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Alterations in Growth Requirements of Kidney Epithelial Cells in Defined Medium Associated With Malignant Transformation

Mary Taub, Ben Ü, Lorraine Chuman, Michael J. Rindler, Milton H. Saier, Jr., and Gordon Sato

Biochemistry Department, State University of New York at Buffalo, Buffalo, New York 14214 (M.T.) and Biology Department, University of California, San Diego, La Jolla, California 92093 (B.U., L.C., M.J.R., M.H.S., G.S.)

The possibility has been investigated that 1) the supplements required for the growth of the Madin Darby Canine Kidney (MDCK) cell line in serum-free Medium K-1 are indeed requirements for the growth of normal kidney cells vitro, and 2) that alterations in these growth requirements are associated wi malignant transformation. Consistent with the hypothesis that MDCK cells resemble normal kidney cells in culture, primary cultures of baby mouse kidney epithelial cells grow in Medium K-1 and respond to the 5 components in the medium. The growth properties of Moloney sarcoma virus (MSV)-transformed MDCK cells in defined media have been examined. Unlike MDCK cells, MSV-transformed MDCK cells form tumors in adult nude mice. Although they still respond to the 5 factors in Medium K-1, the optimal dosage for insulin is lower for the MSV transformants than for MDCK cells. The MSV transformants also have an additional requirement for growth in Medium K-1 – fibronectin. Variants of MDCK cells have been isolated that have lost the PGE_1 requirement for growth in defined medium. These variant cells have acquired 1) the ability to form tumors in adult nude mice and 2) an alteration affecting cAMP metabolism, in addition to PGE_1 independence.

Key words: defined medium, renal epithelium, tumorigenicity, primary culture, PGE, independent variant

Defined tissue culture medium is an important tool for in vitro studies concerning the regulation of animal cell growth. In the serum-free tissue culture media developed by Sato and his associates [1], hormones [2, 4, 7], growth factors [5–7], transport proteins [2, 4, 7], and cell attachment factors [8] are added to tissue culture medium in lieu of fetal calf serum. The mechanisms by which these supplements regulate growth and the possible in vivo significance of such growth-stimulatory effects have not yet been examined. Presumably, alterations in the requirements for these growth supplements may occur upon malignant transformation.

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The Madin Darby Canine Kidney (MDCK) cell line is a good model system in which to examine the mechanisms by which the supplements in defined medium regulate kidney epithelial cell growth. The MDCK cell line closely resembles "normal" kidney tubule cells both functionally and with regard to growth characteristics. Like the cells in the kidney tubule, MDCK cells transport salt and water across the cell layer [11, 12], as indicated by the hemicysts observed in confluent MDCK monolayers. Also like "normal" cells, MDCK cells do not form tumors when injected into adult nude mice [15]. In serum-free medium supplemented with insulin, transferrin, prostaglandin E_1 (PGE_1), triiodothyronine (T_3), and hydrocortisone (Medium K-1) MDCK cells grow over a long term and at a rate equivalent to that observed in serum-supplemented medium [4]. Medium K-1 also permits the growth of primary cultures of kidney epithelial cells in the absence of fibroblasts [4].

In this paper we show that the primary cultures respond to each of the 5 growth factors in Medium K-1, indicating that MDCK cells and Medium K-1 can be used to study molecular changes associated with malignant transformation of kidney epithelial cells. We then investigate this association by examining 1) the growth requirements of virally transformed MDCK cells and 2) the tumorigenicity of variant MDCK cells that have altered growth requirements.

MATERIALS AND METHODS

Cell Culture

MDCK cells were obtained from Dr. John Holland at the University of California, San Diego, and Moloney sarcoma virus-transformed MDCK cells were obtained from Dr. George Todaro at the National Institutes of Health. The kidney cells were maintained in a humidified 5% CO_2/95% air mixture of Dulbecco's Modified Eagle's and Ham's F12 Medium (SFFD), which was supplemented with 10 mM Hepes, sodium bicarbonate at 1.2 mg/ml, ampicillin at 25 μg/ml, and 10 nM Na_2SeO_3 $5H_2O$. Triple-distilled water was used for medium preparation. Stock cultures were maintained in SFFD supplemented with 2.5% horse serum and 7.5% fetal calf serum. Medium K-1 [4] consisted of SFFD supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), PGE_1 (25 ng/ml), triiodothyronine (T_3, 5×10^{-12} M), and hydrocortisone (5×10^{-8} M).

