Stimulation by cAMP of Erythropoietin Secretion by an Established Human Renal Carcinoma Cell Line

By J.B. Sherwood, E.R. Burns, and D. Shouval

We used our recently reported stable, transformed human renal carcinoma cell line as a model system to study the role of 3', 5'-adenosine monophosphate (cAMP) in erythropoietin secretion. The erythropoietin produced by these cells is both biologically active and immunologically cross-reactive with purified native human hormone in our radioimmunoassay. Erythropoietin release by these renal carcinoma cells appears to be stimulated by cAMP as well as by the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (MIX). The response to cAMP involves a rapid and enhanced release of hormone, which occurred within 30 minutes of exposure of the cells to the effector and continued for at least 4 hours. Intracellular erythropoietin was higher in the control cultures than in the cells treated with cAMP, suggesting that cAMP stimulates the release of a storage pool of hormone. The ability of cAMP and MIX to elicit the release of erythropoietin suggests that a cAMP-mediated mechanism is involved in the release of this hormone.

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MATERIALS AND METHODS

Cells. The human renal carcinoma cell line designated as the RC-1 line was used for all studies. This line was derived from a renal cell carcinoma obtained in 1981 from a patient with erythrocytosis. These cells grow as a homogeneous monolayer, with neoplastic characteristics as evidenced by loss of contact inhibition and by the ability to be cloned and grown in soft agar.1,11 The characteristics of this cell line have been fully described.1 The current studies were performed on cells from several different passages (passages 37 through 105).

Cell culture methods. Tumor cells were routinely maintained in Corning tissue culture flasks (Corning Glass, Corning, NY) in complete medium, consisting of Dulbecco's modified Eagle's medium (DMEM) and nutrient mixture F12 (Ham) 1:1 by volume supplemented with 10% fetal bovine serum (FBS), 2 mmol/L of t-glutamine, 0.10 mmol/L nonessential amino acids, 100 U of penicillin, and 100 μg of streptomycin. All media components were obtained from Grand Island Biological (Grand Island, NY).

Radioimmunoassay of erythropoietin. Erythropoietin levels in the culture supernatant fluids and the cell extracts were determined in the radioimmunoassay described by Sherwood and Goldwasser.12 The pure erythropoietin used as the radiolabeled tracer was described by Miyake and co-workers11 and was generously supplied by Dr Eugene Goldwasser (University of Chicago) and by the Blood Diseases Branch, Division of Blood Diseases and Resources of the National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD. The erythropoietin was radiolabeled with 125I, using the water-insoluble oxidizing agent 1, 3, 4, 6-tetrachloro-3a, 6a-diphenylglycoluril (lodogen, Pierce Chemical, Rockford, IL), to a specific activity of 16 to 21 μCi 125I/μg of erythropoietin. The antibodies to erythropoietin were raised in New Zealand White rabbits in response to a preparation of crude human urinary erythropoietin. Human urinary erythropoietin (Toyobo, Osaka, Japan) was used as the standard in the assay. The methods for incubation of samples and antibody and for separation of antibody-bound and free erythropoietin by the second antibody technique were described previously.13 The ratio of bound to total 125I-erythropoietin was determined for the standard curve and for the culture and serum samples. All erythropoietin standards and unknown samples were assayed in triplicate. Medium with and without the experimental additives incubated without cells served as assay controls. Values are expressed as mean ± SEM, representing the replicate cultures.

Preparation of samples for detection of erythropoietin. Supernatant fluids obtained from culture dishes were centrifuged for 10 minutes at 1,000 rpm at 4°C in an IEC Centra 7-R tabletop.
rifugated centrifuge (International Equipment, Needham Heights, MA). The culture fluid was then stored at -20°C to -70°C until assay.

Cells were harvested from the cultures with 0.1% trypsin-0.1 mol/L of EDTA, washed with Earle’s balanced salt solution (BSS), and extracted in 0.1 mol/L of phosphate buffer by homogenization with a Polytron (Brinkmann Instruments, Westbury, NY) as described by Sherwood and Goldwasser.15 The cell extracts were stored at -20°C to -70°C until assayed.

