Role of Endogenous Prostaglandin E_2 in Erythropoietin Production and Dome Formation by Human Renal Carcinoma Cells in Culture

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Abstract. Studies were carried out on the role of endogenous prostaglandin E_2 (PGE_2) in erythropoietin (Ep) production and dome formation in primary monolayer cultures of a human renal carcinoma from a patient with erythrocytosis that has been serially transplanted into BALB/c athymic nude mice. The metabolism of [^{14}C]arachidonic acid (^{14}C-AA) by cultured renal carcinoma cells, which were plated in 25-cm^2 flasks at a density of 2×10^4 cells/cm^2 and grown for 6, 12 (confluence, 13×10^4 cells/cm^2), 16, 24, and 30 d in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum, was examined by using radiometric thin-layer chromatography (TLC). TLC revealed PGE_2 to be the major metabolite of ^{14}C-AA produced by the cultured cells throughout the 30 d of cultivation. In addition, the cultured cells at each time period were incubated for 24 h in 5 ml of serum-free Eagle's MEM and the levels of PGE_2 and Ep in the incubated media were measured via radioimmunoassay. PGE_2 levels in the serum-free media incubated with the cultured cells grown for 6 d were significantly ($P < 0.001$) elevated (174±2.5 pg/ml, $n = 5$), compared with the unincubated control media (1.5±0.19 pg/ml, $n = 5$) and gradually decreased at each time period to 97.6±4.4 pg/ml ($n = 5$) at 30 d. On the other hand, the levels of Ep in the incubated media of the cells grown for 6 d were 11.5±0.52 mU/ml ($n = 5$) compared with 7.6±0.62 mU/ml ($n = 5$) in the control media. However, after the cultured cells became confluent, the levels of Ep in the incubated media showed a marked increase to 222.9±5.26 mU/ml ($n = 5$) at 30 d of cultivation. Multicellular hemicysts (domes) developed after the cultured cells reached confluence and their numbers increased with increasing time in confluence in parallel with the increase in Ep. Meclofenamate (MF) (3×10^{-6}–3×10^{-5} M), a prostaglandin synthesis inhibitor, produced a significant dose-related decrease in PGE_2, Ep, and dome formation without producing a significant effect on cell viability in the 30-d cells. This inhibitory effect of MF on Ep production and dome formation was completely abolished by the addition of 10^{-8} M PGE_2 to the incubation medium. In conclusion, endogenous PGE_2 plays an important role in supporting and/or stimulating Ep production and dome formation in cultured renal carcinoma cells.

Introduction

Elevated plasma and urinary levels of erythropoietin (Ep)[1] and an associated erythrocytosis have been reported in patients with renal carcinoma (1–7). Remission of the erythrocytosis was seen after the removal of the tumor and erythropoietic activity was found in the tumor extracts, suggesting that this tumor is capable of producing Ep. Direct evidence for production of Ep by a human renal carcinoma has been provided by Sherwood and Goldwasser (8) who have shown that human renal carcinomas in cultures can synthesize and secrete an active erythropoietic factor that is structurally similar, though not identical, to human urinary Ep.

Prostaglandin (PG) production by human renal carcinomas has been reported by several investigators (9–12) and PGs produced by these renal carcinomas have been suggested to be involved in the pathogenesis of the cardiovascular manifestation and hypercalcemia associated with this disease.

Received for publication 17 October 1983 and in revised form 18 May 1984.

J. Clin. Invest.

0021-9738/84/10/1252/10 $1.00
Volume 74, October 1984, 1252–1261

1. *Abbreviations used in this paper:* ^{14}C-AA, [^{14}C]arachidonic acid; Ep, erythropoietin; HE, hematoxylin-eosin; LED cell, low electron density cell; MEM, minimum essential medium; PG, prostaglandin; PGD_2, PGE_1, PGE_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$, prostaglandins D_2, E_1, E_2, $F_{1\alpha}$, $F_{2\alpha}$, respectively; TLC, thin-layer chromatography; TXB_2, thromboxane B_2.

A possible relationship between PG production and an associated erythrocytosis in human renal carcinomas has been suggested by Greaves (13) who investigated a patient with renal carcinoma and secondary erythrocytosis and found high levels of prostaglandin E and Ep in the tumor extracts.

The present studies were carried out to investigate PG production and its role in Ep production in cultures of a human renal carcinoma that was obtained from a patient with erythrocytosis and was serially transplanted into athymic nude mice.

Methods

Transplantation of human renal carcinoma into nude mice. The human renal carcinoma used in the present studies was provided by T. Nomura of the Central Institute for Experimental Animals, Kanagawa, Japan and H. Tazaki of Keio University School of Medicine, Tokyo. The renal carcinoma had been initially grown in nude mice after its removal from a patient with erythrocytosis and was kept frozen before being transplanted into two BALB/c strain athymic nude mice and transported from Kanagawa, Japan to our laboratory. Inbred female BALB/c athymic nude mice 6–8 wk old (Charles River Breeding Laboratories, Inc., Wilmington, MA) were used for successive passages of the tumor in nude mice in our laboratory. The nude mice were housed in a laminar air flow hood unit. All food and other materials in contact with the mice were sterilized. When tumor growths of >1 cm in diameter developed, they were aseptically removed, minced into 2-mm^3 fragments, and transplanted to other nude mice subcutaneously on both sides of the back by using a number 12 trocar needle. Two to three pieces of the tumor fragments were inoculated with each transplant. Serial passages were carried out every 4–6 wk.

The tumors used in the present studies were removed from the nude mice after the 21st to 25th passages and the nude mice developed no significant elevation of hematocrit in comparison with control nude mice.