Primary cultures of baby mouse kidney cells were initiated with kidneys from 10-day-old male mice (Balb/c). The kidneys were minced into 1-mm diameter pieces, and a suspension of mince in SFFD was incubated with 1 mg/ml collagenase (Type IV, Worthington #4188) and 1 mg/ml soybean trypsin inhibitor at 37°C for 15 min; nephron fragments released by this procedure were washed by centrifugation. The collagenase treatment of the mince was repeated 2 or 3 times, and then the nephrons were treated with 0.1% trypsin–0.3% EDTA in phosphate-buffered saline. Trypsin action was stopped with soybean trypsin inhibitor.

Mutagenized cultures of MDCK cells were obtained with N-methyl-N′-nitro-N-nitrosoguanidine (NG). Log phase cultures were treated with 2 μg/ml NG for 2 h as described previously [16].

Cell Growth, Colony Formation, and Tumorigenicity in Nude Mice

Single cell suspensions of kidney cells were inoculated into 35-mm dishes containing medium. In the cell growth studies, which were conducted as described by Taub et al [10], the cell number was determined with a Coulter counter. The effect of fibronectin on cell

growth was determined by adding the fibronectin to the dishes in 1 ml of SFFD, and allowing an attachment period of 1 h at 37°C. The SFFD was then removed by aspiration, and the dishes were washed once with SFFD. The growth medium and cells were then added to the dishes. To assay colony number, the colonies were fixed with 10% formalin, stained with 0.5% crystal violet, and counted. Unless otherwise mentioned, all determinations were in triplicate.

To determine tumorigenicity, 2×10^6 cells in 1 ml SFFD were inoculated subcutaneously into adult nude mice. The mice were maintained as described by Stiles et al [15] and were inspected after 4 months for the appearance of nodules.

Determination of Intracellular cAMP

Intracellular cAMP was determined in MDCK cultures grown to confluency in SFFD supplemented with 10% fetal calf serum [17]. To determine the effects of supplements on cAMP levels, the cells were washed twice with PBS, once with SFFD, and then incubated in SFFD with the appropriate supplements. After the appropriate incubation period, the cells were washed 3 times with PBS and treated with cold 1% perchloric acid. The nucleotide was purified in Dowex AG1-X8 200–400 mesh columns, using ^{14}C-cAMP to monitor recoveries as described by Rindler et al [17]. Cyclic AMP content was assayed by the Gilman procedure.

Materials

Insulin, triiodothyronine, hydrocortisone, and human transferrin were obtained from Sigma. Prostaglandin E_1 was a gift from John Pike at Upjohn Corp. Fibronectin was obtained from Collaborative Research, and radiochemicals were from New England Nuclear.

RESULTS

Growth of Primary Kidney Cultures in Defined Medium

Medium K-1 permits the growth of MDCK cells over long periods at the same rate as serum-supplemented medium (1.4 doublings/day) (Fig. 1) [4]. Baby mouse kidney epithelial cells taken directly from the animal also grow in Medium K-1 and serum-supplemented medium at the lower rate of 0.6 doublings/day [20] when plated at 5×10^3 cells per cm^2. When plated at lower densities, baby mouse kidney cells formed epithelial colonies in Medium K-1 (no fibroblasts were observed), and only fibroblast colonies were observed in serum-supplemented medium (Fig. 2) [4].

