Secretion of Erythropoietin-like Activity by Clones of Human Renal Carcinoma Cell Line GKA

Arthur J. Sytkowski, Karen A. Bicknell, Georgianna M. Smith, and Joseph F. Garcia

Department of Hematology and Oncology, Children's Hospital and the Dana-Farber Cancer Institute; Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115 [A. J. S., K. A. B., G. M. S.]; and the Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720 [J. F. G.

ABSTRACT

Human renal carcinoma cell line GKA was derived from a patient with the paraneoplastic syndrome of erythrocytosis and secretes erythropoietin-like activity into its growth medium (Sytkowski, A. J., Richie, J. P., and Bicknell, K. A. Cancer Res., 43: 1415-1419, 1983). In order to derive homogeneous sublines with higher secretory rates, we cloned line GKA. Over 100 clones were generated, and 21 secreted erythropoietin-like activity, up to 6-fold higher than the uncloned line. This activity stimulated the growth and differentiation of CFU-E derived colonies in plasma clot culture. However, the secreted erythropoietin-like activity did not cross-react in a sensitive radioimmuno assay utilizing highly purified 125I-labeled human urinary erythropoietin and heterologous anti-human urinary erythropoietin antiserum. These results suggest that line GKA secretes an erythropoietic stimulating factor distinct from the hormone erythropoietin.

INTRODUCTION

In the nearly 30 years since the discovery of the hormone erythropoietin, its role in the regulation of erythroid differentiation has been studied with increasing intensity. The investigation of erythropoietin secretion has been carried out in studies using intact perfused kidney preparations as well as with kidney slices and liver cells (6, 16, 22, 34). Erythropoietin and other factors which stimulate erythropoiesis have been found in the conditioned medium derived from macrophages (18), spleen cells (5, 10, 17, 33), and a human T-cell line (6). Reports of erythropoietin secretion by renal carcinoma cells in vitro (23) and by renal tumor cells in nude mice (31) have also appeared. Studies of the biochemistry of the hormone have been performed primarily on material derived from sheep plasma and from the urine of anemic human donors (3, 4, 9, 12, 14, 23, 24, 27, 28). A monoclonal antibody (32) and site-specific heterologous antibodies (25) have been described recently.

We have derived recently a continuous human cell line, designated as GKA, from a patient with renal adenocarcinoma and erythrocytosis (29, 30). Our results indicated that the primary uncloned line secreted an erythropoietin-like activity into its growth medium, thereby simulating the biology of the tumor in vivo. This secretory erythropoietin activity is heat stable and has a molecular weight of >10,000. An examination of the karyotype of this cell line revealed substantial heterogeneity among these cells, which suggested that a similar phenotypic heterogeneity might follow; i.e., only a small percentage of the cells in a culture might be secreting erythropoietin-like activity. Hence, a clonal analysis of the original GKA line should result in the preparation of more homogeneous sublines, some with higher secretory rates. In this report, we describe the cloning of the human renal carcinoma line GKA and the resultant activities in the conditioned medium prepared from several GKA clones. The erythropoietin-like activity derived from these clones stimulates CFU-E colony growth in vitro. Using heterologous antiserum prepared against human urinary erythropoietin in a sensitive RIA, we have demonstrated that the secreted erythropoietin-like activity from clonal lines of GKA does not cross-react with human urinary erythropoietin, suggesting that the activity may be distinct from the native hormone.

MATERIALS AND METHODS

Cell Growth. Human renal carcinoma line GKA was maintained in 90% NCTC-109 (Grand Island Biological Co., Grand Island, N. Y.):10% fetal calf serum (MA Bioproducts, Walkersville, Md.) in 75-sq cm tissue culture flasks or 150-mm tissue culture dishes (Falcon, Cockeysville, Md.) in a humidified 95% air:5% CO2 atmosphere at 37°. The growth medium was changed twice weekly, and cells were subcultured every 2 weeks by removal of the culture medium, washing the cells once with Dulbecco's phosphate-buffered saline at 37°, and disaggregating them with 10 ml of 0.02% trypsin (Grand Island Biological Co.):0.02% EDTA. Cells were reseeded at a dilution of 1:10 in growth medium. Line GKA was cryopreserved in 90% fetal calf serum:10% dimethyl sulfoxide at -196°. Recovery from cryopreservation was greater than 90% viability by trypan blue exclusion. Efforts to grow line GKA under serum-free conditions have proven unsuccessful thus far.