Evaluation of effect of cAMP on erythropoietin production. For studies of erythropoietin production, cells were removed from the surface of the flasks by incubation with 0.1% trypsin-0.1 mol/L of EDTA, followed by resuspension in complete medium. The number of viable cells was determined in a hemocytometer, using exclusion of trypan blue dye as an indicator of viability. Each 60-mm Falcon tissue culture dish (Becton Dickinson, Cockeysville, MD) was seeded with 3 x 10^5 viable cells in 5 mL of complete medium and incubated at 37°C in 5% CO2-95% air until confluence (1 to 2 x 10^6 cells per plate) was reached. The cultures were then incubated at 37°C in 5% CO2-95% O2 in a humidified tissue culture incubator (Forma Scientific, Marietta, OH) in 2 mL of “complete medium” containing 5% fetal bovine serum (FBS), and 8-bromo cAMP or the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (MIX) as indicated. Control cells from the same passage were incubated in the same medium (5% FBS), without cAMP or MIX, at the same time. Three replicate cultures were made for each effector concentration and time point. At the indicated times, cells and supernatant media were harvested, the viable cell count was obtained, and the erythropoietin was extracted from the cells as described. Erythropoietin levels in the media and cell extracts were determined by radioimmunoassay.

RESULTS

Effect of cAMP on intracellular and extracellular erythropoietin. Concentrations of 8-bromo cAMP <10^-3 mol/L were ineffective in stimulating erythropoietin production. Therefore, in these studies, cells were incubated in experimental medium with or without 10^-4 mol/L of 8-bromo cAMP for periods of 1 to 4 hours. As shown in Table 1, erythropoietin levels in the supernatant media from cAMP-treated cells were significantly higher (P < .02) than in the media from control cultures from the same passage incubated (at the same time) without cAMP. Conversely, erythropoietin levels in the cell pellets (intracellular erythropoietin) were significantly (P < .02) lower in the cAMP-treated cultures than in the controls. Control medium with and without 10^-4 mol/L of 8-bromo-cAMP, incubated without cells, contained nondetectable amounts of erythropoietin (<10 mU/mL). The cAMP appeared to have no effect on cell morphology, growth, or viability.

Effect of 8-bromo-cAMP on time course of erythropoietin release. Cells from passage 105 were incubated in experimental medium with or without 10^-4 mol/L of 8-bromo-cAMP. As shown in Fig 1, a slight secretory response to 10^-4 mol/L of 8-bromo-cAMP was observed within 15 minutes (13 ± 10 mU/mL of medium) and reached a maximum at 30 minutes (130 ± 3 mU/mL). Erythropoietin levels in

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<th>Table 1. Effect of 8-Bromo-cAMP on Intracellular and Extracellular Erythropoietin</th>
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Cells were incubated at confluence with 10^-4 mol/L of 8-bromo cAMP in 2 mL of F12 Dulbecco’s modified Eagle’s medium. Erythropoietin values were determined in the radioimmunoassay. Each value represents the mean ± SEM for three plates, each measured in triplicate. All values are significantly different from values for control cells.

*ND, not detectable, <10 mU/mL. Control medium without cells had no detectable erythropoietin.
Cells have been maintained in culture since 1981 and for passage-37 cells were incubated for 4 hours in experimental contrast, erythropoietin was not detected in the supernatant medium. For long-term incubation, such as in the regenerative period, the radioimmunoassay. Addition of either MIX or cAMP to the medium remained constant for at least 4 hours (1 hour, 108 ± 27 mU/mL; 2 hours, 107 ± 7 mU/mL; 4 hours, 98 ± 8 mU/mL). The values for these time periods did not differ significantly (P = 0.09) from the 30-minute value. In contrast, erythropoietin was not detected in the supernatant medium of control cultures of the passage 105 cells until 2 hours of incubation (103 ± 3 mU/mL for 2 hours, 142 ± 42 mU/mL for 4 hours). The 4-hour value for the control cultures did not differ significantly (P = 0.27) from that of the cAMP-treated cultures from the same passage, suggesting some constitutive release by cells of this passage. No erythropoietin was detectable in the control media with and without cAMP, incubated without cells.

