New Human Renal Carcinoma Cell Line Established from a Patient with Erythrocytosis

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ABSTRACT

A continuous human renal carcinoma cell line (GKA) has been established from a patient with the paraneoplastic syndrome of erythrocytosis. The cells are epithelioid and anchor-age dependent and have a doubling time in vitro of 48 to 72 hr. They exhibit a modal karyotype of 45,XX with abnormalities in chromosomes 3 and 9 and an absent chromosome 17 as determined by quinacrine mustard staining. Line GKA secretes erythropoietin activity into its growth medium, consistent with the biology of the tumor in vivo. This unique cell line will permit an investigation of the cellular physiology of this carcinoma and should result in clonal sublines with high erythropoietin-secretory activities.

INTRODUCTION

The presence of erythrocytosis in patients with renal carcinoma is hypothesized to be the result of erythropoietin secretion by certain cells within the tumor (1, 8, 12, 16, 18). An alternative view suggests that the hormone may be produced by surrounding normal renal tissue rendered hypoxic by the expanding mass. Increased levels of erythropoietin activity have been measured in such patients, and similar activity has been demonstrated in tumor extracts. One such tumor, when transplanted into athymic mice, resulted in erythrocytosis and an increase in erythropoietin activity (20). However, the study of such tumor cells in vitro has been limited, and there is only one, albeit unconfirmed, report of a cell line derived from such a tumor (7). Moreover, a definitive identification of the secretory product as erythropoietin is lacking. Short-term culture and apparent erythropoietin production by renal carcinoma cells from nonerythrocytotic patients have been reported, but continuous cell lines did not result, and the tumor tissue was not cryopreserved (17).

Recent progress in cell culture methodology has made it possible to derive continuous cell lines from renal adenocarcinoma (21), permitting investigation of the biology of these tumors under controlled conditions. Therefore, it should be feasible to adapt renal carcinoma cells from patients with erythrocytosis to continuous culture and to utilize such cell lines in the study of the biology of erythropoietin-secreting carcinomas. We report herein a new continuous cell line, designated as GKA, derived from the tumor cells of a patient with renal carcinoma and erythrocytosis. Our results indicate that line GKA will prove to be a valuable system with which to investigate the pathophysiology of the paraneoplastic syndrome of renal cancer and erythrocytosis.

MATERIALS AND METHODS

Case Report. A 62-year-old woman was found on routine annual physical examination to have an elevated hemoglobin and hematocrit. She admitted to symptoms of fatigue, dizziness, low-grade fevers, and a 5-kg weight loss over the past 5 months. She denied cardiorespiratory symptoms and was a nonsmoker. The physical examination was within normal limits except for mild plethora. The laboratory evaluation revealed a hematocrit of 54%, a hemoglobin of 17.5 g/dl, a CBC count of 6.35 \( \times 10^9 \) /mm with normal indices, a WBC of 6,200 with normal differential, and a platelet count of 200,000/cu mm. Blood gases were: pO\(_2\), 86 mm Hg; pCO\(_2\), 33 mm Hg (pH 7.47; O\(_2\), saturation, 96%. The serum iron was 33 mg/dl, and the total iron-binding capacity was 249 mg/dl. The serum folate, vitamin B\(_12\), and leukocyte alkaline phosphatase levels were normal. The hemoglobin electrophoresis demonstrated 97% hemoglobin A, 1.5% hemoglobin A\(_2\), and 1.5% hemoglobin F. The bone marrow was mildly hypercellular with a myeloid:erythroid ratio of 2:5:1. Iron was present. The TBC volume was increased to 37.7 ml/kg (predicted normal, 23.4 ml/kg), and the plasma volume was 43 ml/kg (normal, 40 ml/kg), consistent with a true erythrocytosis. A 24-hr urine collection contained 23 units of erythropoietin activity (normal, 2 to 4 units/24 hr). The urinalysis revealed microscopic hematuria. The chest X-ray showed multiple pulmonary nodules. An i.v. pyelogram and abdominal ultrasound revealed a solid mass in the upper pole of the left kidney. A left radical nephrectomy and a pulmonary wedge resection were performed; subsequent histological examination demonstrated a clear cell adenocarcinoma as did the biopsy of one pulmonary nodule. Postoperatively, the patient was treated with an experimental immunotherapy protocol (14) with subsequent disappearance of her metastases and a fall in her hematocrit to 36% and urinary erythropoietin to normal. She is alive and well 4 years after the operation and maintains a hematocrit of 42%.

