

EXHIBIT K

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Erythropoietin Production in a Primary Culture of Human Renal Carcinoma Cells Maintained in Nude Mice

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The present studies report erythropoietin (Ep) production in primary cultures of a human renal carcinoma from a patient with erythrocytosis that has been serially transplanted to BALB/c nude mice. The levels of erythropoietin in the culture media were estimated using the exhypoxic polycythemic mouse assay (EHPCMA), fetal mouse liver erythroid colony-forming technique (FMFC), and a radioimmunoassay (RIA). The spent culture media of the exponentially growing cells contained less than 10 mU/ml of Ep measured by RIA. However, after the cells became confluent, Ep levels (RIA) in the spent media showed a marked increase to approximately 300 mU/ml. Ep levels estimated using the FMFC and EHPCMA were approximately 2/3 and 1/10, respectively, of those measured by RIA. Rabbit antiserum to highly purified human urinary Ep (70,400

U/mg protein) was utilized for immunocytochemical (peroxidase-antiperoxidase method) localization of Ep in the cultured cells. Very few of the cells in exponential growth exhibited Ep-like immunoreactivity, whereas intense Ep-like immunoreactivity was observed in the cytoplasm of the cells maintained in culture for a prolonged period after reaching confluency. The most intense staining was observed in some of the cells forming domes. The domes developed after the cells reached confluency, and their numbers increased with increasing time in confluent culture, in parallel with the increase in Ep levels in the spent media. This primary cell culture system of a renal cell carcinoma maintained in nude mice, which produces immunologically and biologically active Ep, may provide a useful model for studies of the mechanism of Ep production.

ERYTHROPOIETIN (Ep) production has been demonstrated in cultures of normal human¹ and bovine² kidney cells, human renal carcinoma cells,^{3,4} goat and rat renal glomerular cells,^{5,6} mouse fetal liver cells,⁷ and mouse macrophages.⁸ However, the problem of Ep production in these cell culture systems has been discussed by other investigators,⁹ emphasizing the need for a reproducible cell culture system as a model to study the mechanism of Ep production.

An Ep-producing human renal carcinoma from a patient with erythrocytosis has been successfully transplanted into nude mice previously by Toyama et al.,¹⁰ and growth of this carcinoma in monolayer cultures has been reported by Katsuoka et al.¹¹ The present studies were undertaken to develop an erythropoietin-producing cell culture system with this renal carcinoma that might be useful as an *in vitro* model for studies of the mechanism of Ep production and as a possible source of messenger RNA for *in vitro* production of Ep using recombinant DNA technology.

MATERIALS AND 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, Japan. The renal carcinoma had been initially grown in nude mice after its removal from a patient with erythrocytosis and was kept frozen prior to 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 Co., Wilmington, MA), were used for successive passages of the tumor in our laboratory. The nude mice were housed in a laminar air flow hood unit. Food and other materials in contact with the mice were sterilized, and the water was treated with antibiotics. When tumor growths of more than 1 cm in diameter developed, they were aseptically removed, minced into 2 cu mm fragments, and transplanted to other nude mice subcutaneously on both sides of the back using a no. 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, resulting in a significantly ($p < 0.01$) higher mean hematocrit level ($53.8\% \pm 5.1\%$, $n = 4$) in mice bearing the tumor after the sixth passage than that of nongrafted control nude mice studied over the same time interval ($46.5\% \pm 1.2\%$, $n = 4$). However, nude mice transplanted with the tumor after the eighth passage no longer developed a significant elevation in hematocrit in comparison with the control mice. The tumors used in the present studies were removed from the nude mice after the 14th passage.

Preparation of Antiserum to Nude Mouse Spleen Cells

The method described by Okabe et al.¹² was utilized to prepare the antiserum against nude mouse spleen cells. Briefly, approximately 10^8 spleen cells from BALB/c nude mice were suspended in 1 ml of Eagle's minimum essential medium (Eagle's MEM) (GIBCO, Grand Island, NY) and injected intravenously into a rabbit 3 times at 2-wk intervals. Blood was withdrawn 2 wk after the third injection and the serum separated and heated at 56°C to inactivate the complement. Complement-dependent cytolytic activity of the antiserum was determined using the trypan blue exclusion test. The mixtures of 50 μ l of nude mouse spleen cell suspension (3×10^6 cells/ml), 50 μ l of the antiserum in serial dilutions and 50 μ l of a tenfold diluted rabbit serum as the source of complement were incubated at 37°C for 45 min, and the surviving cells were counted following the addition of 150 μ l of 0.5% trypan blue dye solution. The