Preparation of antiserum to nude mouse spleen cells. The method described by Okabe et al. (14) was used to prepare the antiserum against nude mouse spleen cells. Briefly, $\sim 1 \times 10^8$ spleen cells from BALB/c nude mice were suspended in 1 ml of Eagle's minimum essential medium (Eagle's MEM) (Gibco Laboratories, Grand Island, NY) and injected intravenously into a New Zealand white rabbit three times at 2-wk intervals. Blood was withdrawn 2 wk after the third injection and the serum was separated and heated at 56°C to inactivate the complement. Complement-dependent cytolytic activity of the antiserum was determined by using the trypan blue dye exclusion test. The mixtures of 50 μl of nude mouse spleen cell suspension (3×10^6 cells/ml), 50 μl of the antiserum in serial dilutions, and 50 μl of a 10-fold diluted rabbit serum as a source of complement were incubated at 37°C for 45 min, and the surviving cells were counted after the addition of 150 μl of 0.5% trypan blue dye solution. The antiserum titer for 50% cytolysis was 1:384 and the antiserum diluted to 1:96 was sufficient for 100% cytolysis.

Cell culture technique. The tumors were removed aseptically from the nude mice, minced, and pressed with a spatula through a No. 100 stainless steel sieve with continuous rinsing with cold Eagle's MEM. The resulting cell aggregate suspension was passed through a series of hypodermic needles of different sizes (19–25 gauges) to dissociate the cell aggregates. The disaggregated cells were then washed once with Eagle's MEM and resuspended in Eagle's MEM containing 4% each of normal rabbit serum and the nude mouse spleen cell antiserum. After incubation at 37°C for 45 min, the cells were washed three times and viable cells excluding trypan blue were counted in a hemocytometer counting chamber. Aliquots of 0.5×10^6 cells were plated in 25-cm^2 plastic tissue culture flasks (Falcon Labware, Oxnard, CA) with 5 ml of Eagle's MEM, to which 10% fetal bovine serum (Flow Laboratories, Inc., Rockville, MD; Lot No. 29101685), 0.1 mM nonessential amino acids (Gibco Laboratories), 1 mM sodium pyruvate (Gibco Laboratories), 100 μg/mg streptomycin, and 100 U/ml penicillin had been added. Incubation was carried out in a humidified atmosphere of 5% CO_2 in air at 37°C. The culture medium was renewed every 3 d.

Characterization of cultured cells. The tumors passaged in nude mice were removed and fixed overnight at 4°C in 0.1 M phosphate buffer (pH 7.4) containing 3% glutaraldehyde. These materials were postfixed for 30 min in 0.1 M phosphate buffer containing 2% O_sO_4, dehydrated in a graded series of ethanols and propylene oxide, and infiltrated with a 1:1 mixture of propylene oxide and Epon 812 followed by infiltration with Epon 812. The Epon 812 was polymerized at 60°C for 48 h. Thick sections (0.5 μm) were cut, dried on glass slides, and stained with toluidine blue O and hematoxylin-eosin (HE) for light microscopic examinations. Ultrathin sections (80 nm) were cut and stained with uranyl acetate and lead citrate. Observations and micrographs of ultrathin sections were made with a JEM 100 B transmission electron microscope operated at 60 kV.

The growth of the cultured cells was quantitated every 6 d by counting viable cells detached and disaggregated by treatment for 15 min with a solution of 0.125% trypsin and 0.05% ethylenediaminetetraacetic acid (trypsin-EDTA) (Gibco Laboratories).

The morphology of the cultured cells was examined by means of an Olympus inverted phase microscope and the electron microscope. The cultured cells were fixed in situ for 2 h in 0.1 M phosphate buffer containing 3% glutaraldehyde and postfixed for 30 min in 0.1 M phosphate buffer containing 2% O_sO_4. The fixed cells were then dehydrated in a graded series of ethanols and infiltrated with a 1:1 mixture of absolute ethanol and Epon 812 followed by infiltration with Epon 812. After the Epon 812 was polymerized at 60°C for 48 h, the plastic-embedded cell layer was separated mechanically from the culture flask. Thick sections were cut parallel and perpendicular to the cell growth plane and stained with toluidine blue O and HE for light microscopic examinations. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined under the electron microscope.

The cultured cells grown to confluence were harvested after treatment for 15 min with trypsin-EDTA and $\sim 20 \times 10^6$ cells were inoculated into each of four nude mice subcutaneously on the back, using a No. 12 trocar needle. The tumors formed in nude mice by the cultured cells were processed for electron microscopic examination in the same way as the original tumors passaged in nude mice.

Assay of [^{14}C]arachidonic acid (^{14}C-AA) metabolism by cultured cells. Radiometric thin-layer chromatography (TLC) similar to that reported by Skidgel and Printz (15) and previously used in our laboratory (16, 17) was used with some modifications to study the metabolism of arachidonic acid by the cultured cells. The reaction was initiated in the culture flask by a medium change with 5 ml of Eagle's MEM containing 0.2 μg/ml ^{14}C-AA with a specific activity of 52.7 mCi/mM (Amersham Corp., Arlington Heights, IL). After incubation for 20 min in the dark at 37°C in 5% CO_2-air, the medium was collected in a 10-ml glass tube and 1 ml of ethyl acetate/methanol/0.2 M citric acid, pH 2.0 (15:2:1) was added to extract the products. The

tube was vortexed; then the aqueous and organic layers were allowed to separate and placed on dry ice. The upper organic layer (containing 85–95% of the radiolabel) was spotted for TLC on Analtech silica gel GHL plates (Analtech, Inc., Newark, DE). The lower aqueous layer was thawed and residual products were extracted and spotted on the same TLC plate as above. PGs were identified by co-migration with authentic PG standards (The Upjohn Co., Kalamazoo, MI). The plates were developed by using the solvent system ethyl acetate/acetic acid/hexane/water (54:12:25:60, organic phase). PG standards were located by exposing plates to iodine vapor. Radioactivity on TLC plates was monitored with the use of a radiochromatogram scanner (7201B; Packard Instrument Co., Inc., Downer's Grove, IL) equipped with a disc integrator.

