Journal of Supramolecular Structure 11:207–216 (1979)
Tumor Cell Surfaces and Malignancy 153–162

Growth of Kidney Epithelial Cells in Hormone-Supplemented, Serum-Free Medium

Mary Taub and Gordon H. Sato

Department of Biology, University of California, San Diego, La Jolla, California 92093

Madin Darby canine kidney cells can grow in synthetic medium supplemented with 5 factors – insulin, transferrin, prostaglandin E_1, hydrocortisone and triiodothyronine – as a serum substitute. These 5 factors permit growth for one month in the absence of serum, and a growth rate equivalent to that observed in serum-supplemented medium. Dibutyryl cAMP substitutes for prostaglandin E_1 in the medium, suggesting that increased growth of Maden Darby canine kidney cells results from increased intracellular cAMP. Potential applications of the serum-free medium are discussed. The medium permits the selective growth of primary epithelial cell cultures in the absence of fibroblast overgrowth, and a defined analysis of the mechanisms by which hormones regulate hemicyst formation.

Key words: renal epithelium, primary cultures, prostaglandins, mammalian cell growth

Hormones are important regulators of kidney epithelial cell growth and function in vivo. They play an important role in regulating transepithelial solute transport by renal tubule cells [1], and have been implicated in regulating kidney growth during development [1, 2] and in response to injury [2]. However, studies concerning the mechanisms by which hormones affect these processes have been difficult because of the complex structure of the kidney.

Cultured kidney epithelial cells provide convenient systems to study hormonal regulation of kidney functions. The Madin Darby canine kidney cell line (MDCK) for example, functions in culture like tubular epithelial cells [3–5]. At confluency MDCK cells form multicellular hemicysts (groups of cells slightly raised from the tissue culture dish surface) [3]; hemicyst formation has been attributed to the transport of salt and water from the mucosal surface of the cells (facing the culture medium) through the serosal surface of the cells (facing the dish surface) [4]. The tubule segment from which MDCK cells are derived was not identified. However, the MDCK cell line responds to

Received April 25, 1979; accepted July 6, 1979.

0091-7419/79/1102-0207$02.00

arginine vasopressin by producing cAMP [6]. The latter response is distinctive of distal tubule cells.

Other kidney epithelial cell lines are also available, which form hemicysts, including monkey kidney ($LLC\text{-}MK_2$) and bovine kidney (MDBK) cells [8]. However, the available lines are relatively "leaky" epithelia [4, 5], as the transepithelial potential generated by such cells is low [4, 5]. As a consequence the utility of such cell lines for studies concerning transepithelial solute flux is limited. Thus the development of additional culture systems of kidney epithelial cells, and hormone-supplemented, serum-free media may prove to be an important tool towards these ends.

Previously, serum in tissue culture medium has impeded studies concerning the regulation of animal cell growth and differentiated function in vitro by hormones. However, Sato and coworkers have demonstrated that serum can be replaced as a growth requiring supplement by specific hormones and accessory factors, which differ according to cell type [9–11]. This paper reports the long-term growth of MDCK cells in a serum-free culture medium supplemented with insulin, transferrin, PGE_1, hydrocortisone, and triiodothyronine (T_3). These 5 components are the minimal number required to attain the growth rate observed in serum-supplemented medium [12]. The utilization of the medium to support the growth of primary kidney epithelial cell cultures, as well as established kidney lines has been demonstrated. The medium should also prove useful to study regulation of vectorial solute transport processes in cultured kidney cells.

MATERIALS AND METHODS

Cells and Maintenance

The canine kidney epithelial cell line MDCK [3] was obtained from Dr. John Holland at the University of California at San Diego. Stock cultures were routinely incubated in a humidified 5% CO_2/95% air mixture at 37°C; the growth medium for stock cultures was a 50:50 mixture of Dulbecco's Modified Eagle's Medium (DME) and Ham's F12 Medium supplemented with 5% horse serum and 2.5% fetal calf serum. Serum-free growth experiments were also conducted using a 50:50 mixture of DME and F12(SF-DME/F12) based upon the observation that the growth rate of MDCK cells in serum-free DME/F12 supplemented with 5 μg/ml insulin and 5 μg/ml transferrin was maximal when using this combination of the two media. All media were supplemented with 10 mM N-2-hydroxypiperazine-N'2-ethane-sulfonic acid (HEPES), sodium bicarbonate at 1.1 mg/ml, 92 IU/ml penicillin, 200 μg/ml streptomycin, 25 μg/ml ampicillin, and 10^{-8}M selenium. Triple-distilled water was used for medium preparation.