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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 removed aseptically from the nude mice were minced and pressed through a no. 100 stainless steel sieve with a spatula and continuously rinsed with cold Eagle's MEM. The resulting cell aggregate suspension was passed through a series of hypodermic needles of different sizes (19–25 gauge) to dissociate the cell aggregates. The disaggregated cells were then washed once with Eagle's MEM and resuspended in Eagle's MEM containing normal rabbit serum and the nude mouse spleen cell antiserum, both at a final concentration of 4%. After incubation at 37°C for 45 min, the cells were washed 3 times and the viable cells excluding trypan blue were counted in a hemocytometer counting chamber. Aliquots of 1.5×10^6 cells were plated in 75 sq cm plastic tissue culture flasks (Falcon, Oxnard, CA) with 15 ml of Eagle's MEM, to which 10% fetal bovine serum (Flow Laboratories, Inc., Rockville, MD; lot 29101607), 0.1 mM nonessential amino acids (GIBCO), 1 mM sodium pyruvate (GIBCO), 100 µg/ml streptomycin, and 100 U/ml penicillin had been added. Incubation was carried out in a humidified atmosphere of 5% CO₂ in air at 37°C. The culture medium was renewed every 3 days, and the spent medium was stored at -76°C until the time of assay for Ep.

Characterization of Morphology and Growth of Cultured Cells

The morphology of the cultured cells was examined by means of a Zeiss inverted phase-contrast microscope, and the cell growth quantitated every 3 days 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). The cultured cells were fixed in situ for 2 hr in 0.1 M phosphate buffer containing 3% glutaraldehyde and postfixed for 30 min in 0.1 M phosphate buffer containing 2% OsO₄. The fixed cells were dehydrated in a graded series of ethanols and infiltrated with a 1:1 mixture of absolute ethanol and Epon 812, followed by infiltration with pure Epon 812. The Epon 812 was polymerized at 60°C for 48 hr. The Epon 812 embedded cell layer was separated mechanically from the plastic culture flask. Ultrathin sections (80 nm) were cut parallel and perpendicular to the cell growth plane, stained with uranyl acetate followed by lead citrate, and then examined under JEM 100B electron microscope operated at 60 kV. Thick sections (0.5 µm) were cut, dried on glass slides, and stained with toluidine blue 0 for light microscopic examination.

Inoculation of Cultured Cells Into Nude Mice

Confluent monolayers of the cultured cells were harvested after treatment for 15 min with trypsin-EDTA, and approximately 2×10^7 cells were inoculated into each of 5 nude mice subcutaneously on the back, using a no. 12 trocar needle.

Assays for Erythropoietin

Exhypoxic polycythemic mouse assay. A modification of the exhypoxic polycythemic mouse assay method described by Cotes and Bangham¹³ was used. CD-1 strain virgin female mice (Charles River Co.) were made polycythemic by exposure to 0.42 atmospheres of pressure for 22 hr/day for 2 wk. The mice were injected subcutaneously on days 6 and 7 after their removal from the hypobaric chamber with one-half of the total dose of either saline, human urinary Ep standardized against the International Reference Preparation (IRP-B), or test culture medium. On the eighth posthypoxic day, each mouse received 0.5 µCi of radioactive iron (⁵⁹Fe) intrave-

nously through the tail vein. Two days later (posthypoxic day 10), the mice were exsanguinated via cardiac puncture, 0.5 ml of blood per mouse was counted on a Packard Auto-Gamma spectrometer, and the percentage of radioactive iron incorporation into red blood cells (RBC) was determined. Five mice were injected with each sample and the mean 48-hr ⁵⁹Fe incorporation into RBC was determined. All mice with hematocrits below 51% were excluded from the assay. The ⁵⁹Fe incorporation into RBC increased in a linear fashion as a function of the log dose of standard Ep between 0.05 and 2.0 U. Ep activity in the culture medium was calculated from the standard log dose-response regression line.

Fetal mouse liver erythroid colony-forming technique. Fetal mouse (CD-1) liver cells from fetuses 13–14 days old were disaggregated by passing the livers through hypodermic needles of decreasing diameter (21–25 gauge) and suspended in alpha medium without ribosides or deoxyribosides (Flow Laboratories). Following two washes with alpha medium, the number of nucleated cells was counted in a hemocytometer counting chamber.

Erythroid colony-forming units (CFU-E) were assayed in a methylcellulose culture system using the technique developed by Iscove and Sieber¹⁴ and modified further by Ogawa et al.¹⁵ The mouse fetal liver cells were suspended at a concentration of 10⁵ cells/ml in alpha medium containing 0.8% methylcellulose (Fisher Scientific Co., Norcross, GA), 30% fetal bovine serum (Flow Laboratories; lot 29101607), 10⁻⁴M β-mercaptoethanol (Sigma Chemical Co., St. Louis, MO), 200 µg/ml streptomycin, 200 U/ml penicillin, and either human urinary Ep standardized against IRP-B or 5% test culture medium. One milliliter of the suspension was pipetted into 35 × 10 mm petri dishes (Falcon) prepared in duplicate. The petri dishes were incubated for 48 hr at 37°C in a humidified atmosphere of 5% CO₂ in air. The cultures were stained with diaminobenzidine (Sigma) according to the method of Ogawa et al.¹⁵ Benzidine-positive colonies of 8 or more cells were considered to represent colonies derived from CFU-E and were counted in 1/16.8 of the total surface area of the dish with an Olympus inverted microscope. The number of CFU-E-type colonies/10⁵ cells plated increased in a linear fashion as a function of the log dose of Ep standard between 3.13 and 100 mU/ml. Ep activity in the test culture medium was calculated from the standard log dose-response regression line.