Assays of prostaglandin E_2 (PGE_2) and Ep production by cultured cells. The cultured cells in each flask, after removal of the spent medium, were washed three times in serum-free Eagle's MEM containing 0.1 mM nonessential amino acids, 100 μg/ml streptomycin, and 100 U/ml penicillin and incubated in 5 ml of the serum-free medium for 24 h at 37°C in 5% CO_2-air. The incubated media were collected and stored at −76°C until the time of assay for PGE_2 and Ep. The cultured cells were detached and disaggregated in trypsin-EDTA and the viable cells that excluded trypan blue were counted in a hemocytometer counting chamber.

The levels of PGE_2 in the incubated media were determined using a New England Nuclear (Boston, MA) radioimmunoassay kit. The cross-reactivities of prostaglandin E_1 (PGE_1), 13,14-dihydro-15-keto-PGE_2 and prostaglandin A_2 at the 50% bound/free (B/B_0) point were 3.7, 0.4, and 0.4%, respectively. The cross-reactivities of some other compounds including linoleic acid, arachidonic acid, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 6-keto-prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$), thromboxane B_2 (TxB_2,) and prostaglandin D_2 (PGD_2) were <0.02%. The sensitivity of the assay was ~1.3 pg/ml. The test culture media were assayed directly with the radioimmunoassay without purification.

The incubated media were also assayed for Ep by using a radioimmunoassay. The details of the radioimmunoassay for Ep used in our laboratory have been published previously (18). Briefly, highly purified human urinary Ep (70,400 U/mg protein) obtained from the National Heart, Lung, and Blood Institute, Bethesda, MD and prepared by Dr. Eugene Goldwasser's laboratory at the University of Chicago (19) was labeled with ^{125}I by the chloramine T method of Greenwood and Hunter (20). Antiserum to Ep was prepared in a New Zealand white rabbit by a modification of the procedure of Vaitukaitis et al. (21). A human urinary Ep preparation with a specific activity of 80 U/mg protein[2] was used for the immunization.

This Ep antiserum, 1 ml of which neutralized the biologic activity of 100 U of Ep in the exhypoxic polycythemic mouse assay, was used in a dilution of 1:1,600. Goat anti-rabbit gamma globulin was used for the separation of bound from free labeled antigen. The log dose-response curve using the highly purified human urinary Ep as the standard was linear between 5 and 400 mμ/ml. The sensitivity of this assay was ~6.6 mμ/ml.

Results were expressed as the mean±SEM of four to five experiments. Each experiment was carried out in duplicate cultures. Statistical analyses were made by the application of the Dunnett test for comparing several treatments with a single control and the *t* test.

Results

Morphologic and growth characteristics of cultured cells. The tumors removed from nude mice after the 21st passage, when their thick sections stained with HE were examined under a light microscope, were composed of cells with a granular cytoplasm arranged in solid patterns. The cells were polygonal to ovoid in shape with large nuclei showing a marked invagination. Electron microscopic studies revealed the tumors to be composed predominantly of cells with a cytoplasm containing diffusely scattered glycogen particles, sparse lipid droplets, abundant free ribosomes, sparse rough endoplasmic reticulum and Golgi apparatus, and a number of mitochondria. The nuclei of these cells showed a marked infolding of nuclear membrane and nucleochromatin was more abundant in the periphery. Microvilli were seen in areas where several cells were joined with attenuated junctional complexes.

The cultured cells of these tumors, when examined under a phase microscope, grew in a monolayer, producing colonies with epithelioid morphology. Their growth was exponential with an approximate population doubling time of 3.5 d up to a confluent density of 13×10^4 cells/cm^2. The cells in the confluent monolayer assumed a pavement-like arrangement. After reaching confluence, the cells piled up and gradually increased their population to $>40 \times 10^4$ cells/cm^2. Multicellular hemicysts (domes) resembling those observed in other cell culture systems (22–26) developed after the cells reached confluence (Fig. 1, *a* and *b*), and became abundant as the cells were further maintained in confluent cultures. The domes in the cultures maintained for 30 d, when examined over a 6-d period using time-lapse photography, gradually expanded and some of the domes fused with each other. However, these domes, unlike those in other cell culture systems that periodically expanded and collapsed in various regions of the cultures (22, 24), did not collapse (at least during the 6 d in which we observed the domes).

The morphology of cultured cells was further characterized by correlative light and electron microscopic studies. The cells grown for 12 d to confluence retained ultrastructural features closely resembling those of the original tumor passaged in nude mice, containing scattered glycogen particles, sparse lipid droplets, abundant free ribosomes, sparse rough endoplasmic reticulum and Golgi apparatus, and a number of mitochondria. The nuclei, which contained more abundant chromatin in the periphery, showed a marked infolding of the nuclear membrane. Microvilli were prominent on the cell surface interfacing the medium.

The cells, which were maintained in culture for prolonged periods after reaching confluence, were densely arranged and joined with a continuous series of tight junctions, retaining the ultrastructural characteristics of the cells grown for 12 d.