To initiate primary kidney cultures, the kidneys from 10-day-old mice (Balb/c) were minced into 1 mm diameter pieces. Kidney cell suspensions were prepared from the mince following the method of Leffert and Paul [13] with the modification that the cells were incubated in a 0.3% EDTA, 0.1% trypsin solution containing 1 mg/ml collagenase (Worthington) and 0.1% soybean trypsin inhibitor. Prior to use, cells were pelleted by centrifugation and resuspended in SF-DME/F12.

Cell Growth and Plating Efficiency

Logarithmically growing MDCK cells were trypsinized using a 0.3% EDTA, 0.1% trypsin solution. The cells were then treated with an equal volume of 0.1% soybean trypsin inhibitor, pelleted by centrifugation, and resuspended in SF-DME/F12. After repeating this procedure, the cells were inoculated into tissue culture dishes containing medium. After

an appropriate incubation period, the cells were trypsinized and counted with a Coulter counter. The dosage response to added factors was assayed by measuring cell number after a 4-day incubation period. Unless otherwise mentioned, the determinations of cell number were made in triplicate.

To determine the plating efficiency of MDCK cells, 500 cells were inoculated into 60 mm dishes containing 4 ml of medium (determinations were in triplicate); one week later colonies were fixed with formalin, stained with 0.5% crystal violet, and counted.

Materials

Hormones, including prostaglandins (J. Pike, Upjohn Co.), epidermal growth factor (EGF) and fibroblast growth factor (FGF) (Collaborative Research), purified human transferrin (original source Behring diagnostics, obtained from R. W. Holley, Salk Institute), purified bovine insulin (original source Eli Lilly, obtained from J. Lever, Salk Institute), and selenium (R. Ham, University of Colorado) were gifts. Dibutyryl cAMP, isobutyl methylxanthine (IBMX), bovine insulin, human transferrin, triiodothyronine and hydrocortisone were obtained from Sigma Co.

RESULTS

Growth of MDCK Cells in a Hormone-Supplemented, Serum-Free Medium

MDCK cells grew at 1.5 doublings per day in SF-DME/F12 supplemented with 10% fetal calf serum (Fig. 1). When serum was deleted from the medium the growth rate was

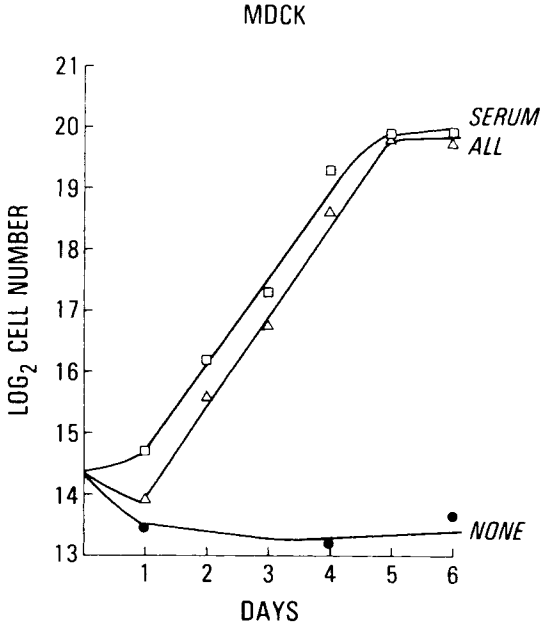

Fig. 1. The effect of medium supplementation of MDCK cell growth. MDCK cells were inoculated at 2.5×10^4 cells/dish into 60 mm dishes containing SF-DME/F12 supplemented with a) 10% fetal calf serum △; b) PGE_1, 25 ng/ml; T_3, 5×10^{-12}M; hydrocortisone, 5×10^{-8}M; insulin 5 μg/ml; transferrin, 5 μg/ml □; or no additional factors ●.

less than 0.4 doublings per day. However, MDCK cells grew at 1.5 doublings per day when SF-DME/F12 was supplemented with PGE_1, hydrocortisone, triiodothyronine, insulin and transferrin (Medium K-1) in the absence of serum (Fig. 1). The ability of Medium K-1 (SF-DME/F12 supplemented with the 5 components) to support clone formation, and the long-term growth of MDCK cells was also studied. The plating efficiency of MDCK cells in Medium K-1 was 70% of that observed in the serum-supplemented medium, and MDCK cells also successfully grew over a 5-week test period (through 4 passages) in Medium K-1.