Cloning of Line GKA. GKA cells from cultures in early to midlog phase (less than 50% confluence) were disaggregated exhaustively into a suspension of 0.02% trypsin (Grand Island Biological Co.):0.02% EDTA. Cells were seeded at a dilution of 1:10 in growth medium. Line GKA was cryopreserved at -196°. The cloning medium was changed twice weekly, and cells were subcultured every 2 weeks by removal of the culture medium, washing the cells once with Dulbecco's phosphate-buffered saline at 37°, and disaggregating them with 10 ml of 0.02% trypsin (Grand Island Biological Co.):0.02% EDTA. Cells were reseeded at a dilution of 1:10 in growth medium. Line GKA was cryopreserved in 90% fetal calf serum:10% dimethyl sulfoxide at -196°. Recovery from cryopreservation was greater than 90% viability by trypan blue exclusion. Efforts to grow line GKA under serum-free conditions have proven unsuccessful thus far.

Received May 10, 1983; accepted September 19, 1983.

1 Supported in part by Grants CA 26105 and AM 29992.
2 Recipient of Research Career Development Award AM 01001. To whom requests for reprints should be addressed, at Children's Hospital, 300 Longwood Avenue, Boston, Mass. 02115.
Erythropoietin Bioassay. Conditioned medium from GKA clones was assayed routinely for erythropoietin activity in a highly sensitive in vitro bioassay as described (19, 30). Triplicate determinations containing 0, 5, 10, 20, and 50% of conditioned medium in the assay wells were made. For all clones secreting erythropoietin-like activity, the responses were linear from 0 to 20%. For most clones, 50% of conditioned medium resulted either in no further increase or an actual decrease in observed $^{55}$Fe incorporation, presumably due to other products and metabolites in the medium. Erythropoietin activities in the conditioned medium samples were calculated by extrapolation from a standard curve of human urinary erythropoietin (0 to 100 milliunits/ml) included in each assay and normalized to milliunits/ml of conditioned medium. The erythropoietin standard (7 units/mg) was calibrated by RIA against the Second International Reference Standard.

CFU-E Growth. The effect of conditioned medium from GKA clones on rabbit CFU-E growth was assessed in plasma clot culture utilizing a modification of the method of McLeod et al. (13) as described by Salvado and Sytkowski (20). In all cases, 20% of conditioned medium was used, since it had resulted in the highest activity (milliunits/ml) within the linear response in the in vitro bioassay (see above). Clots contained 1 x $10^4$ nucleated rabbit marrow cells/0.1 ml (1 x $10^5$ cells/ml) and were incubated for 48 hr prior to fixation, staining with dimethoxybenzidine and hematoxylin, and counting by microscopic examination. Six clots were scored for each experimental point.

Erythropoietin RIA. Selected samples of conditioned medium were subjected to RIA utilizing rabbit antiserum produced against partially purified human urinary erythropoietin and $^{125}$I-erythropoietin (7). Suitable standard curves as well as human urinary erythropoietin controls were included routinely to permit comparison with GKA clonal supernatants.

RESULTS

The conditioned medium from the primary GKA cell line and over 100 GKA clones were assayed for erythropoietin activity by the in vitro cryopreserved rabbit bone marrow technique (Table 1). The medium from the primary GKA line contained 5 milliunits/ml. The erythropoietin-like activity values for most of the clones were below our limits of detection as illustrated by clones 23, 31, and 63. In contrast, 21 of the clones, exemplified by clones 9 through 107, secreted erythropoietin-like activity from 7 to 29 milliunits/ml of conditioned medium. The highest value detected, clone 55 = 29 milliunits/ml, was almost 6-fold higher than the level found in the primary, uncloned line, 5 milliunits/ml, supporting our hypothesis that a clonal analysis of line GKA would reveal substantially different rates of erythropoietin activity production among the derived clones. The conditioned media from several clones, illustrated by clones 54 and 81, were toxic to the assay, resulting in $^{55}$Fe incorporation below base-line values. These results serve to emphasize the need to work with clonal lines in order to derive especially suitable sublines with higher secretory rates.