Cell Culture. A 1-g slice of renal tumor tissue obtained at operation was minced with sterile scissors in 5 ml of Dulbecco’s phosphate-buffered saline (8.0 g NaCl per liter: 0.2 g KCl per liter: 1.15 g Na\(_2\)HPO\(_4\) \( \cdot 2H_2O \) per liter: 0.2 g KH\(_2\)PO\(_4\) per liter), pH 7.4. One hundred ml of 0.25% trypsin (Grand Island Biological Co., Grand Island, N. Y.): 0.02% EDTA were added, and the mixture was incubated at 37° for 15 min with swirling to effect cell disaggregation. The resulting cell suspension was decanted, and cells were harvested by centrifugation at 800 x g for 5 min at 4°. The cells were resuspended in 90% NCTC-109, NCTC-135, Dulbecco’s modified Eagle’s medium, or Medium F-12 (Grand Island Biological Co.): 10% fetal calf serum (MA Bioproducts, Walkersville, Md.), and incubated at 37° in a humidified atmosphere of 95% air:5% CO\(_2\). The growth medium was changed once weekly.

Continuous cell line GKA (see “Results”) was maintained in 90% NCTC-109:10% fetal calf serum in tissue culture dishes or flasks. The growth medium was changed twice weekly, and cells were passed every 2 weeks by disaggregation with 0.01% trypsin:0.02% EDTA and...
reinoculation in a dilution of 1:10. Cell counts were performed with a hemacytometer.

**Chromosomes.** Exponentially growing cultures of line GKA (passage 7) were prepared for chromosome analysis by incubation for 6 hr in the presence of 0.05 μg Colcemid per ml (Sigma Chemical Co., St. Louis, Mo.). Mitotic cells were detached from the flask bottom, removed by swirling, washed once, and treated with Hanks’ solution (8.0 g NaCl per liter: 0.4 g KCl per liter: 0.14 g CaCl2 per liter: 0.1 g MgSO4-7H2O per liter: 0.1 g MgCl2-6H2O per liter: 0.06 g Na3HPO4·2H2O per liter: 0.06 g KH2PO4 per liter: 1.0 g glucose per liter: 0.35 g NaHCO3 per liter) distilled H2O (1:4). Chromosome spreads were made on clean glass slides by fixing the hypotonic cells with methanol and acetic acid, dropping the cell suspension onto the slides, and permitting the chromosomes to disperse. The chromosome preparations were stained with quinacrine mustard (Sigma) (2) and examined and photographed with a fluorescence photomicroscope.

**Erythropoietin Assay.** Erythropoietin activity was determined with a new, highly sensitive in vitro bioassay utilizing cryopreserved rabbit bone marrow2 (15). Briefly, 1 × 106 rabbit bone marrow cells are incubated in the absence or presence of known concentrations of human urinary erythropoietin standard (1 to 100 milliunits/ml) or unknown samples, i.e., conditioned medium from line GKA, for 48 hr in 90% NCTC-109:10% fetal calf serum in 0.5-ml Multiwells (Falcon). Two μCi of 59Fe (New England Nuclear, Boston, Mass.) and 25 μg of rabbit transferrin are added, and the incubation is continued for 24 hr. The cells are harvested, washed with Dulbecco’s phosphate-buffered saline, and lysed with 1 ml of distilled water. The hemoglobin is denatured with 2 ml of 0.1 N HCl, and the [59Fe]heme is converted to the stable cyanmetheme form by the addition of 2 ml of Drabkin’s solution (Fisher Scientific Co., Pittsburgh, Pa.). [59Fe]Cyanmetheme is extracted into 5 ml of cyclohexanone (Fisher) and counted in a γ counter (Tracor, Elk Grove Village, Ill.). Unknowns are calculated from a standard curve run with each assay. Erythropoietin is expressed in milliunits/ml of test sample or units/mg of protein. For the experiments presented below, all standards and samples were run in triplicate. 59Fe radioactivity was linear with no added erythropoietin (60 to 100 cpm) to 100 milliunits/ml (4000 to 5000 cpm). Standard deviations for both known standards and unknown samples were ±10 to 15%. In addition to the laboratory human urinary erythropoietin standard, other samples from human urine assayed previously were usually included to verify accuracy. In vitro assays with similar sensitivities using 121I incorporation into mouse bone marrow (9) and rabbit bone marrow (10) erythroid colony-forming unit (CFU-E) counts have been reported recently.