2. Supplied by the Department of Physiology, University of the Northeast, Corrientes, Argentina. The material was further processed and assayed by the Hematology Research Laboratories, Children's Hospital of Los Angeles, under U. S. Public Health Service Research grant HE-10880 (National Heart, Lung, and Blood Institute).

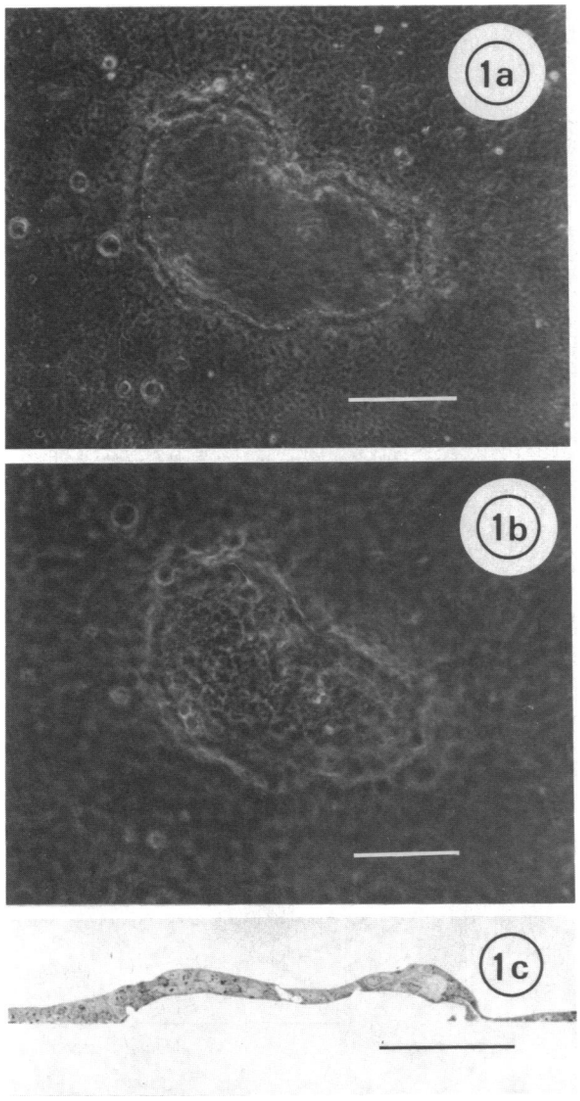


Figure 1. Light microscopic appearance of a dome observed in a culture of human renal carcinoma cells grown for 24 d. The phase-contrast micrographs were focused both on the cells on the surface of the flask (*a*) and at the top (*b*) of the dome. Light micrograph of a thick section of the dome cut perpendicular to the cell growth plane and stained with toluidine blue O (*c*) shows a focal area of a cell layer raised off the surface of the flask. Bars: *a* and *b*, 50 μm; *c*, 100 μm.

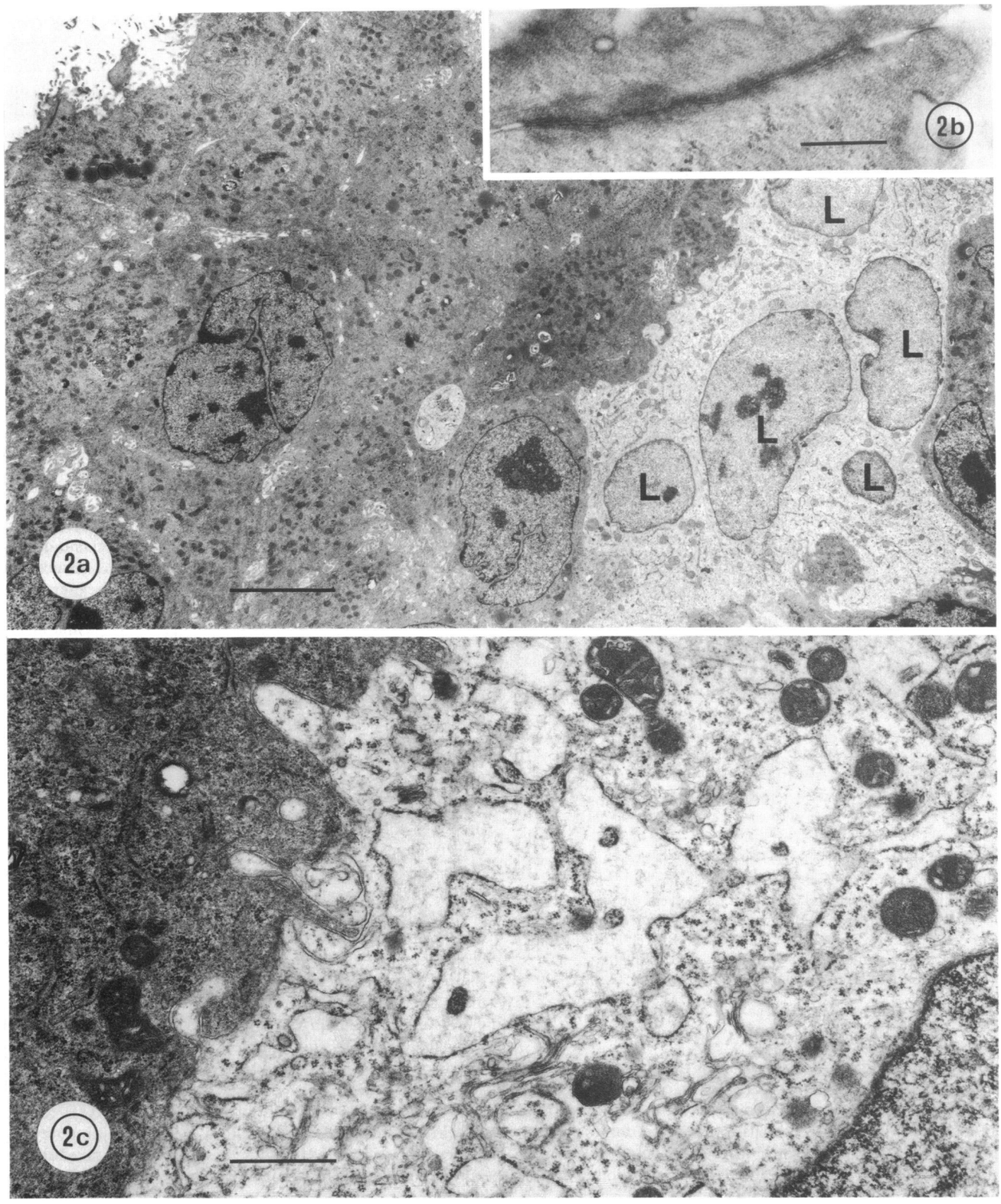
However, the domes, which were focal regions of a cell layer raised off the surface of the flask (Fig. 1 *c*), contained a distinct type of cell with a cytoplasm of markedly low electron density (LED cells), while the other cells forming the domes showed an ultrastructural resemblance to the cells in the confluent monolayer (Fig. 2, *a* and *b*). LED cells in the domes in the cultures maintained for 24 d contained abundant rough endoplasmic reticulum and Golgi apparatus and very few glycogen particles in the cytoplasm. Their nuclei contained dispersed chromatin and prominent nucleoli with less marked nuclear invagination. These cells were joined with tight junctions and showed less prominent microvilli than the other cells. LED cells in the cultures maintained for 30 d had more abundant and dilated rough endoplasmic reticulum than those in the cultures at 24 d (Fig. 2 *c*). LED cells were easily distinguishable from the other cells under a light microscope because of their less marked nuclear invagination and pale cytoplasmic staining with toluidine blue O and HE in comparison with the other cells. LED cells had a tendency to be located at the crest of the domes and arranged in groups.