Since erythropoietin stimulates the growth and differentiation of erythroid progenitors in vitro, we examined the effects of secreted erythropoietin-like activity from 4 GKA clones on rabbit CFU-E growth in plasma clot culture. As shown in Chart 1A, 20% of conditioned medium from clones 49A, 55, 96, and 105 stimulated CFU-E growth. The values were 115, 175, 170, and 140 colonies/10$^5$ nucleated cells, respectively. Control cultures performed in the same experiment (Chart 1B) showed that no added erythropoietin resulted in only 60 CFU-E colonies/10$^5$ cells, whereas sheep plasma erythropoietin stimulated CFU-E growth as expected. Similar results were obtained with human urinary erythropoietin. The stimulation of CFU-E growth by conditioned medium from these GKA clones found positive in the bioassay suggests a similarity between the biological actions of GKA-secreted erythropoietin activity and hormonal erythropoietin. The erythropoietin-specific activities (milliunits/ml) of the conditioned media calculated from CFU-E colony erythropoietin dose-response curves were: clone 49A, 11.5; clone 55, 18; clone 96, 15; and clone 105, 13. These values were similar to those obtained from the in vitro bioassay.

Selected samples of conditioned medium from both negative and positive clones were subjected to RIA. This highly sensitive assay is able to detect human urinary erythropoietin in concentrations as low as 0.5 milliunits/ml of sample. Although both the $^{125}$I-erythropoietin used in the assay and the rabbit antiserum were derived from human urinary erythropoietin, both serum and

Table 1

<table>
<thead>
<tr>
<th>Cells</th>
<th>Erythropoietin activity (milliunits/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary line GKA</td>
<td>5.0 ± 1.0$^a$</td>
</tr>
<tr>
<td>Clone 23</td>
<td>0</td>
</tr>
<tr>
<td>Clone 31</td>
<td>0</td>
</tr>
<tr>
<td>Clone 53</td>
<td>0</td>
</tr>
<tr>
<td>Clone 9</td>
<td>16 ± 3.0</td>
</tr>
<tr>
<td>Clone 20</td>
<td>16 ± 3.0</td>
</tr>
<tr>
<td>Clone 49A</td>
<td>12 ± 2.5</td>
</tr>
<tr>
<td>Clone 55</td>
<td>29 ± 2.5</td>
</tr>
<tr>
<td>Clone 56</td>
<td>20 ± 3.0</td>
</tr>
<tr>
<td>Clone 67</td>
<td>12 ± 2.0</td>
</tr>
<tr>
<td>Clone 71</td>
<td>7 ± 1.5</td>
</tr>
<tr>
<td>Clone 96</td>
<td>14 ± 2.0</td>
</tr>
<tr>
<td>Clone 104</td>
<td>11 ± 1.5</td>
</tr>
<tr>
<td>Clone 105</td>
<td>14 ± 2.0</td>
</tr>
<tr>
<td>Clone 107</td>
<td>16 ± 2.5</td>
</tr>
<tr>
<td>Clone 54</td>
<td>Toxic</td>
</tr>
<tr>
<td>Clone 81</td>
<td>Toxic</td>
</tr>
</tbody>
</table>

$^a$Expressed as milliunits of activity per ml of conditioned medium. Calculated from in vitro bioassay incorporating 20% of conditioned medium, which was shown to give the maximal response (see "Materials and Methods").

$^b$Mean ± S.E.
Erythropoietin levels in conditioned medium of GKA clones measured by bioassay

Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Erythropoietin (milliunits/ml)</th>
<th>Bioassay</th>
<th>RIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone 9</td>
<td>16 ± 2.0*</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td>Clone 20</td>
<td>19 ± 3.0</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>Clone 67</td>
<td>12 ± 2.0</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>Control growth medium</td>
<td>0</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td>Sheep plasma erythropoietin</td>
<td>10 ± 2.0</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>Human urinary erythropoietin</td>
<td>14 ± 2.5</td>
<td>12.0</td>
<td></td>
</tr>
</tbody>
</table>

* In vitro rabbit bone marrow technique.
* Mean ± S.E.