**RESULTS**

After 6 to 8 weeks of incubation, small colonies of cells were visible in the primary culture which utilized NCTC-109 in the growth medium. No cell growth was visible in NCTC-135, Dulbecco’s modified Eagle’s medium, or Medium F-12. Microscopic examination of the cells revealed an epithelioid appearance with large nuclei containing one to 2 nucleoli (Fig. 1). Occasionally, multinucleate cells were seen. The cytoplasm frequently contained small refractile bodies or granules. These cells exhibited a strikingly different morphology from normal human fibroblasts and normal kidney cells grown in our laboratory under identical conditions. The cells have been serially subcultured intermittently for 3.5 years (over 50 passages) and have been cryopreserved. We have designated this cell line as GKA.

Line GKA grows relatively slowly. Under 2 conditions of fetal calf serum concentration and CO2 content of the atmosphere, the cells exhibited a doubling time of 48 to 72 hr (Chart 1). The slight deviation from linearity of the logarithmic growth curve may indicate partial contact inhibition of the cell line, since refeeding does not alter its slope. However, a microscopic examination reveals some piling up of the cells in cultures, consistent with a malignant or transformed character. The morphological appearance of the cells has not changed despite long-term culture.

A chromosome analysis demonstrated a modal chromosome number of 45 with some degree of aneuploidy and some tendency toward tetraploidy, also reflecting their cancerous nature (Table 1). The modal karyotype (Fig. 2) was 45,XX, consistent with the origin of this cell line in a female patient. The marker chromosomes characteristic of HeLa cells were not present (13). Instead, the modal karyotype exhibited abnormalities in chromosomes 3 and 9 and an absent chromosome 17. An examination of the abnormal chromosomes from 3 cells with this modal karyotype (Fig. 3) confirmed the absence of chromosome 17. There was an apparent translocation of part or all of the chromosome 17 onto chromosome 3. In addition, the abnormal chromosome 9 may have part of the missing material from chromosome 3 translocated onto it. This interpretation requires confirmation by additional chromosome-banding techniques.

To determine whether line GKA exhibits the same biological property as does the tumor from which it was derived, i.e., secretion of erythropoietin activity, we measured erythropoietin levels in samples of fresh growth medium (NCTC-109:10% fetal calf serum), conditioned medium from confluent cultures (Table 2, Experiments 1 to 3), and conditioned medium from a sparse culture (10% confluent; Table 2, Experiment 4) of the

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cells. As expected, fresh growth medium contained no detectable erythropoietin activity. However, erythropoietin activity was found in the conditioned medium, suggesting that it was secreted by GKA cells. This is supported by the increases observed corresponding to time of conditioning, 24 versus 72 hr. Moreover, as seen in Experiment 4, conditioned medium prepared from a sparse culture contained only barely detectable levels, consistent with a secretory rate proportional to the cell number present. The secretory activity appears constant despite serial passage of the cells. Assays of conditioned medium from human fibroblasts, Friend erythroleukemia cells, Rauscher erythroleukemia cells (19), and primary cultures of mouse kidney cells did not detect erythropoietin activity, providing further evidence that the activity observed was specific for an erythropoietic factor secreted by GKA cells and not simply reflective of a nonerythropoietic growth-enhancing substance.

**DISCUSSION**

Our data demonstrate that a continuous cell line has been derived from the renal tumor of a patient exhibiting the paraneoplastic syndrome of erythrocytosis. The elevated urine erythropoietin activity preoperatively and the correction of the erythrocytosis after removal of the tumor and subsequent immunotherapy support the thesis of a causal relationship between the tumor and the increased erythropoiesis.

The chromosomal markers characteristic of the 45,XX modal karyotype of line GKA appear unique to this human renal carcinoma line. Giard et al. (5) established line A709 which was hyperdiploid to hypertriploid. Line DU5873 derived by Pozner et al. (23) exhibited a modal number of 70. Fogh and Trempe (3) reported line Caki-1 to be hypertriploid with one marker chromosome and Caki-2 to be hypopentaploid to hypohexaploid, also with one marker. Hagemeijer et al. (6) described an extensive cytogenetic analysis of 3 clonal renal carcinoma lines (NC65-Sp, NC65-R, and NC65-V), all of which were derived from the same primary line NC65. These lines exhibited characteristic modal numbers and a variety of markers. In addition, Williams et al. (22) have derived 2 interesting lines, 786-O and 769-P, each with highly characteristic karyotypes. Finally, OUR-10, reported by Matsuda et al. (11), has a hypoploid karyotype with no markers. None of these tumors was reported to be associated with erythrocytosis. As yet, there are insufficient data in the literature to permit an assignment of a karyotype to a particular “type” of renal carcinoma. Ideally, it would be of interest to correlate a karyotype or marker chromosome with an erythropoietin-secreting tumor. However, karyotypes can vary widely among clonal lines derived from the same primary cell line (6).