Effect of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine on erythropoietin release. Cultures of passage-37 cells were incubated for 4 hours in experimental medium with 10^-4 mol/L of MIX. After the 4-hour incubation period, the supernatant fluids were harvested; erythropoietin levels in the samples and in control medium containing the MIX but not incubated with cells were measured in the radioimmunoassay. Addition of either MIX or cAMP to the cultures was associated with erythropoietin levels in the supernatant medium that were significantly greater than in the control cultures: cultures with 10^-4 mol/L of MIX, 48 ± 4 mU erythropoietin/10^6 cells; cultures with 10^-4 mol/L of 8-bromo cAMP, 110 ± 1 mU/10^6 cells; control cultures, no detectable erythropoietin; and no detectable erythropoietin in the control medium incubated without cells.

Effect of long-term incubation on erythropoietin production by passage-37 cells. Cells from the passage 37 were incubated in experimental medium with and without 10^-4 mol/L of 8-bromo cAMP, and the supernatant fluids were harvested after 4 hours and 24 hours of incubation. After 4 hours of incubation, erythropoietin levels in the supernatant media from cAMP-treated cells (110 ± 1 mU/10^6 cells) were significantly higher (P < 0.02) than in the medium from control cultures from passage 37 incubated without cAMP (nondetectable erythropoietin levels), whereas intracellular erythropoietin was higher in the controls (310 ± 38 vs 118 ± 39 mU/10^6 cells). Control passage-37 cells did not appear to show constitutive erythropoietin release until 24 hours of culture; after 24 hours of incubation, erythropoietin levels in the medium were 38 ± 14 mU/10^6 cells in cAMP-treated cultures and 34 ± 10 mU/10^6 cells in controls (cAMP vs control, P = NS). The cell content was higher in the cAMP-treated cultures (474 ± 84 mU/10^6 cells), however, than in the controls (118 ± 10 mU/10^6 cells).

DISCUSSION

This report describes the effect of 8-bromo-cAMP on the production of erythropoietin in vitro by our stable, transformed human renal carcinoma cell line and provides evidence that cAMP is an intermediate in the regulation of erythropoietin production.

We described this cell line in a recent publication. The cells have been maintained in culture since 1981 and for >150 passages and retain an abnormal human karyotype, ultrastructural features characteristic of human renal clear cell carcinoma, and the growth characteristics of transformed neoplastic cells. The erythropoietic factor produced by this cell line appears to be biologically active in vitro, in the mouse CFU-E assay, and in vivo, as demonstrated by the development of significant erythrocytosis in athymic Balb/c mice transplanted with cells from this line. In our radioimmunoassay, displacement curves produced by increasing amounts of culture supernatant fluids were parallel to those produced by pure native human urinary erythropoietin, suggesting that the native human hormone and the tumor factor are similar.

Little information exists on regulation of erythropoietin synthesis and secretion at the cellular level, in part due to the difficulty of establishing a suitable in vitro model system. Previously reported in vitro systems include cultures of normal kidney cells such as goat renal glomeruli and rat mesangial cells, as well as transformed cells such as the human renal carcinomas reported by Hagiwara and colleagues, Sherwood and Goldwasser, Sherwood and Shoulav, and Sytkowski and coworkers, a human testicular carcinoma, and mouse erythroleukemia cells. Our studies represent the first reported use of a long-term stable human cell line to study regulation of erythropoietin production at the cellular level.