The effect of the 5 supplements in Medium K-1 on the formation of MDCK and baby mouse kidney epithelial colonies is illustrated in Figure 3. The individual deletion of any one of the 5 supplements from Medium K-1 resulted in a decrease in the frequency of both baby mouse kidney and MDCK colonies. When either PGE_1 or transferrin was individually removed from Medium K-1, fewer baby mouse kidney epithelial colonies were observed than when any of the other 3 components was deleted from Medium K-1. A similar observation was made with MDCK cells. This study indicates that, with respect to their growth responses to hormones, MDCK cells closely resemble kidney cells taken directly from the animal. Thus, the MDCK cell line is appropriate for studying the alterations that occur in the in vitro growth requirements for kidney epithelial cells following malignant transformation.

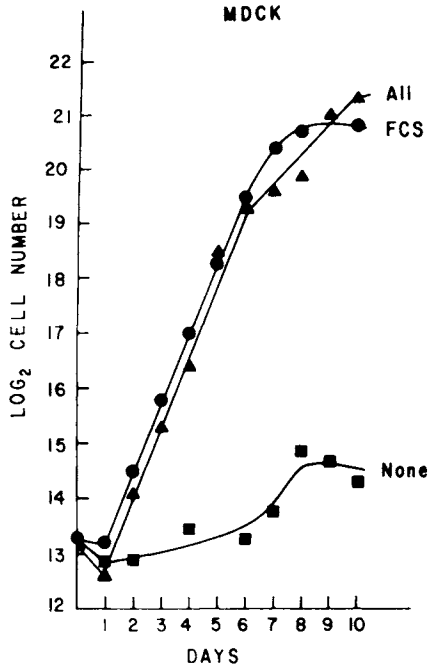

Fig. 1. Growth of MDCK cells in defined medium. MDCK cells were inoculated at 10^4 cells per 60-mm dish into SFFD supplemented with (a) 10% fetal calf serum (FCS), (b) insulin (5 μg/ml), transferrin (5 μg/ml), PGE_1 (25 ng/ml), T_3 (5×10^{-12} M) and hydrocortisone (5×10^{-8} M) (all), or (c) with no supplements (none). The cells were counted in duplicate dishes on a daily basis. Medium was changed on days 4, 6, and 8.

Growth of Viral Transformants of MDCK Cells in Defined Medium

Previous studies of Moloney sarcoma virus-transformed MDCK cells (MSV-MDCK cells) [18] have indicated that in serum-supplemented medium, MSV-MDCK cells have a similar growth rate and saturation density to MDCK cells. However, unlike MDCK cells, MSV-MDCK cells form tumors in adult nude mice [18]. Thus, MSV-MDCK cells were examined to determine whether alterations in growth requirements in defined medium occur concomitantly with malignant transformation.

Table I illustrates that transformation may be accompanied by the addition of growth requirements in defined medium. MSV-MDCK cells required fibronectin-coated dishes, as well as a relatively high cell inoculum (2×10^3 cells/cm^2) in order for cell growth to occur in SFFD with the 5 supplements. This latter observation suggests that MSV-MDCK-conditioned medium may contain a factor required for the growth of this transformant in the absence of fetal calf serum.

The effect of the 5 supplements in Medium K-1 was also studied (Table I). Like MDCK cells, MSV-MDCK cells require all of the factors in Medium K-1 in order to obtain maximal growth in serum-free medium (ie, to grow at the rate obtained in serum-supplemented medium). Although insulin at the concentration in Medium K-1 (5 μg/ml) was not growth-stimulatory to MSV-MDCK cells, a growth-stimulatory affect of insulin was observed at 0.1 μg/ml. This latter concentration was significantly below the dosage of insulin that has