The optimal concentrations of the hormones in Medium K-1 were determined by assaying the effect of hormone concentration on MDCK cell growth (Table I). With the exception of insulin, all hormones caused maximal growth stimulation at physiological concentrations (Table I). The effects of different prostaglandins on growth were similarly compared between 1 and 1,000 ng/ml [12]. PGE_2 caused equivalent growth stimulation to PGE_1 while $PGF_2\alpha$ and PGA_1 did not have significant growth stimulatory effects.

The relative effects of the individual components in Medium K-1 on cell growth were compared by means of a hormone deletion study (Fig. 2). Omission of either PGE_1 or transferrin from Medium K-1 was more deleterious to cell growth (growth was inhibited by over 50%) than the omission of hydrocortisone, T_3 or insulin; the growth inhibition resulting from the removal of T_3 was observed only after a 6-day time interval. These observations indicate that PGE_1 and transferrin are the most critical of the 5 factors for MDCK cell growth in serum-free medium. However, no growth stimulation was observed when SF-DME/F12 was supplemented with only one of these factors. Increased growth was observed, however, when SF-DME/F12 was supplemented with 2 components, either transferrin and PGE_1, or transferrin and insulin (Table II).

The Mechanism of Action of PGE_1

As PGE_1 a) is one of the most critical components in Medium K-1 for growth, and b) specifically stimulates growth of MDCK cells, rather than the other cell types studied [8], the mechanism by which PGE_1 enhances MDCK cell growth was of interest. The possibility that PGE_1 stimulates growth as a result of its stimulatory effects on cAMP production was also examined with respect to its effects on cell growth in SF-DME/F12 sup-

TABLE I. Concentration Optima of Supplements in Medium K-1

Medium supplement	Optimal concentration range	Concentration in Medium K-1
Insulin	5–10 μg/ml	5 μg/ml
Transferrin	5–20 μg/ml	5 μg/ml
PGE_1	25–1,000 ng/ml	25 ng/ml
T_3	5×10^{-12}M	5×10^{-12}M
Hydrocortisone	5×10^{-9}–10^{-7}M	5×10^{-8}M

The dependence of MDCK cell growth on hormone concentration was determined as follows. The optimal dosage of each factor was determined in the presence of the other 4 factors (insulin, 5 μg/ml; transferrin, 5 μg/ml; T_3, 5×10^{-12}M; hydrocortisone, 5×10^{-8}M; PGE_1, 25 ng/ml), with the exception that the T_3 optima were determined in the absence of hydrocortisone. Determinations were made in triplicate after 4 days. The effect of each hormone on cell growth was expressed as the percentage of the control cell number (the cell number in 35 mm dishes lacking the hormone being studied) as described in Taub et al [12].