Radioimmunoassay for erythropoietin. The details of the radioimmunoassay (RIA) used in our laboratory have been published elsewhere.¹⁶ 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 laboratory¹⁷ at the University of Chicago, was labeled with iodine 125 by the chloramine T method of Greenwood and Hunter.¹⁸ Antiserum to Ep was prepared in rabbits by a modification of the procedure of Vaitukaitis et al.¹⁹ A human urinary Ep preparation with a specific activity of 80 U/mg protein, obtained from the National Heart, Lung and Blood Institute, was used for the immunization. Rabbit antiserum to this human urinary Ep, which neutralized the biologic activity of 45 U of Ep in the exhypoxic polycythemic mouse assay, was used in a dilution of 1:2,000. 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 10 mU and 400 mU/ml. The minimum amount of Ep detectable by this assay was 10 mU/ml.

Immunocytochemical Studies

Antiserum to Ep. Antiserum to Ep was prepared in a rabbit that was initially immunized with a total of 256 U Ep (69 U/mg protein) over a period of 4 wk, then rested for 12 mo and finally boosted with 40 U of the highly purified human urinary Ep (70,400 U/mg

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protein) described previously. One milliliter of this antiserum (HPEAS) was found to neutralize the biologic activity of 100 U Ep, measured by the exhypoxic polycythemic mouse assay.

Fixation. After collection of the spent medium, the monolayer of cells was washed with 3 changes of phosphate-buffered saline (PBS) and fixed initially with 0.1 M phosphate buffer containing 4% paraformaldehyde, 0.05% glutaraldehyde, and 0.05% saponin for 10 min at 25°C. The cells were subsequently fixed with the saponin-depleted fixative for 2 hr at 4°C.

Immunocytochemical method. The peroxidase-antiperoxidase method described by Sternberger et al.²⁰ was utilized with some modifications. After treatment for 20 min with 80% methanol, containing 1% H₂O₂ to block endogenous peroxidase, the cells were washed with 3 changes of PBS for 30 min and incubated for 48 hr at 4°C with HPEAS Ep antiserum diluted to 1:2,000 in PBS or normal rabbit serum with the same dilution. The cells were washed with 3 changes of PBS for 30 min and incubated with a 1:100 dilution of goat anti-rabbit gamma globulin (Miles Laboratories, Elkhart, IN) for 30 min at 37°C. Following 3 washes with PBS, the cells were incubated with a 1:200 dilution of horseradish peroxidase-rabbit anti-horseradish peroxidase soluble immune complex (PAP) (Miles Laboratories) for 30 min at 37°C and washed again with 3 changes of PBS. The cells were finally incubated for 10 min with 0.05% diaminobenzidine and 0.03% H₂O₂ in 0.05 M Tris buffer, pH 7.6.

RESULTS

Morphological and Growth Characteristics of Cultured Cells

The cultured cells grew in a monolayer, producing colonies with epithelioid morphology. The cells were trigonal to polygonal in shape, with a distinct cell border. Their growth was exponential, with an approximate population doubling time of 3.5 days up to a confluent density of 10⁷ cells/flask. The cells in the confluent monolayer assumed a pavement-like arrangement and contained vacuoles with nonuniform size in the cytoplasm. After reaching confluency, the cells piled up and gradually increased their population to more than 2 × 10⁷ cells/flask. Multicellular hemicysts (domes), resembling those observed in other cell culture systems,²¹⁻²³ developed after the cells reached confluency (Fig. 1, A and B), and became abundant as the confluent monolayer was further maintained in culture. The morphology of the cultured cells was

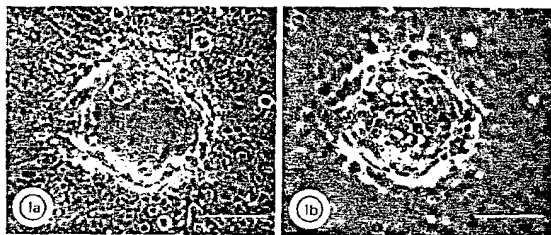


Fig. 1. Phase-contrast micrographs of a dome observed in a culture of human renal carcinoma cells grown for 24 days. The micrographs were focused both on the cells on the bottom (a) and the top (b) of the dome. Bars = 40 µm.

further characterized by means of correlative light and electron microscopic studies. The cells in the confluent monolayer were joined by a continuous series of tight junctions. Microvilli were localized exclusively to the upper surface of the cultured cells, which faced the medium. The cytoplasm contained variable sized lipid droplets and numerous glycogen particles. Light microscopic observations of the perpendicular thick sections of the domes confirmed that the domes consisted of one or more layers of cells that were raised from the surface of the flask (Fig. 2A). Electron microscopic studies revealed cells forming the domes that were similar to, but more polarized than, those attached to the surface of the flask (Fig. 2, B and C).