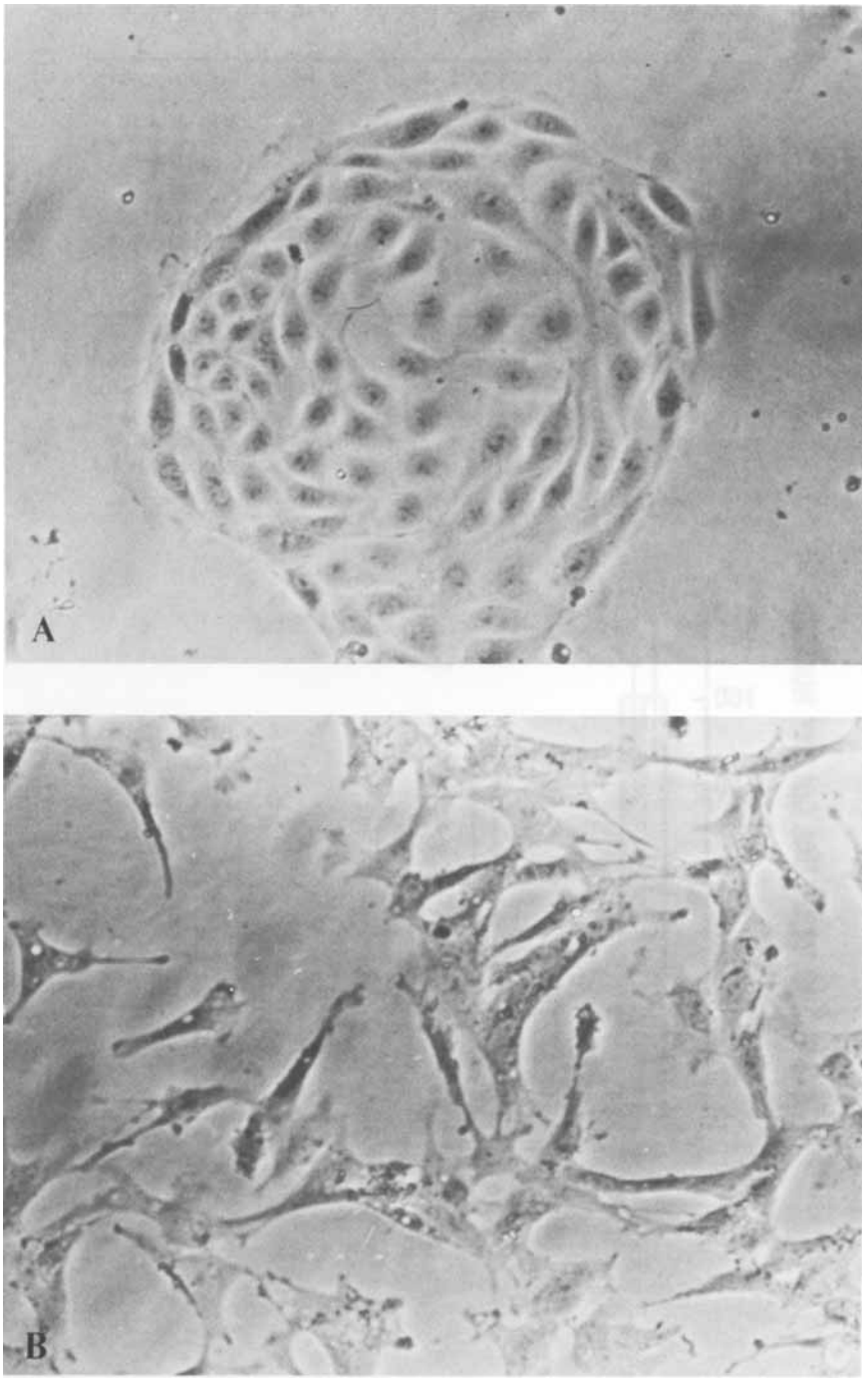

Fig. 2. Colony formation by baby mouse kidney epithelial cells. Baby mouse kidney cells were inoculated at 5×10^3 cells/35-mm dish into (A) Medium K-1 or (B) SFFD supplemented with 10% fetal calf serum. Representative colonies were photographed at 100× magnification ten days later.

