDCK cells) and human (HKC). The variable levels of biologic activity in cyst fluid may ultimately be accounted for by (1) activity levels of cystogen/secretagogue below the limits of detection by bioassay, (2) the presence in cyst fluid of cystogenesis and fluid secretion inhibitors, (3) cyst wall permeability in situ that precludes developing a gradient for the biologically active materials between cavity and interstitium, (4) intermittent production or transport of the cystogen/secretagogue, and (5) processes to inactive the cystogen/secretagogue that are more active in some kidneys and cysts than in others. Renal insufficiency is not a consideration to explain the variable detection of cystogen/secretagogue among kidneys and cysts because fluids strongly positive for cystogenic and secretory activity were obtained from both nonazotemic and azotemic subjects.

Secretory activity was detected in the serum of subjects with ADPKD of biologic activity that promotes in vitro cystogenesis and fluid secretion by renal epithelial cells supports the hypothesis that factors extrinsic to the primary, genetically defined alterations in tubule biology may modulate the progressive enlargement of polycystic kidneys.

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