Tumor Formation of Cultured Cells in Nude Mice

Tumors developed at the site of inoculation in all 5 nude mice inoculated and grew to more than 1 cm in

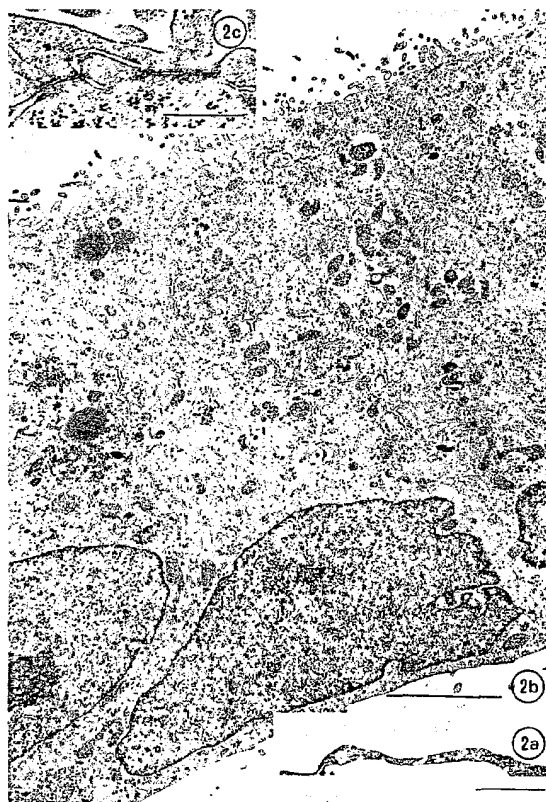


Fig. 2. Light and electron micrographs of perpendicular sections of a dome. Light micrograph of a thick section (0.5 µm) of the dome stained with toluidine blue 0 (a) shows a focal area of a cell layer raised from the surface of the flask. An electron micrograph of the cells forming a dome (b) shows polarized cells with prominent microvilli, numerous cytoplasmic glycogen particles, and tight junctions (c). Bars 2A = 100 µm; 2B = 2 µm; 2C = 0.3 µm.

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diameter within 5 wk of inoculation. Histologic examination of the tumors confirmed their morphological resemblance to the original tumors passed in nude mice.

Ep Activity in Culture Media

Ep activity, estimated by radioimmunoassay (RIA), was less than 10 mU/ml in the spent culture media of the exponentially growing cells, when Ep activity in the control media (14.2 ± 3.4 mU/ml, $n = 4$) was subtracted from that of the spent media. However, after the cells became confluent, Ep activity in the spent media showed a marked increase, reaching approximately 300 mU/ml (Fig. 3). The dilution regression line for the spent medium containing a high level of Ep activity was parallel to that of the standard Ep (Fig. 4), indicating the immunologic similarity of Ep activity in the culture media to the Ep standard.

Ep activity was also estimated using the fetal mouse liver erythroid colony-forming technique (FMLC). The test culture media were concentrated 5 times using an Amicon PM-10 membrane (Amicon Co., Lexington, MA). The control medium did not contain detectable Ep activity. Ep activity in the spent media, estimated using the FMLC, correlated with, but was approximately two-thirds of, the activity measured by RIA (Fig. 3). This discrepancy in Ep activity between the two assay methods was considered to be due to the

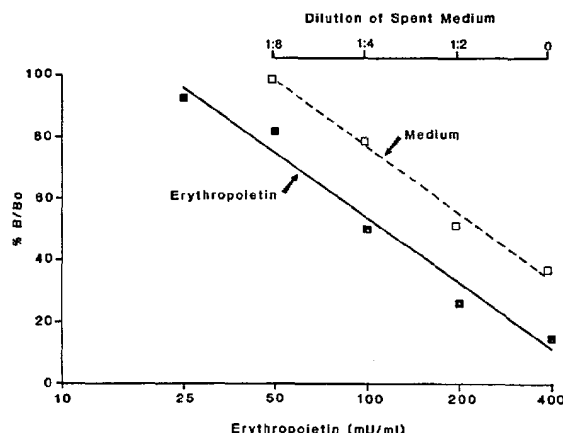


Fig. 4. Parallel radioimmunoassay dilution regression lines for spent culture medium containing a high level of erythropoietin (Ep) (□---□) and highly purified human urinary EP (70,400 U/mg protein).

concentration procedure used for the test samples for the FMLC, in that the Ep activity determined using the FMLC was approximately the same as that measured by RIA when the test samples for the FMLC contained a high Ep activity and were not concentrated (Table 1).