TABLE I. Supplements in SFFD Required for Optimal Kidney Cell Growth

Supplement	Concentrations which permit maximal growth	
	MDCK	MSV-MDCK
Insulin	5 μg/ml	0.1 μg/ml
Transferrin	5 μg/ml	5 μg/ml
Hydrocortisone	10^{-8}–10^{-7} M	10^{-8}–10^{-7} M
PGE_1	25 ng/ml	25 ng/ml
T_3	5×10^{-12} M	5×10^{-12} M
Fibronectin	–	2 μg/ml

The effects of insulin, transferrin, hydrocortisone, PGE_1, and T_3 on MDCK and MSV-MDCK growth were determined after 4 days of growth in SFFD supplemented with the other 4 components (insulin at 5 μg/ml, PGE_1 at 25 ng/ml, hydrocortisone at 50 nM, and T_3 at 5 pM) with the exception that hydrocortisone was omitted from the T_3 dose response [4]. In studies with MSV-MDCK the dishes were also coated with fibronectin, as described in Materials and Methods. Determination of cell number was made in triplicate dishes.

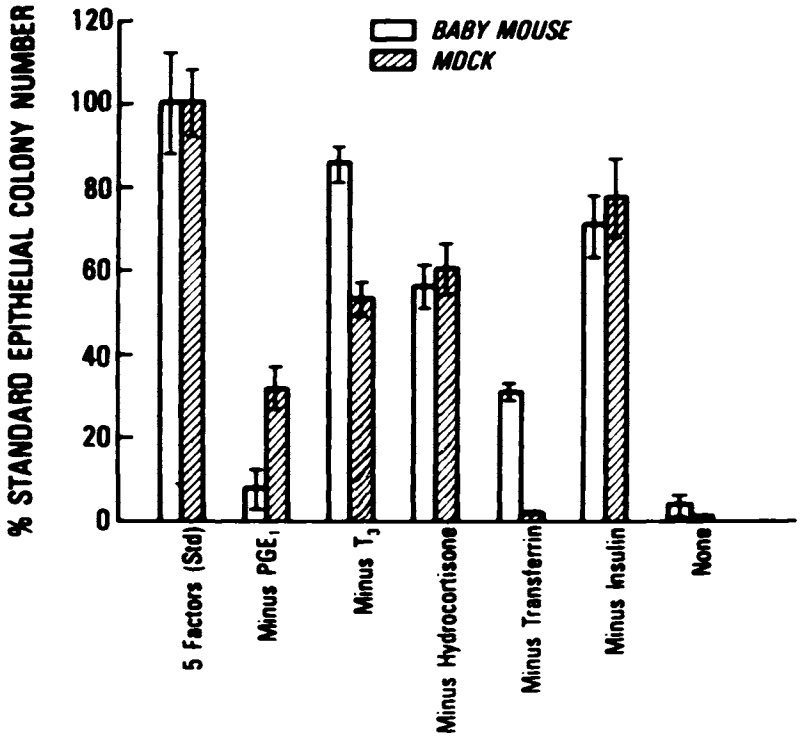

Fig. 3. Effect of hormone deletion on the formation of kidney colonies. MDCK cells and baby mouse kidney cells were inoculated into 35-mm dishes at 10^3 and 5×10^3 cells per dish, respectively. The dishes contained Medium K-1, and Medium K-1 lacking either insulin, transferrin, PGE_1,T_3, or hydrocortisone. Ten days later, colony number in the dishes was determined in triplicate, as described in Materials and Methods.

a significant affect on MDCK cell growth [4]. The other 4 supplements were growth-stimulatory to MSV-MDCK cells at the concentrations present in Medium K-1. Thus, a decrease in the hormone dosage required for kidney cell growth may occur concomitantly with malignant transformation, although the growth responses of kidney epithelial cells to hormones are not necessarily lost.