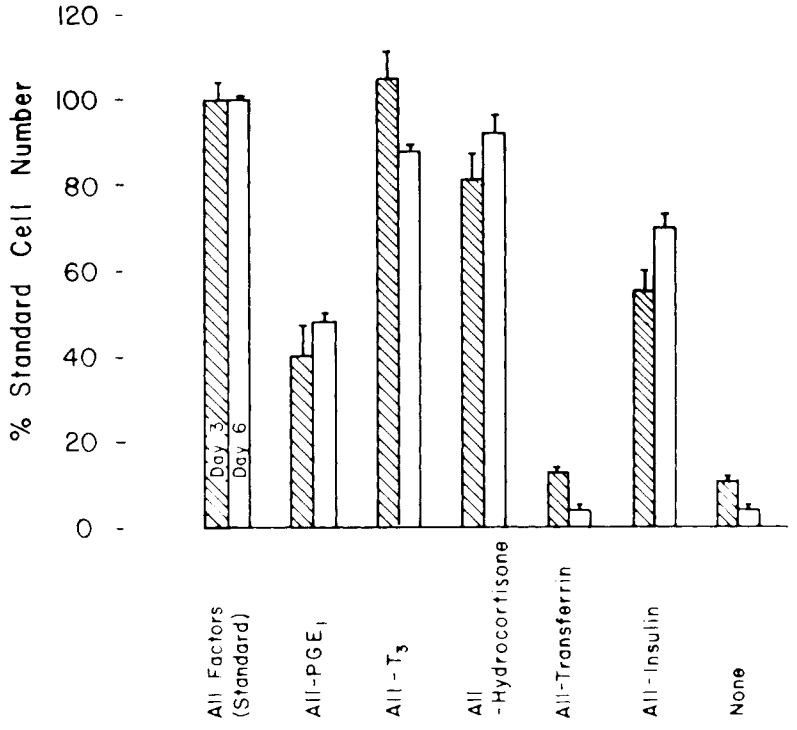

Fig. 2. The effect of deletion of a factor from hormone-supplemented medium on MDCK cell growth. MDCK cells were inoculated at 5×10^4/dish into 60 mm dishes containing a) SF-DME/F12 and 5 factors (PGE_1, 25 ng/ml; T_3, 5×10^{-12}M; hydrocortisone, 5×10^{-8}M; transferrin, 5 μg/ml; insulin, 5 μg/ml); b) SF-DME/F12 and 4 of the above factors or c) SF-DME/F12 (alone). Cell number was assayed in triplicate on days 3 and 6. In Medium K-1 the cell number was 1.1×10^6 on day 3 and 3.7×10^6 on day 6.

TABLE II. Synergistic Effects of Medium Supplements on Cell Growth

Medium supplement	Percentage standard cell number
None	2 ± 0
Insulin	2 ± 0
PGE_1	2 ± 0
T_3	1 ± 0
Transferrin	2 ± 0
Hydrocortisone (HC)	1 ± 0
Transferrin, PGE_1	22 ± 6
Transferrin, insulin	29 ± 6
Insulin, PGE_1	3 ± 0
Insulin, transferrin, PGE_1	82 ± 7
Insulin, transferrin, PGE_1, HC, T_3 (Std)	100 ± 7

MDCK cells were inoculated into dishes containing SF-DME/F12 supplemented with different combinations of the 5 factors in Medium K-1 (insulin, 5 μg/ml; transferrin, 5 μg/ml; T_3, 5×10^{-12}M; hydrocortisone, 5×10^{-8}M; PGE_1, 25 ng/ml). Growth was assayed in dishes in triplicate after 4 days, and was compared to the cell number in Medium K-1 (Standard).

TABLE III. Effect of Hormones on Hemicyst Formation

Added components	Hemicysts/field	Percentage of monolayer as hemicyst
Insulin, transferrin (Standard)	0	0
Standard + PGE_1	2.7 ± 2.1	8 ± 5
Standard + PGE_1 + hydrocortisone	10.4 ± 3.4	32 ± 10
Standard + PGE_1 + T_3	6.0 ± 3.8	18 ± 9
Standard + T_3	0	0
10% Fetal calf serum	1.3 ± 1.3	12 ± 31

MDCK cells were grown to confluency in SF-DME/F12 supplemented with the components indicated above (insulin, 5 μg/ml; transferrin, 5 μg/ml; PGE_1, 25 ng/ml; hydrocortisone, 5×10^{-8}M; T_3, 5×10^{-12}). The average number of hemicysts per field was estimated by counting 10 microscope fields at 100× magnification using a Nikon microscope. The hemicyst size was estimated from the diameter hemicysts, determined using a Nikon microscope grid, and compared to the total field size, also using the grid. The above estimations permitted a calculation of the percentage of monolayer of hemicyst.

plemented with insulin (5 μg/ml), and transferrin (5 μg/ml). Dibutyryl cAMP (0.5 mM) was not only growth stimulatory to MDCK cells, but also substituted for PGE_1 in Medium K-1, permitting optimal growth to occur. At a similar concentration sodium butyrate had no effect on growth. Three other factors which affect cAMP metabolism in MDCK cells were also studied. While isobutyl methylxanthine (0.5 mM) (a phosphodiesterase inhibitor) and glucagon (5 μg/ml) were also growth stimulatory, arginine vasopressin had no significant growth-enhancing effect.