The spent media containing Ep activity above 300 mU/ml (RIA) did not significantly stimulate ⁵⁹Fe incorporation into RBC in the exhypoxic polycythemic mice. However, the spent media, when concentrated on the Amicon PM-10 membrane, showed significant Ep activity in the exhypoxic polycythemic mouse assay (EHPCMA). Ep activity in the media estimated using the EHPCMA was approximately 1/10 of that measured by RIA (Table 1). Pretreatment of the media with rabbit antiserum to Ep (HPEAS) and goat anti-rabbit gamma globulin completely abolished their Ep activities in the EHPCMA. Furthermore, the dose-response regression line for the concentrated spent medium was parallel to the regression line for the standard Ep in the EHPCMA (Fig. 5).

Treatment of the cultured cells producing Ep with the antiserum to nude mouse spleen cells did not affect Ep production in these cells.

Immunocytochemical Localization of Ep in Cultured Cells

Very few of the cells in exponential growth were stained positively for Ep-like immunoreactive material, whereas intense positive staining was seen in the cytoplasm of the cells maintained in culture for a prolonged period after reaching confluency. The most intense staining was observed in some of the cells forming the dome (Fig. 6). Absorption of the antise-

7. 30 mU/100 cells - 45

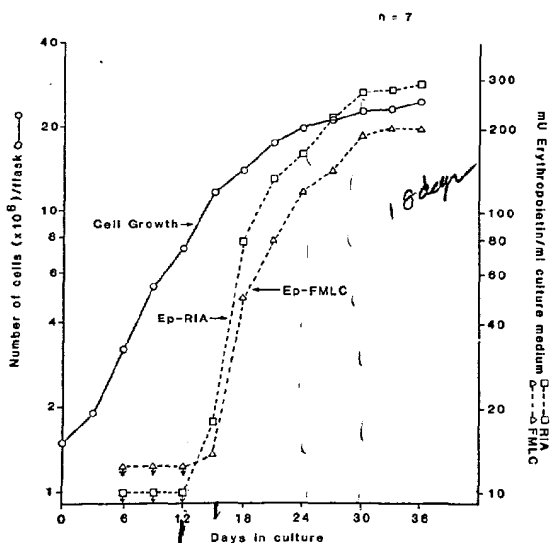


Fig. 3. Correlation between renal carcinoma cell growth (O—O) and erythropoietin (Ep) activity in spent culture media estimated using a radioimmunoassay (Ep-RIA, □---□) and a fetal mouse liver erythroid colony-forming technique (Ep-FMLC, △---△). Ep activity in spent media was undetectable when the cells were growing exponentially, but showed a marked increase after they reached a confluent density of 10^7 cells/flask.

Table 1. Comparison Between Immunologic and Biologic Activities of Erythropoietin in the Culture Media of Renal Carcinoma Cells

Days in Culture	RIA (mU/ml)	FMLC (mU/ml)		PCMA (mU/ml) 5 x Concentrated
		5x Concentrated	Unconcentrated	
Control	14.9	Undetectable	Undetectable	Undetectable
24 Days	158.7	522	168	77
36 Days	403.8	1,384	422	190

Note that the Ep activity measured by RIA was similar to that using the FMLC in unconcentrated culture medium.

rum to Ep (HPEAS) with a partially purified side fraction of human urinary Ep (2,052.6 U/mg protein)²⁴ completely abolished the positive staining.

Correlation Between Number of Domes and Ep Activity in Culture Medium

Domes were counted every 6 days using an inverted microscope in a 9.6 sq cm area. The domes were not observed in cultures of the exponentially growing cells. The domes developed after the cells reached confluency, and their numbers increased exponentially with increasing time in confluent cultures, in parallel with the increase in Ep activity in the culture media (Fig. 7).