PGE_1-Independent Variants of MDCK Cells

These observations do not exclude the possibility that loss of a growth requirement for a supplement in defined medium may result in malignant transformation. In order to examine this possibility, and the mechanism by which hormones increase growth, variants of the MDCK cell line that lack the PGE_1 requirement for growth were isolated as follows. MDCK cells from NG-mutagenized cultures were plated at 5×10^3 cells/cm^2 into SFFD supplemented with 10% fetal calf serum. The following day, the cells were washed twice with SFFD, and then cultured in Medium K-1 without PGE_1. Although MDCK cells can grow well over a month in Medium K-1 [4], the majority of MDCK cells died after a two-week culture period in Medium K-1 lacking PGE_1 (which included one subculturing). Viable clones remained in the dishes after this time, however, and were isolated in cloning cylinders. These clones, described as PGE_1-independent clones, due to the selection procedure, were grown up in SFFD supplemented with 10% fetal calf serum.

Figure 4 compares the growth rate of (I) MDCK cells and (II) a PGE; independent clone of MDCK cells (PGE_1-independent clone I) in serum-supplemented and serum-free media. MDCK cells grew at 1.4 doublings/day in SFFD supplemented with 10% fetal calf serum

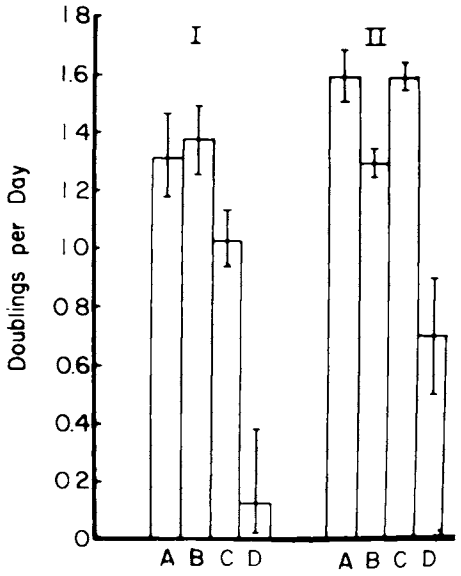

Fig. 4. Growth of (I) MDCK cells, and (II) PGE_1-independent MDCK cells in defined medium. MDCK and PGE_1-independent MDCK cells were grown in (A) SFFD supplemented with 10% fetal calf serum, (B) Medium K-1, (C) Medium K-1 lacking PGE_1, and (D) SFFD alone over a 10-day period. Cells were counted in duplicate dishes on a daily basis, and medium was changed on days 4, 6, and 8. The growth rates were estimated from the slope of plots of $\log_2$ cell number as a function of time.

and in Medium K-1. When PGE_1 was removed from Medium K-1, the MDCK cell growth rate was lower – 1.1 doublings per day; consequently, after 5 days the MDCK cell number in Medium K-1 lacking PGE_1 was only 14% of the cell number observed in Medium K-1.

In contrast, PGE_1-independent clone I grew at 1.6 doublings/day, both in SFFD supplemented with 10% fetal calf serum and in Medium K-1 lacking PGE_1. The growth rate of this PGE_1-independent clone in Medium K-1 (which contains PGE_1), however, was considerably lower – 1.1 doublings/day. This observation indicated that PGE_1 was growth-inhibitory, rather than growth-stimulatory to PGE_1-independent clone I. Indeed, an inhibitory effect of PGE_1 on the growth of this independent clone was observed at concentrations of 1–100 ng/ml. These growth properties of PGE_1-independent clone I were stable over a 6-month culture period.

If PGE_1 were growth limiting to MDCK cells not only in vitro but also in vivo, then the loss of this growth requirement might cause these cells to become tumorigenic in animals. Thus, the tumorigenic potential of PGE_1-independent MDCK cells in adult nude mice was examined. Four months after subcutaneous injection of PGE_1-independent MDCK cells into adult nude mice, tumors were observed in all injected animals (6/6). However, no tumor formation was observed with the parental MDCK cell line [15].