Our results on the uncloned line GKA demonstrate that it secretes erythropoietin or an erythropoietin-like factor into the growth medium. We have attempted to concentrate the erythropoietin activity by ultrafiltration from conditioned complete growth medium without success. Although the activity does not pass through an M, 10,000 cutoff membrane (Amicon UM-10), other substances present in the fetal calf serum or secreted by the cells are also concentrated and are toxic to the erythropoietin assay. We have obtained similar results when using DEAE-cellulose to concentrate the activity. The possibility that erythropoietin activity secretion will continue in the presence of serum-free medium with concomitant loss of the toxic substance remains to be explored. However, the relatively low levels of activity secreted by line GKA under standard culture conditions almost certainly preclude substantial purification from the conditioned medium of the uncloned line.

The genetic heterogeneity of this cell line (Table 1) suggests that a similar phenotypic heterogeneity among the cells may be expected. Only a small percentage of the cells of line GKA may, in fact, be secreting erythropoietin. This suggestion can be tested by cloning the line and thus deriving sublines of single-cell origin. One would expect that such sublines would exhibit marked differences in their erythropoietin-secretory activity and that lines with much higher secretory activity could be produced. These studies are currently in progress.

The availability of a continuous erythropoietin-secreting cell line is important not only because it will increase our understanding of the paraneoplastic syndrome from which it is derived, but also because it would permit an array of unique experimental approaches not presently available. An erythropoietin-secreting cell line could be used to produce intrinsically radiolabeled, biologically active hormone by incubating the cells with radiolabeled amino acids and harvesting the product accordingly. Such a radiolabeled hormone could then be used as a probe for erythropoietin receptors, thereby allowing the identification of such receptor-bearing cells in the bone marrow. In addition, radiolabeled hormone could be utilized in a radioimmunoassay (4). This is especially important since the availability of highly purified erythropoietin suitable for radio-labeling is severely restricted. Were lines with sufficiently high secretory rates available, the hormone could be purified and characterized from such a source. In addition, the cellular physiology of its secretion would be available to study in detail. Finally, a line with high secretory activity would presumably be rich in erythropoietin mRNA, thereby providing an important tool needed for ultimate cloning of the erythropoietin gene. Clearly, further work is needed to ascertain whether line GKA or clonal lines derived from it will fulfill these possibilities. It must be determined whether the erythropoietin activity secreted by line GKA is identical to the hormone erythropoietin or whether this activity represents a different molecule that stimulates erythropoietin differentiation. Nevertheless, line GKA appears to be an important starting point from which such inves-

### Table 2

Erythropoietin activity of conditioned medium from line GKA

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Addition</th>
<th>Erythropoietin activity (milli-units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fresh medium</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Conditioned medium, 24 hr</td>
<td>1.0 ± 0.5a</td>
</tr>
<tr>
<td></td>
<td>Conditioned medium, 72 hr</td>
<td>5.0 ± 1.0</td>
</tr>
<tr>
<td>2</td>
<td>Fresh medium</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Conditioned medium, 24 hr</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Conditioned medium, 72 hr</td>
<td>8.0 ± 2.0</td>
</tr>
<tr>
<td>3</td>
<td>Fresh medium</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Conditioned medium, 24 hr</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Conditioned medium, 72 hr</td>
<td>6.0 ± 1.5</td>
</tr>
<tr>
<td>4</td>
<td>Fresh medium</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Conditioned medium, 72 hr (10% confluent culture)</td>
<td>1.0 ± 0.5a</td>
</tr>
</tbody>
</table>

*Mean ± S.E.*
tigations may proceed. Further, it represents a significant contribution to the increasing number of renal carcinoma lines available for study in vitro.

ACKNOWLEDGMENTS

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REFERENCES


Fig. 1. Human renal carcinoma line GKA cells, phase-contrast view. Original magnification, x 200.
Fig. 2. Modal karyotype (45,XX) of line GKA. Note abnormal chromosomes 3 and 9 and absent chromosome 17.

Fig. 3. Abnormal chromosomes from 3 GKA cells, each exhibiting the modal karyotype of 45,XX.
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