Tumors developed at the site of inoculation of the cultured cells in all of four nude mice inoculated and grew to >1 cm in diam within 5 wk of inoculation. Light and electron microscopic examinations of the tumors confirmed their morphological resemblance to the original tumors passaged in nude mice, and the cells that morphologically resembled LED cells in culture were not observed in these tumors.

^{14}C-AA metabolism by cultured cells. The metabolism of ^{14}C-AA by the cultured cells was examined every 6 d for 30 d of cultivation by using radiometric TLC. TLC revealed PGE_2 to be the major metabolite of ^{14}C-AA of the cultured cells throughout 30 d of cultivation (Fig. 3). The conversion of ^{14}C-AA to PGE_2 by the cultured cells was markedly reduced in the presence of a cyclooxygenase inhibitor, meclofenamate (Warner-Lambert Co., Ann Arbor, MI).

PGE_2 formation, Ep production, and dome formation by cultured cells. The levels of PGE_2 in the serum-free media incubated for 24 h with the cultured cells grown for 6 d were significantly ($P < 0.001$) elevated (174.0±2.5 pg/ml, $n = 5$) in comparison with the unincubated control media (1.5±0.19 pg/ml, $n = 4$). The dilution regression line for the incubated medium was parallel to the standard dose-response regression line, indicating that the radioimmunoassay was detecting PGE_2. The levels of PGE_2 in the 24-h incubation media gradually decreased to <100 pg/ml as the cultured cells grew and their density increased (Fig. 4).

On the other hand, the levels of Ep in the 24-h incubation media of the cells grown for 6 d were 11.5±0.52 mμ/ml ($n = 5$), while the Ep activity in the unincubated control media was 7.6±0.62 mμ/ml ($n = 5$). However, after the cultured cells became confluent the levels of Ep in the incubated media showed a marked increase, reaching a level of 222.9±5.26 mμ/ml ($n = 5$) at 30 d (Fig. 4). The dilution regression line for the incubated medium containing a high level of Ep activity was parallel to the radioimmunoassay standard dose-response regression line, indicating the immunologic similarity of Ep activity produced by the cultured cells to the standard Ep, which was a highly purified human urinary Ep (70,400 U/mg protein). As reported elsewhere (27, 28), the Ep produced in this renal carcinoma cell culture system, when assayed in the exhypoxic polycythemic mouse assay, showed only 10% of the activity seen in the radioimmunoassay, while its in vitro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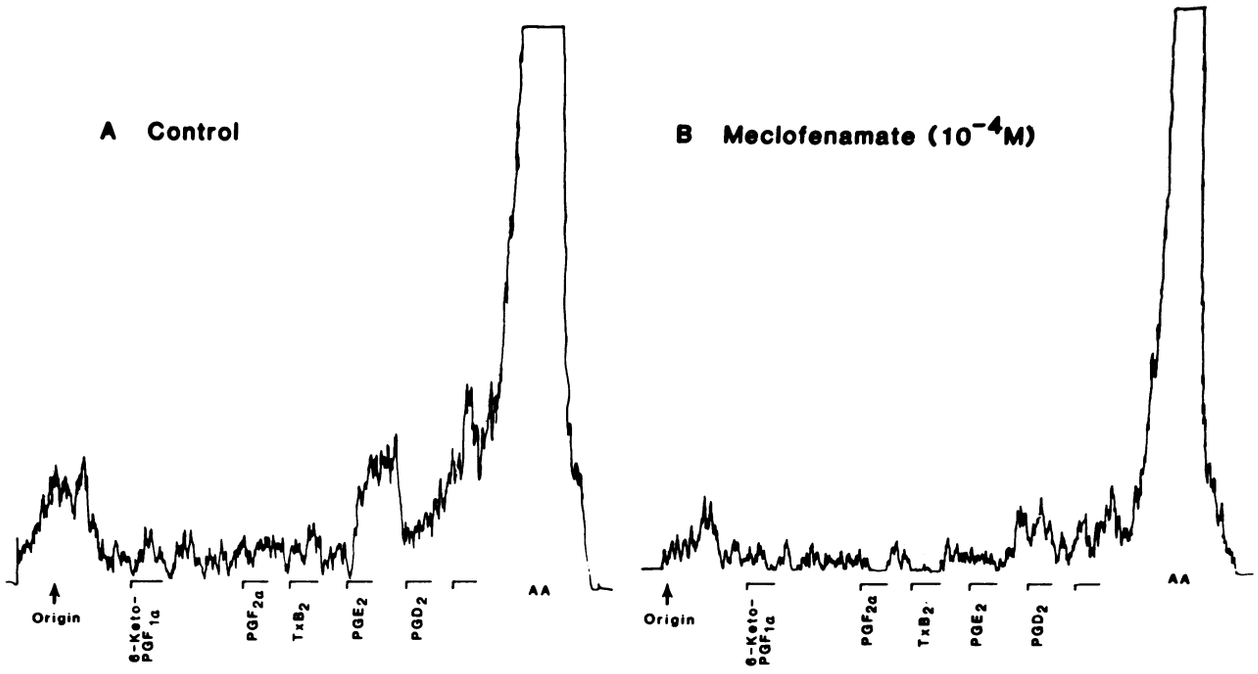