The PGE_1-independent cells were also studied to determine the mechanism by which PGE_1 affects growth. PGE_1 has been postulated to increase MDCK cell growth by elevating intracellular cAMP levels [4]. If this hypothesis is true, then loss of the growth-stimulatory effect of PGE_1 may be caused by defective cAMP metabolism. Table II illustrates the effect of PGE_1 and isobutyl methylxanthine on intracellular cAMP levels in MDCK and PGE_1-independent cells. The maximal intracellular cAMP level observed in MDCK cells (0.17 nmoles/mg protein) was after 2 minutes with PGE_1. After PGE_1-independent cells were treated for either 2 or 45 minutes with PGE_1, intracellular cAMP levels were either 5-fold or 17-fold higher, respectively, than the maximal cAMP level attained in MDCK cells (Table II). Extracellular cAMP levels were also elevated in the variant cells (data not presented), indicating that the increased intracellular cAMP did not simply result from a defect in cAMP efflux.

DISCUSSION

The MDCK cell line and Medium K-1, the defined medium for MDCK, have been used to study alterations in the growth requirements of kidney cells that occur upon malignant transformation. Conclusions from such studies depend on the assumption that the

TABLE II. Intracellular cAMP in MDCK and in PGE_1-Independent MDCK Cells*

Agent	MDCK (nmoles cAMP/mg protein)	PGE_1-independent clone 1
None	0.074 ± 0.011	0.085 ± 0.025
IBMX for 2 min	0.130 ± 0.046	0.074 ± 0.009
IBMX + PGE_1 for 2 min	0.17 ± 0.032	0.85 ± 0.11
IBMX + PGE_1 for 45 min	0.094 ± 0.012	2.88 ± 0.03

*Intracellular cAMP levels in confluent monolayers of MDCK cells were determined 0, 2, or 45 minutes after the addition of the agents shown above. PGE_1 was at 1 μg/ml and IBMX at 0.1 mM. The determination of intracellular cAMP level was as described in Materials and Methods and by Rindler et al [17].

MDCK cell line closely resembles normal kidney cells with regard to its growth regulation. Thus, the similarity of MDCK cells to normal kidney epithelial cells was evaluated. Primary cultures of baby mouse kidney epithelial cells not only grow in Medium K-1 [20] but also form epithelial colonies when inoculated at low densities into Medium K-1 [4]. The effect of each of the 5 factors in Medium K-1 on the formation of such colonies by baby mouse kidney and MDCK cells was compared by means of a hormone deletion study. The individual deletion of any of the 5 supplements from Medium K-1 resulted in a decrease in the number of baby mouse kidney and MDCK colonies. However, the individual deletion of either PGE_1 or transferrin resulted in a more dramatic decrease in the number of baby mouse kidney colonies and MDCK colonies than the individual deletion of either insulin, hydrocortisone, or T_3. These observations indicate 1) that the MDCK cell line closely resembles "normal" kidney cells with regard to its growth properties in defined medium, and consequently 2) that MDCK is a good cell line for studies concerned with the effect of malignant transformation on growth in defined medium.

Malignant transformation may be associated with the loss of a growth requirement in defined medium. This possibility was examined by studying 1) the growth properties of a viral transformant of MDCK cells in defined medium and 2) the properties of a variant of MDCK cells, isolated by its ability to grow in defined medium lacking one required supplement, PGE_1.

A Moloney sarcoma viral transformant of MDCK cells (MSV-MDCK cells), which has acquired a number of properties often associated with malignant transformation, was studied. Rather than losing a requirement for the 5 supplements in Medium K-1, MSV-MDCK cells acquired additional requirements for growth in defined medium, including fibronectin, and a high plating density. Although insulin was still growth stimulatory, the concentration required to attain optimal growth decreased. If a hormone such as insulin actually affects kidney epithelial cell growth in vivo and is present in plasma at a suboptimal dosage, then a reduction (or even a loss) in the cellular requirement for insulin may result in malignancy.