Applicability of Serum-Free Medium to Examine Regulation of Hemicyst Formation

In serum-supplemented medium confluent monolayers of MDCK cells form domes or hemicysts, a process which depends upon the vectorial transport of salt and water across the monolayer. MDCK cells maintained in Medium K-1 not only were similar morphologically to cells in serum-supplemented medium, but also formed hemicysts.

The effect of the 5 components in Medium K-1 on hemicyst formation was examined (Table III). No hemicysts were observed in monolayers grown to confluency in SF-DME/F12 containing only insulin and transferrin. However, when SF-DME/F12 was supplemented with PGE_1 in addition to insulin and transferrin, hemicyst formation was apparent. The addition of hydrocortisone further increased the frequency of hemicysts, and this effect of hydrocortisone was observed only when PGE_1 was present.

Applicability of Medium K-1 for Growth of Primary Kidney Cultures

The simultaneous growth of both fibroblasts and epithelial cells in primary kidney cultures has complicated studies of tubular transport functions. The possibility that Medium K-1 would permit the selective growth of epithelial cells in primary kidney cultures was examined.

When baby mouse kidney cells were inoculated at 5×10^3 cells/cm^2 into either Medium K-1 or SF-DME/F12 supplemented with 10% fetal calf serum, 25% of the cells attached, and grew exponentially (0.6 doublings per day). Microscope examinations indicated that initially over 99% of the attached cells were epithelial in morphology under both culture conditions. However, in serum-supplemented medium after 5 days 13% of the cells were fibroblasts and after 11 days the majority of the cell population was fibroblastic (Fig.