DISCUSSION

A human renal carcinoma from a patient with erythrocytosis, which was transplanted into nude mice, was successfully grown in culture and was demonstrated to produce significant levels of Ep. The Ep produced by this renal carcinoma cell culture was detectable in three different assays, including the exhypoxic polycythemic mouse assay (EHPCMA), fetal mouse liver erythroid colony-forming technique (FMLC), and a radioimmunoassay (RIA). The Ep detected in the spent medium of this renal carcinoma cell culture was found to have immunologic properties

similar to that of highly purified human urinary Ep (70,400 U/mg protein), as evidenced by the dilution regression line for the spent medium being parallel to that of the highly purified Ep in our RIA. The Ep values, which were estimated using the FMLC, were approximately the same as those measured by the RIA, unless the test samples for the FMLC assay were concentrated using ultrafiltration with Amicon membranes. Approximately one-third of the Ep activity in the test samples was lost with our concentration procedure. It is quite possible that the spent media could contain biologically active Ep fragments which passed through the ultrafiltration membranes with a 10,000 molecular weight cutoff. It seems very likely that most of the Ep in the spent media has physicochemical properties that are different from native Ep in that the spent media containing more than 300 mU/ml Ep (RIA) did not significantly stimulate ⁵⁹Fe incorporation into RBC in the EHPCMA, unless the media were concentrated several-fold by membrane ultrafiltration.

Sherwood and Goldwasser⁴ have reported that human renal carcinoma cells in culture may produce desialylated Ep that is biologically active in vitro but 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 in the spent culture media of this renal carcinoma.

Recently, Rich et al.⁸ reported Ep production by murine macrophages. They detected Ep up to 25 mU/ml in culture media of murine macrophages using

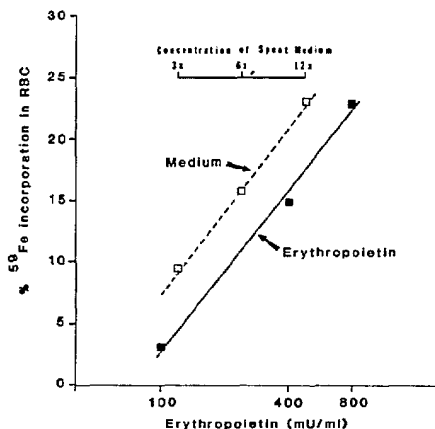


Fig. 5. Parallel dose-response regression lines for concentrated spent medium and laboratory standard erythropoietin (22 U/mg protein) in the exhypoxic polycythemic mouse assay.

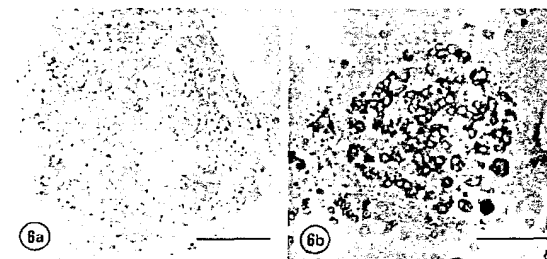


Fig. 6. Light micrograph illustrating negative staining with normal rabbit serum (a) and intense positive cytoplasmic staining (diaminobenzidine) with rabbit erythropoietin antiserum in some cells forming the dome (b). Bars = 50 μm.

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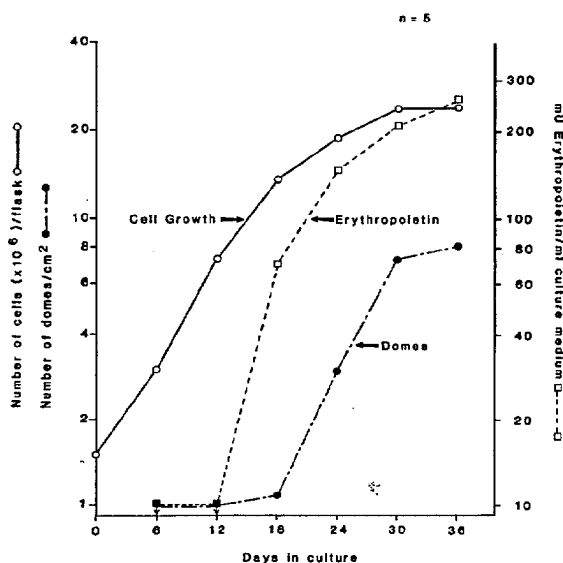


Fig. 7. Correlation between renal carcinoma cell growth (○—○), erythropoietin (Ep) activity in spent media measured by a radioimmunoassay (□---□), and number of domes (●---●). Note the parallel increase in number of domes and Ep activity in the spent media.

the fetal mouse liver erythroid colony-forming technique. Thus, macrophages grown in tissue culture are apparently capable of producing Ep. 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.¹² Therefore, it is possible that the Ep detected in our cell culture system was produced by macrophages derived from the host nude mice and not Ep derived from the renal carcinoma cells. However, this is probably not a tenable hypothesis, in that the cells in our culture system retained several morphological features characteristic of epithelial cells. For example, when the cells were studied with the use of light and electron microscopy, dome formation was seen in the confluent monolayers of these cultured cells. Microvilli and tight junctions were also observed in these cells. The cells that were in confluent monolayers, when reinoculated back into nude mice, produced tumors that had histologic features closely resembling the original renal carcinoma. Furthermore, treatment of the cultured cells with the antiserum to nude mouse spleen cells did not affect their ability to produce Ep. In addition, the immunocytochemical studies suggested the presence of an immunoreactive form of Ep in the cells forming the domes, as well as in the cells in the confluent monolayer. These several lines of evidence support the conclusion that the cells producing Ep in our cell culture system were derived from the renal carcinoma

cells transplanted into nude mice, not from nude mouse stromal cells, including macrophages.