Figure 3. A thin-layer chromatogram of the metabolites of ^{14}C-AA produced by cultured human renal carcinoma cells grown for 30 d (*A*) showing PGE_2 to be the major metabolite of ^{14}C-AA. Note the significant inhibition of ^{14}C-AA conversion to PGE_2 in the presence of 10^{-4} M meclofenamate (*B*).

biologic activity estimated using the fetal mouse liver erythroid colony-forming technique was very similar to its immunologic activity.

The domes were counted by using an inverted microscope in a 9.6-cm^2 area after completion of the 24-h incubation. The domes were observed only in the cultures maintained for a prolonged period of time after the cells reached confluence and their numbers increased with increasing time in confluence in parallel with the increase in Ep activity in the incubated media (Fig. 4).

Effects of meclofenamate and indomethacin on PGE_2 formation, Ep production, and dome formation by cultured cells. The cultured cells grown for 30 d were incubated for 24 h in serum-free medium containing an increasing dose (3×10^{-6}–3×10^{-5} M) of meclofenamate. Meclofenamate inhibited PGE_2 formation, Ep production, and dome formation by the cultured cells in a dose-related fashion without any significant effect on cell viability, as determined by the trypan blue exclusion test (Fig. 5). Indomethacin, which is another cyclooxygenase inhibitor, produced a similar dose-related inhibitory effect on PGE_2 formation, Ep production, and dome formation in the renal carcinoma cell cultures in a dose-range of 10^{-5}–10^{-4} M ($n = 3$). However, when the cultured cells were incubated for 24 h in the medium containing meclofenamate (10^{-5} M) and increasing concentrations of exogenous PGE_2 (10^{-9}–10^{-6} M), the inhibitory effect of meclofenamate on Ep production and dome formation was completely abolished by the addition of 10^{-8} M and higher of PGE_2 in the incubation medium (Fig. 6).

Discussion

A human renal carcinoma derived from a patient with erythrocytosis that has been serially transplanted into nude mice was successfully grown in culture. Electron microscopic studies revealed an ultrastructural resemblance of the cultured cells to the original tumor cells passaged in nude mice. These cultured cells, when reinoculated back into nude mice, produced tumors

Figure 2. Electron micrographs of an ultrathin section of a dome cut parallel to the cell growth plane and stained with uranyl acetate and lead citrate. Low power electron micrograph of a dome at 24 d of cultivation (*a*) reveals a distinct type of cell with a cytoplasm of markedly low electron density (LED cells = *L*). Note the less marked nuclear invagination, more dispersed nucleochromatin and less prominent microvilli in the LED cells than the other cells. A high power electron micrograph (*b*) shows tight junctions. A high power electron micrograph of the LED cell at 30 d of cultivation (*c*) shows an abundance of dilated rough endoplasmic reticulum and Golgi apparatus in the cytoplasm. Bars: *a*, 5 μm; *b*, 0.5 μm; *c*, 1 μm.

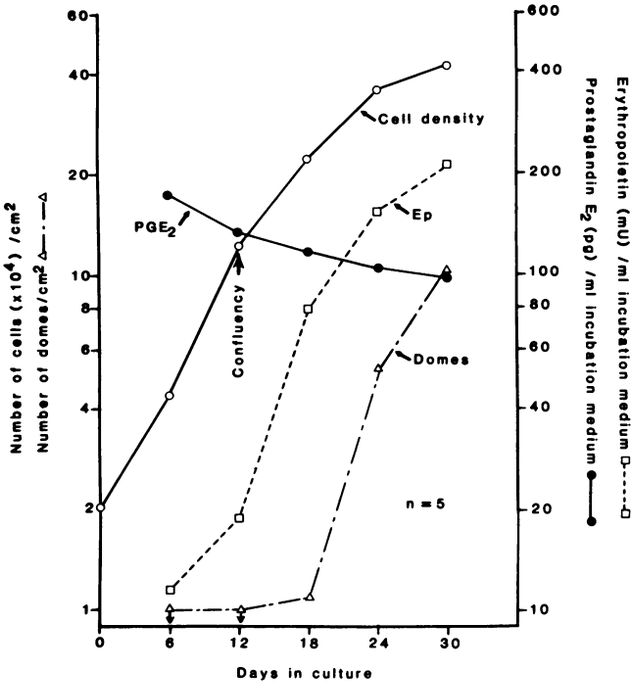

Figure 4. Time course of cell density (○), PGE_2 formation (●), Ep production (□), and dome formation (△) in cultures of human renal carcinoma cells. Note the parallel increase in the number of domes and Ep activity in the incubation media.