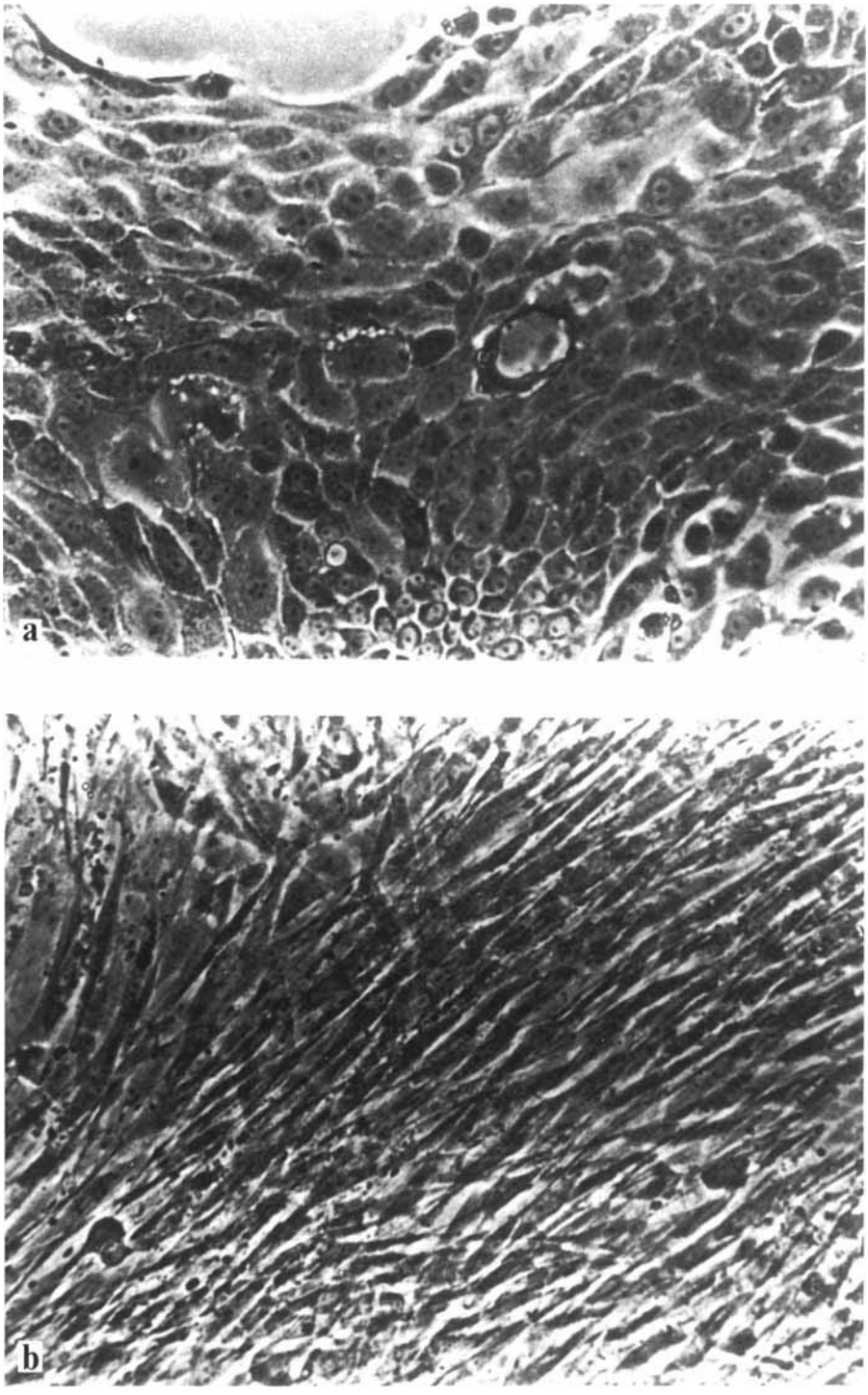

Fig. 3. Primary baby mouse kidney cultures. Baby mouse kidney cells were distributed at 10^4 cells/35 mm dish into dishes containing either a) Medium K-1 or b) SF-DME/F12 supplemented with 10% fetal calf serum. Eleven days later the cells were photographed. The suspension of baby mouse kidney cells was prepared as described in Materials and Methods.