Leighton et al.^{21,22} first described the occurrence of multicellular structures, called hemicysts, blisters, or domes, in a confluent monolayer of a Madin-Darby canine kidney (MDCK) cell line. Light and electron microscopic studies have demonstrated that the walls of the domes and the contiguous monolayers consist of cells retaining the morphological features unique to epithelial cells involved in transport functions, with microvilli extending upward into the medium and tight junctions joining adjacent cells at the apical surfaces of the cells. Electrophysiologic studies have demonstrated that the confluent monolayers of the MDCK cells retain transport and permeability properties of renal tubular epithelium.²⁵ These findings have confirmed the conclusion that the domes in the MDCK cell line are focal regions of fluid accumulation between the cell layer and the culture flask, owing to active transepithelial fluid transport.

The domes observed in primary cultures of human renal carcinoma cells in the present studies closely resemble those in the MDCK cell line and could reflect the expression by the renal carcinoma cells of the differentiated functions of the normal renal tubular epithelium, from which the renal carcinoma is believed to originate.²⁶ It is of interest that these renal carcinoma cells in culture are capable of producing Ep as well as forming domes. Ep production and dome formation both became detectable only after the cultured cells reached confluency and increased exponentially with time in culture. Furthermore, immunocytochemical studies have localized Ep-like immunoreactivity in the cells forming the domes. These findings might indicate the possibility that Ep production in these renal carcinoma cells reflects the expression of one differentiated function that is coupled to the expression of another differentiated function—dome formation. Alternatively, these two differentiated functions may be dissociated and expressed by two distinct types of cells forming the dome. One type of cell may be responsible for Ep production and a second type of cell, which is negative for Ep, may be responsible for dome formation. Furthermore, it seems quite possible that the cells forming the domes may not have been able to produce Ep, but only to absorb or concentrate the Ep produced by the cells in the monolayer. Further studies are needed before any conclusions can be reached concerning the possible coupling of Ep production and dome formation. Further studies are also needed to preclude the possibility that the positive staining with our Ep antiserum may reflect the presence of an antigen other than Ep. Highly purified Ep for absorption studies and/or monoclonal antibodies

specific for Ep would be useful to confirm our immunocytochemical findings.

Murphy et al.³ have demonstrated that a primary culture of human renal carcinoma cells produced Ep to a greater extent initially than after more prolonged culture periods, which were characteristically associated with progressive loss of cellular differentiation. Our results are opposite to their findings. The differentiated functions of our cultured cells, namely Ep production and dome formation, were not evident initially during the period of exponential growth but became very marked after the cells reached confluency, indicating an inverse relationship between cell growth rate and expression of the differentiated functions. The inverse relationship between cell growth rate and expression of differentiated functions has been well documented in other cell culture systems^{22,23,27-30} and seems to be a well-established principle of these cell lines.

Sherwood and Goldwasser⁴ have reported that the amount of Ep produced in cultures of human renal carcinomas decreased in the course of successive pas-

sages of their tumor cells. The same phenomenon was observed in our cell culture system. Both Ep production and dome formation were less marked in subcultures than in primary cultures of these renal carcinoma cells ongoing in our laboratory (unpublished data). Subcultivation of these cells could have resulted in their dedifferentiation, or else the selection of less differentiated clones, which grow more rapidly than the more differentiated cells, may have occurred. Our cell culture system, utilizing primary cultures of human renal carcinoma cells maintained in nude mice, may provide a useful in vitro model for studies of the mechanisms of Ep production and could also provide a source for messenger RNA for large-scale in vitro production of Ep using recombinant DNA technology.