PGE_1-independent variants of MDCK cells (cells that lack the PGE_1 requirement for growth) were selected by their ability to grow over a long term (more than 1 month) in Medium K-1 lacking PGE_1. Normal MDCK cells can survive over a long period in Medium K-1, but they die if PGE_1 is deleted. One PGE_1-independent clone, examined in detail, grows in Medium K-1 lacking PGE_1 at the rate observed in serum-supplemented medium. When PGE_1 is added to the medium, decreased rather than increased growth was observed with these PGE_1-independent cells. Furthermore, this PGE_1-independent variant also forms tumors in adult nude mice, unlike normal MDCK cells. This observation is consistent with the hypothesis that PGE_1 (or PGE_2) is required for kidney cell growth in vivo, and that in adult animals the PGE_1 concentration is below the minimal dosage required for growth to occur. According to this hypothesis, loss of the PGE_1 requirement for growth would result in tumor formation.

The biochemical defect that results in the altered growth regulation in the PGE_1-independent clone was also studied. The growth-stimulatory effect of PGE_1 on MDCK cells has been postulated to be mediated via an increase in intracellular cAMP levels. If this hypothesis is correct, then the loss of the PGE_1 requirement for growth may be associated with an alteration in cAMP metabolism. Indeed, PGE_1-independent cells were observed to have much higher levels of intracellular cAMP than MDCK cells following treatment with PGE_1.

Both the PGE_1-independent variants and the viral transformants of MDCK cells should be useful in future studies concerning the changes in growth regulation that are associated with tumorigenicity. The viral transformant studied here has a decreased insulin requirement for growth. This alteration may result from a modification of the insulin receptors in MDCK cells. The PGE_1-independent cells may have lost the PGE_1 growth requirement as the result of an alteration affecting the PGE_1 receptor, adenylate cyclase, or phosphodiesterase. Future studies with PGE_1-independent cells, and with virally transformed MDCK cells, should elucidate the role of insulin and PGE_1 in regulating growth, as well as the molecular changes that have caused the altered growth requirements for these factors.

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REFERENCES

1. Sato GH, Reid L: In Rickenberg N (ed): "Biochemistry and Mode of Action of Hormones II." Baltimore: University Park Press, 1978, pp 219–251.
2. Hayashi I, Sato G: Nature 259:132, 1976.
3. Rizzino A, Crowley C: Proc Natl Acad Sci USA (in press).
4. Taub M, Chuman L, Saier MH, Sato G: Proc Natl Acad Sci USA 76:3338, 1979.
5. Hutchings SE, Sato G: Proc Natl Acad Sci USA 75:901, 1978.
6. Serrero G, McClure D, Sato G: In Ross R, Sato G (eds): "Hormones and Cell Culture, Cold Spring Harbor Conferences on Cell Proliferation, 1980," vol 6. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory (in press).
7. Hayashi I, Larner J, Sato G: In Vitro 14:23, 1978.
8. Orly J, Sato G: Cell 17:295, 1979.
9. Gaush CR, Hard WL, Smith TF: Proc Soc Exp Biol Med 122:931, 1966.
10. Taub M, Saier MH: In Jacoby W, Pastan I (eds): "Methods in Enzymology, 1979," vol 38, 1979, pp 552–560.
11. Misfeldt DS, Hamamoto SJ, Pitelka DR: Proc Natl Acad Sci USA 73:1212, 1976.
12. Cerrejido M, Robbins ES, Dolen WJ, Rotunno CA, Sabatini DD: J Cell Biol 77:853, 1978.
13. Leighton JL, Estes W, Mansukhani J, Braday Z: Cancer 26:7022, 1970.
14. Dulbecco R, Elkington J: Nature 246:197, 1973.
15. Stiles CD, Desmond W, Chuman LM, Saier MH, Sato G: Cancer Res 36:3300, 1976.
16. Taub M: Somatic Cell Genet 4:609, 1978.
17. Rindler MJ, Chuman L, Schaeffer L, Saier MH: J Cell Biol 81:635, 1979.
18. U B, Rindler MJ, Stiles C, Saier MH: J Cell Biol 79:74a, 1978.
19. Pastan IH, Johnson GS, Anderson WD: Annu Rev Biochem 44:491, 1975.
20. Taub M, Sato G: J Supramol Struct 11:207, 1980.