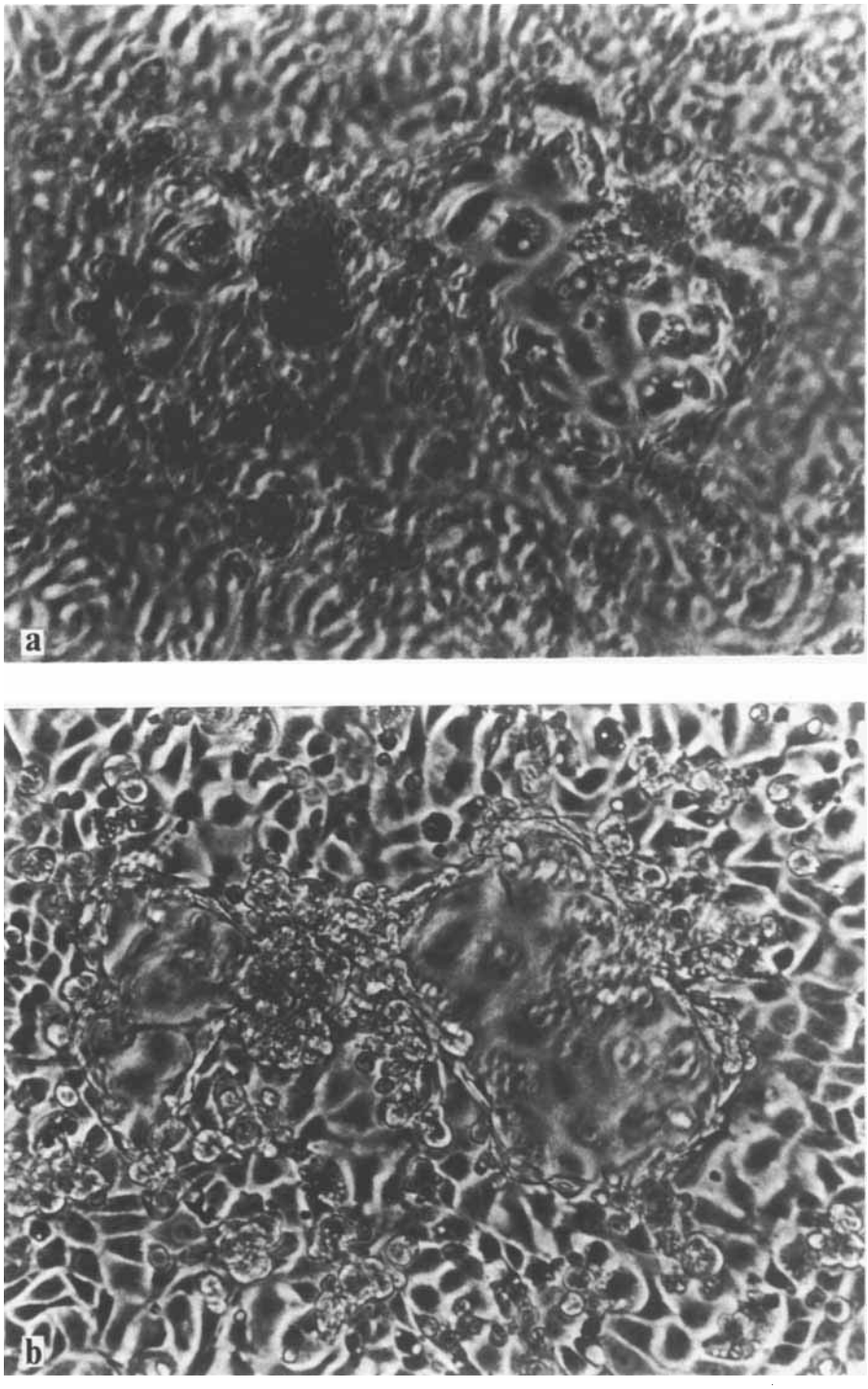

Fig. 4. Hemicyst formation by primary cultures. Baby mouse kidney cells were distributed at 5×10^4/ 35 mm dish into Medium K-1. Hemicyst formation was observed in confluent cultures.

TABLE IV. Growth of Kidney Cell Lines in Medium K-1

Cell type		Growth rate (doublings/day)	
Cell line	Species of origin	SF-DME/F12 + FCS	Medium K-1
LLC-MK_2 (7)	Monkey	1.6	1.6
MDBK (8)	Bovine	0.8	1.0
BSC-1	Monkey	0.7	0.4
NRK (15)	Rat	1.3	0.1
RAG (16)	Rat	1.6	1.2
MDCK (3)	Canine	1.5	1.5

Cells were inoculated at 2.5×10^4/60 mm dish into dishes containing SF-DME/F12 supplemented with 10% fetal calf serum. The next day the medium was removed by aspiration, the cells were washed three times with SF-DME/F12, and then incubated with Medium K-1 or SF-DME/F12 supplemented with 10% fetal calf serum. The growth rate was estimated from daily cell counts in duplicate dishes over a 5-day period.

3b). During this time interval no equivalent fibroblast overgrowth was observed in Medium K-1 (Fig. 3a). Although hemicyst formation was observed in primary cultures maintained in Medium K-1 (Fig. 4), it is particularly interesting that no such hemicysts were observed in SF-DME/F12 supplemented with 10% fetal calf serum.

The observation that baby mouse kidney cells grew in Medium K-1 (a dog kidney medium) suggested that Medium K-1 could be used for the maintenance of kidney epithelial cells derived from a number of animal species. Indeed, primary cultures of human fetal, baby rabbit and adult wolf kidney cells were all maintained in Medium K-1 in the absence of fibroblast overgrowth.

However, these observations do not indicate whether all types of epithelial cells in mammalian kidneys can grow in Medium K-1. To test this possibility, the effect of Medium K-1 on the growth of a number of kidney epithelial cell lines was examined. Table IV illustrates that 4 of the 6 kidney cell lines tested grew at equivalent rates in Medium K-1 and SF-DME/F12 supplemented with 10% fetal calf serum after a serum preincubation overnight. However, the BSC-1 cell line grew at only 57% of the rate observed in serum-supplemented medium.

DISCUSSION

When SF-DME/F12 was supplemented with insulin, transferrin, PGE_1, T_3 and hydrocortisone (Medium K-1), the kidney epithelial cell line MDCK was maintained one month in the absence of serum. MDCK cells proliferated at equivalent rates in Medium K-1 and in SF-DME/F12 supplemented with 10% fetal calf serum.

Although the addition of each of these 5 components was necessary to obtain optimal growth of MDCK cells, the deletion of either PGE_1 or transferrin from Medium K-1 had the most severe inhibitory effects on growth, as compared to the deletion of any other factor. Of interest in regard to the PGE_1 requirement is the observation that prostaglandins are produced in large quantities by the renal medulla [17]. Thus in vivo prostaglandins may regulate renal growth as well as systemic blood pressure [17].