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REFERENCES

- Ozawa S: Erythropoietin from the kidney cells cultured in vitro. *Keio J Med* 16:193, 1967
- McDonald TP, Martin DH, Simmons ML, Lange RD: Preliminary results of erythropoietin production by bovine kidney cells in culture. *Life Sci* 8:945, 1969
- Murphy GP, Brendler H, Mirand EA: Erythropoietin release from renal carcinomas grown in tissue culture. *Res Commun Chem Pathol Pharmacol* 1:617, 1970
- Sherwood J, Goldwasser E: Erythropoietin production by human renal carcinoma cells in culture. *Endocrinology* 99:504, 1976
- Burlington H, Cronkite EP, Reinecke U, Zanjani ED: Erythropoietin production in cultures of goat renal glomeruli. *Proc Natl Acad Sci USA* 69:3547, 1972
- Kurtz A, Jelkmann W, Bauer C: Mesangial cells derived from rat glomeruli produce an erythropoiesis stimulating factor in cell culture. *FEBS Lett* 137:129, 1982
- Zucali JR, Stevens V, Mirand EA: In vitro production of erythropoietin by mouse fetal liver. *Blood* 46:85, 1975
- Rich IN, Heit W, Kubanck B: Extrarenal erythropoietin production by macrophages. *Blood* 60:1007, 1982
- Ogle JW, Lange RD, Dunn CDR: Production of erythropoietin in vitro: A review. *In Vitro* 14:945, 1978
- Toyama K, Fujiyama N, Suzuki H, Chen TP, Tamoaki N, Ueyama Y: Erythropoietin levels in the course of a patient with erythropoietin-producing renal cell carcinoma and transplantation of this tumor in nude mice. *Blood* 54:245, 1979
- Katsuoka Y, Baba S, Hata M, Tazaki H: Transplantation of human renal cell carcinoma to the nude mice: As an intermediate of in vivo and in vitro studies. *J Urol* 115:373, 1976
- Okabe T, Suzuki A, Ohsawa N, Kosaka K, Terasima T: Immune elimination of host fibroblasts for the cultivation of human tumors transplanted into nude mice. *Cancer Res* 39:4189, 1979
- Cotes PM, Bangham DR: Bioassay of erythropoietin in mice made polycythemic by exposure to air at a reduced pressure. *Nature* 191:1065, 1961
- Iscoe NN, Sieber F: Erythroid progenitors in mouse bone marrow detected by microscopic colony formation in culture. *Exp Hematol* 3:32, 1975
- Ogawa M, Parmley FT, Bank HL, Spicer SS: Human marrow erythropoiesis in culture. I. Characterization of methyl cellulose assay. *Blood* 48:408, 1976
- Rege AB, Brookins J, Fisher JW: A radioimmunoassay for erythropoietin: Serum levels in normal human subjects and patients with hemopoietic disorders. *J Lab Clin Med* 100:829, 1982
- Miyake T, Kung CKH, Goldwasser E: Purification of human erythropoietin. *J Biol Chem* 252:5558, 1977
- Greenwood FC, Hunter WM: The preparation of ¹³¹I-labelled human growth hormone of high specific radioactivity. *Biochem J* 89:114, 1963
- Vaitukaitis J, Robbins JB, Nieschlay E, Ross GT: A method for producing specific antisera with small doses of immunogen. *J Clin Endocrinol* 33:988, 1971
- Sternberger LA, Hardy PH Jr, Cuculis JJ, Meyer HG: The unlabeled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horse-radish peroxidase-antihorse-radish peroxidase) and its use in identification of spirochetes. *J Histochem Cytochem* 18:315, 1970
- Leighton J, Brada Z, Estes LW, Justh G: Secretory activity and oncogenicity of a cell line (MDCK) derived from canine kidney. *Science* 163:472, 1969
- Leighton J, Estes JW, Mansukham S, Brada Z: A cell line derived from normal dog kidney (MDCK) exhibiting qualities of papillary adenocarcinoma and of renal tubular epithelium. *Cancer* 26:1022, 1970
- Smith RE, Ellis LF, Hull RN: Light, scanning, and transmission electron microscopy of "dome like" structures in a continuous pig kidney cell strain. *J Cell Biol* 63:322a, 1974
- Espada J, Brandan N, Li YT, Li SC, Fisher JW: Purification of human urinary erythropoietin. *Fed Proc* 41:1159, 1982
- Misfeldt DS, Hamamoto ST, Pitelka DR: Transepithelial transport in cell culture. *Proc Natl Acad Sci USA* 73:1212, 1976

AM 27 011317

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835

26. Bennington JL, Beckwith JB: Tumors of the kidney, renal pelvis, and ureter, in Firminger HI (ed): The Atlas of Tumor Pathology, vol 12. Washington DC, AFIP, 1975, p 93

27. Lever JE: Expression of a differentiated transport function in apical membrane vesicles isolated from an established kidney epithelial cell line. *J Biol Chem* 257:8680, 1982

28. Scott RE, Florine DL, Wille JJ Jr, Yun K: Coupling of

growth arrest and differentiation at a distinct state in the G₁ phase of the cell cycle: GD. *Proc Natl Acad Sci USA* 79:845, 1982

29. Blume A, Gilbert F, Wilson S, Farber J, Rosenberg R, Ninenberg M: Regulation of acetylcholinesterase in neuroblastoma cells. *Proc Natl Acad Sci USA* 67:786, 1970

30. Laskin JD, Piccini L, Engelhardt DL, Weinstein IB: Control of melanin synthesis and secretion by B16/C3 melanoma cells. *J Cell Physiol* 113:481, 1982

AM 27 011318

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