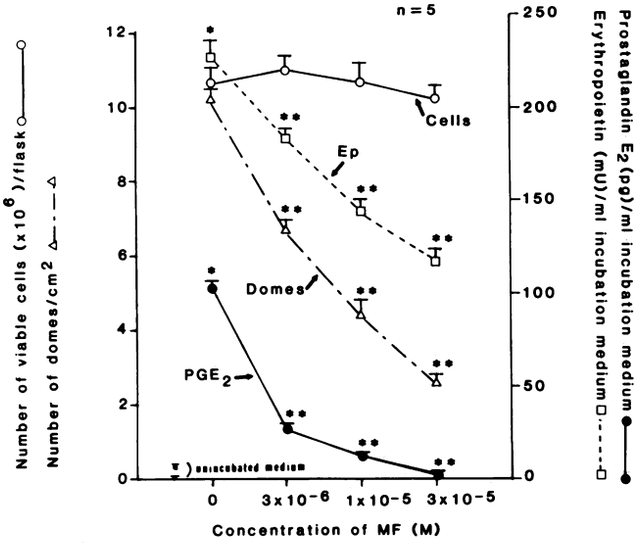

Figure 5. Effect of meclofenamate (MF) on PGE_2 formation (●), Ep production (□), and dome formation (△) by cultured human renal carcinoma cells grown for 30 d. The cultured cells were incubated for 24 h with serum-free medium containing increasing concentrations (3×10^{-6}–3×10^{-5} M) of meclofenamate. After the completion of incubation, the numbers of viable cells and domes were determined and the incubation media were assayed for Ep and PGE_2 via radioimmunoassay. Note the dose-related decrease in PGE_2 formation, Ep production, and dome formation. Each point is the mean of five experiments carried out in duplicate cultures.

that had histological features closely resembling the original tumors. However, the domes that developed after the cells reached confluence and became abundant with increasing time in confluent culture, contained a distinct type of cell with a cytoplasm of markedly low electron density (LED cells). LED cells contained more abundant and dilated rough endoplasmic reticulum in their cytoplasm than the other cells in culture and the original tumor cells in nude mice. The nuclei of the LED cells contained dispersed chromatin and prominent nucleoli with less marked nuclear invaginations. To our knowledge, no previous reports have described the human renal carcinoma cells that ultrastructurally resembled the LED cells found in our cell culture system while the ultrastructural features of the other cells in culture and the tumors passaged in nude mice were compatible with those of human renal carcinoma described by several other investigators (29–32). It could be postulated that the LED cells were not derived from the human renal carcinoma but from the host nude mouse. In fact, cultures of human tumors transplanted into nude mice are known to contain stromal cells derived from a nude mouse that sometimes grow along with the tumor cells (14). However, this hypothesis is probably not tenable in that treatment of the cultured cells with the antiserum to nude mouse spleen cells, which is known to be cytolytic to nude mouse stromal cells (14), did not affect the development of LED cells. Furthermore, some microvilli and tight junctions were seen in LED cells, supporting the conclusion that LED cells were derived from the human renal carcinoma. In considering the reason why LED cells were found only in culture but not in the tumors passaged in nude mice nor in the tumors formed in nude mice by the cultured cells, the tumors in nude mice might contain LED cells that were not contained in the sample taken for the electron microscopic studies. Furthermore, it seems possible that LED cells, which were not found in the tumors in nude mice because of their small population in these tumors, grew preferentially in culture. Alternatively, the renal carcinoma cells might have differentiated into LED cells in culture but were not capable of doing so in nude mice because of factors that were contained in the culture media and were not present in sufficient amounts in our nude mice in vivo. Further studies using cloned cells are needed to test this hypothesis.

Dome formation, which is believed to reflect a fluid-transport function or a secretory activity of cultured cells in other cell culture systems (22–26), might reflect the secretory activity of LED cells in our cell culture system. Alternatively, the other cells forming domes might have a fluid-transport function and could be responsible for dome formation. How-