plasma samples can be measured successfully by the RIA, and we therefore presumed that the secreted erythropoietin-like activity which we demonstrated would share antigenic determinants with the human urinary and plasma hormones. Samples were assayed in 2 separate assays utilizing 2 protocols. The first was a less sensitive (1 to 2 milliunits/ml) but more rapid protocol using 100 µl of sample per assay. As shown in Table 2, none of the conditioned media from 3 positive clonal lines contained immunoassayable erythropoietin significantly higher than the control growth medium. The 5.1 milliunits/ml found by RIA in the control growth medium represented presumably erythropoietin in the fetal calf serum. In the bioassay, this control medium is defined as 0 milliunits/ml so that added erythropoietin values from unknown samples could be calculated directly by extrapolation from the standard curve (30) (see "Materials and Methods"). Both sheep plasma erythropoietin and human urinary erythropoietin samples were included in this RIA, and the results were similar to those obtained by bioassay. The second RIA protocol was more sensitive (0.1 to 0.2 milliunits/ml) and used 1.0 ml of sample. As shown in Table 3, none of the 3 clones tested in this fashion contained immunoassayable erythropoietin above control growth medium despite their biological activity.

DISCUSSION

The present study demonstrates that the primary line and clonal lines of human renal carcinoma line GKA secrete an erythropoietin-like activity. Our initial results (30) suggested that a clonal analysis of the primary line would produce sublines with higher secretory activity, and the present study confirms this hypothesis. This erythropoietin-like activity stimulates the production of hemoglobin in the in vitro bone marrow bioassay as well as the growth of CFU-E colonies in plasma clot culture. The GKA cell line was derived originally from a patient with the paraneoplastic syndrome of human renal cancer and erythrocytosis. This syndrome, like similar ones associated with other neoplasms, is believed to be due to secretion of erythropoietin by certain cells within the tumor. It must be noted that, in virtually all of the published reports of this syndrome (1, 2, 11, 15, 21, 26), the hormone erythropoietin has been identified functionally by bioassay and not through any parameter of its chemical structure. Functional bioassays are subject to the potential error of measuring more than one molecule which exhibits the same biological activity in the assay test system. An analogous fashion, the RIA, an extremely powerful analytical tool, is subject to certain criteria of interpretation. It depends upon the epitopes identified by the antisera prepared against the antigen of interest and upon the expression of these antigenic determinants in the unknown sample. Thus, a certain discordance between biological activity and antigenic determinants can be observed in such systems.

The RIA designed to detect the hormone erythropoietin failed to detect GKA-secretory erythropoietin-like activity. As expected, the same anti-erythropoietin antisera used in the RIA failed to neutralize the activity in the bioassay (data not shown). At least 2 potential explanations exist. (a) This activity may be a precursor to or similar to hormonal erythropoietin but may have the antigenic determinates recognized by the RIA masked in some way. At present, it is unknown what roles proteolysis or carbohydrate hydrolysis play in posttranslational modification of erythropoietin during passage from its cells of origin in the kidney into the general circulation. Moreover, filtration through the glomeruli and movement through the renal tubules also occur before it is purified and utilized as an immunogen. Thus, GKA-secretory erythropoietin-like activity may indeed be hormonal erythropoietin in a relatively unprocessed form. For these same reasons, its identity or nonidentity with hormonal erythropoietin cannot be demonstrated definitively by analytical techniques based upon molecular size or change. (b) It may be distinctly different from the hormone erythropoietin. It must still be determined whether GKA-secretory erythropoietin-like activity, if different from hormonal erythropoietin, interacts with the same erythropoietin receptor on the surface of erythroid progenitors. If so, it could serve as an important research tool in the study of erythropoiesis and of the biochemistry of erythropoiesis. Alternatively, it may stimulate erythropoiesis through an entirely different mechanism. Moreover, the question of whether this instance of the neoplastic syndrome of erythrocytosis and all other such erythrocytosis syndromes are due to secretion of similar molecules is still unanswered. Further studies of GKA-secretory erythropoietin-like activity and its interaction with the erythroid progenitors should enhance our understanding of the control of erythroid differentiation and of this paraneoplastic syndrome.

ACKNOWLEDGMENTS

We thank Dr. David G. Nathan for constant encouragement and Jeanne Clenon for expert secretarial work.

REFERENCES

A. J. Sytkowski et al.


Secretion of Erythropoietin-like Activity by Clones of Human Renal Carcinoma Cell Line GKA


*Cancer Res* 1984;44:51-54.

Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/44/1/51

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.