These studies introduce the possibility that cAMP mediates the growth-stimulatory effects of PGE_1 on MDCK cells. Dibutyryl cAMP could substitute for PGE_1 in the medium;

moreover, other factors which affect cAMP production in MDCK cells, including PGE_2, glucagon, and isobutyl methylxanthine also enhanced growth [12], with the exception of arginine vasopressin. However, increased intracellular cAMP may still be correlated with growth, if arginine vasopressin affects a distinct compartment of intracellular cAMP, as compared to the other factors. The proposed correlation between increased intracellular cAMP levels and growth in MDCK cells is contrasted with the correlation between decreased intracellular cAMP levels and growth previously made in fibroblast cultures [18].

The extent to which Medium K-1 can be applied to cultured kidney cells has been examined. The studies indicate that the hormone-supplemented medium has little species specificity, as kidney epithelial cells from mouse, monkey, and man were maintained in Medium K-1. Second, in primary kidney cultures the hormone-supplemented medium permitted the selective growth of the epithelial cells, rather than the fibroblasts. Thus, the problem of fibroblast overgrowth which occurs in serum-supplemented medium can be avoided. Finally, Medium K-1 is apparently not universally applicable for all epithelial cells in the kidney. Only one of the two monkey kidney epithelial lines tested grew at equivalent rates in Medium K-1 and serum-supplemented medium. Conceivably, alternative media may be developed for the selective growth of different types of kidney epithelial cells in primary cultures. The availability of such hormone-supplemented media should make primary kidney cultures a more powerful tool for studies concerning kidney function.

ACKNOWLEDGMENTS

The investigators thank Dr. Jane Bottenstein for critical reading of the manuscript. The work was supported by NIH-GM 17019, NIH-CA 19731, NIH-GM 17702, and NSF PCM 76-80785 to G.S. M.T. was supported by an NIH postdoctoral fellowship (NIH 1 F32 CA05917-01).

REFERENCES

1. Katz A, Lindheimer MD: Ann Rev Physiol 39:97, 1977.
2. Reiter RJ: In Nowinski WW, Goss RS (eds): "Compensatory Renal Hypertrophy." New York: Academic Press, p 183, 1969.
3. Leighton JL, Estes W, Mansukhani S, Brada Z: Cancer 26:7022, 1970.
4. Misfeldt DS, Hamamoto SJ, Pitelka DR: Proc Natl Acad Sci USA 73:1212, 1976.
5. Cereijido M, Robbins ES, Dolan WJ, Rotundo CA, Sabatini DD: J Cell Biol 77:853, 1978.
6. Rindler MJ, Chuman L, Schaeffer L, Saier MH: J Cell Biol (In press).
7. Hull RN, Cherry WR, Johnson IS: Anat Rec 124:450, 1956.
8. Madin SH, Darby NB: Proc Soc Exp Biol Med 122:931, 1966.
9. Sato GH, Reid L: In Rickenberg HV (ed): "Biochemistry and Mode of Action of Hormones II." Baltimore: University Park Press, Vol 20:215, 1978.
10. Hayashi I, Sato G: Nature 259:132, 1976.
11. Sato GH: In Litwak G (ed): "Biochemical Action of Hormones." New York: Academic Press, Vol III:391, 1975.
12. Taub M, Chuman L, Saier MH, Sato G: Proc Natl Acad Sci USA (In press).
13. Leffert HL, Paul D: J Cell Biol 52:559, 1972.
14. Hopps HE, Bernheim BC, Nisalak A, Hin Tjio S, Smadell SE: J Immunol 91:416, 1963.
15. Duc-Ngugen H, Rosenblum EN, Zeigel RF: J Bacteriol 92:1133, 1966.
16. Klebe RS, Chen T, Ruddle FH: J Cell Biol 45:74, 1970.
17. Lee JB, Attalah AA: In Kurtzmann NA, Martinez-Maldonado M (eds): "Pathophysiology of the Kidney." Springfield: Charles C. Thomas, 1977.
18. Pastan IH, Johnson GS, Anderson WD: Ann Rev Biochem 44:491, 1975.