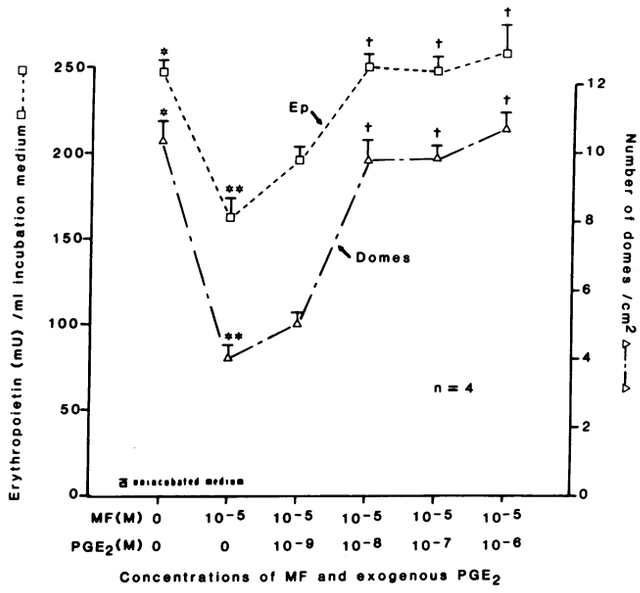

Figure 6. Effects of meclofenamate (MF) and exogenous PGE_2 on Ep production (□) and dome formation (△) in cultured human renal carcinoma cells grown for 30 d. The cultured cells were incubated for 24 h with serum-free medium containing 10^{-5} M MF and increasing concentrations (10^{-9}–10^{-6} M) of exogenous PGE_2. After the completion of incubation, the number of domes was determined and the incubation media were assayed for Ep via radioimmunoassay. Note that 10^{-8} M and higher concentrations of PGE_2 completely abolished the MF-induced decrease in Ep production and dome formation. Each point is the mean of four experiments carried out in duplicate cultures. Vertical lines at each point indicate SEM. $*P < 0.001$ when compared with unincubated medium. $**P < 0.01$ when compared with control. $\dagger P < 0.01$ when compared with MF alone.

ever, the former hypothesis seems more likely in that all of the domes thus far examined contained LED cells, very few LED cells were found in monolayer, and the other cells forming the domes showed ultrastructural features closely resembling the cells in monolayer.

The Ep produced by these cultured renal carcinoma cells was found to have immunologic properties similar to that of highly purified human urinary Ep (70,400 U/mg protein) in that the dilution regression line for the incubated medium was parallel to that of highly purified Ep in our radioimmunoassay. However, it seems most likely that most of the Ep produced by these cultured cells has physicochemical properties that are different from native Ep in that its biologic activity in the exhypoxic polycythemic mice was approximately $^1/_{10}$ that of its immunologic activity (27, 28). Sherwood and Goldwasser (8) have reported that human renal carcinoma cells in culture may produce desialylated Ep, which is inactive in vivo probably because of its short in vivo half-life. Further physicochemical characterization studies are needed to elucidate the structural differences between native Ep and Ep produced in our renal carcinoma cell culture system.

Ep production and dome formation by the cultured renal carcinoma cells were not evident initially but became very marked after the cells reached confluence. The inverse relationship between cell growth and expression of differentiated functions has been well documented in other cell culture systems (32–36). This well-established principle of these cell lines might hold true for our cell culture system; and Ep production and dome formation could be best interpreted as the expression of a differentiated function of the cultured renal carcinoma cells.

These cultured renal carcinoma cells have been shown to transform ^{14}C-AA mainly into PGE_2 and to release a significant amount of PGE_2 into the serum-free incubation medium. It seems clear that the endogenous PGE_2 played an important role in supporting and/or stimulating Ep production and dome formation in the serum-free cultures of these renal carcinoma cells in that meclofenamate, a PG synthesis inhibitor, produced a dose-related decrease in Ep production and dome formation by these renal carcinoma cells. This inhibitory effect of meclofenamate on Ep production and dome formation was completely abolished by adding PGE_2 to the incubation medium.

It seems most likely that the effect of endogenous PGE_2 on Ep production and dome formation by these renal carcinoma cells was permissive or supportive, in that exogenous PGE_2 did not increase either Ep production or dome formation above control levels. Taub et al. (37) and Taub and Sato (38) have demonstrated that PGE_1 or PGE_2 is an essential requirement of the Madin-Darby canine kidney (MDCK) cell line and mouse kidney epithelial cells to grow and form domes in a hormone-supplemented serum-free medium. PGE_2, which was produced endogenously in our renal carcinoma cell culture system, might be an essential requirement of the renal carcinoma cells to maintain their cellular functions, including Ep production and dome formation.

Alternatively, we can postulate that Ep production and dome formation by these renal carcinoma cells, which were stimulated to a maximum extent by endogenous PGE_2, were reduced to basal levels by treatment with meclofenamate. A- and E-series PGs have been reported to stimulate iron incorporation into erythrocytes of plethoric mice and their erythropoietic effects have been shown to be inhibited by the concomitant administration of anti-Ep immunoglobulin (39–42). Accordingly, renal arterial infusions of PGE_1, PGE_2, or arachidonic acid have been shown to elicit the release of Ep from isolated perfused canine kidneys (41–43). Furthermore, PGE_1 or PGE_2 has been shown not only to support the growth of the Madin-Darby canine kidney cell line in a defined medium but also to stimulate dome formation in this cell line (37, 44). Therefore, spontaneous Ep production and dome formation in our renal carcinoma cell culture system could reflect the expression of the differentiated functions that were stimulated to a maximum extent by endogenous PGE_2.

It is of great interest that Ep production was closely correlated with dome formation in our renal carcinoma cell culture system. Both Ep production and dome formation

became very marked with increasing time in culture after the renal carcinoma cells reached confluence, and meclofenamate decreased Ep production and dome formation in parallel and in a dose-related fashion. In addition, our preliminary immunocytochemical studies have localized Ep-like immunoreactivity in LED cells in the domes (unpublished data). These data led us to postulate that dome formation reflects the secretory activity of LED cells, namely Ep production, in our renal carcinoma cell culture system. However, further immunocytochemical studies using a highly specific Ep antiserum or a monoclonal antibody to Ep are needed to prove this hypothesis.

Acknowledgments

The authors gratefully acknowledge the expert technical assistance of Mr. Jesse Brookins and Mr. Eugene Maulet.

This work was supported by U. S. Public Health Service grants AM-13211 and HL-29456 and private funds.

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