The membrane enzyme adenylate cyclase and its product cAMP are intermediates in the regulation of secretion of some polypeptide hormones. The secretory effects of cAMP are mediated through activation of a cAMP-dependent protein kinase, leading to exocytosis of secretory granules. Erythropoietin production by our renal carcinoma cell line was stimulated by 8-bromo-cAMP and MIX. MIX, a cyclic nucleotide phosphodiesterase inhibitor, prevents the degradation of intracellular cAMP, thus allowing its accumulation. The 8-bromo-cAMP used in these studies is a derivative of cAMP with an 8 substitution of the purine ring. Although these derivatives are able to inhibit cyclic nucleotide phosphodiesterase and to activate cAMP-dependent protein kinase, their primary mechanism of action at the cellular level is considered to occur by protein kinase activation.

In these studies, cells were incubated with and without cAMP or with and without MIX, and erythropoietin production by cells of the same passage was measured by radioimmunoassay. Comparisons were made between cells of the same passage, not between cells of different passages. In all experiments and cell passages, erythropoietin levels in medium from control cultures were lower than those of the control cultures of the same passage. In the short-term cAMP-treated cultures, the increased erythropoietin in the medium was associated with a concomitant decrease in intracellular hormone, in contrast to the control cultures—which had high intracellular and low media levels of erythropoietin.

CAMP produced a rapid and enhanced release of erythropoietin with 15 minutes of exposure of the cells to the nucleotide. Erythropoietin release continued to increase for ~30 minutes, after which the levels in the medium remained relatively constant (erythropoietin levels at the other times were harvested after 4 hours and 24 hours of incubation. Cells from the same passage were measured by radioimmunoassay. Addition of either MIX or cAMP to the cultures was associated with erythropoietin levels in the supernatant medium that were significantly greater than in the control cultures: cultures with 10^-4 mol/L of MIX, 48 ± 4 mU erythropoietin/10^6 cells; cultures with 10^-4 mol/L of 8-bromo cAMP, 110 ± 1 mU/10^6 cells; control cultures, no detectable erythropoietin; and no detectable erythropoietin in the control medium incubated without cells.

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did not differ significantly from the 30-minute value). Erythropoietin release after 2 hours by the control cells of this passage suggests some constitutive production.

This pattern of erythropoietin release in response to elevated intracellular cAMP is similar to the response that has been observed for other hormones. Addition of dibutyryl (db) cAMP at concentrations of \( \geq 10^{-4}\) mol/L to parathyroid gland explants resulted in significant stimulation of parathyroid hormone release 1 hour after incubation. Growth hormone release from bovine pituitary gland anterior lobe explants and cultures of rat adenohypophyseal somatotrophs was stimulated within 30 minutes in response to elevated extracellular and intracellular cAMP and remained relatively constant during the 2-hour incubation period. cAMP also stimulates release of thyroid hormone, thyroid-stimulating hormone, insulin, and calcitonin in similar fashion.

cAMP stimulation may also lead to increased synthesis of some proteins. In each experiment the total amount of erythropoietin in each culture (cellular content plus released some proteins) in each experiment the total amount of erythropoietin in each culture (cellular content plus released some proteins) in each experiment the total amount of erythropoietin in each culture (cellular content plus released some proteins) in each experiment the total amount of erythropoietin in each culture (cellular content plus released some proteins).

The increase in intracellular erythropoietin in the cAMP-treated cultures may be due to direct stimulation of synthesis by cAMP or may involve a feedback stimulus caused by depletion of an intracellular pool by the cAMP-induced hormone release. There may also be two intracellular pools of erythropoietin responsive to different physiological stimuli, one pool responsive to agents that elevate cellular cAMP and another pool responsive to other factors, as suggested also for parathyroid hormone. As support for this, we showed that this cell line produces erythropoietin under control conditions (Table I and Fig 1), an observation that was important to us in developing this erythropoietin-producing line in amounts and at time intervals that may differ for each cell passage. Some erythropoietin may also be released in a constitutive fashion, as has been reported for other culture systems.

The short-term effects described in this report, increased erythropoietin in the medium within 15 minutes after cAMP stimulation associated with a concomitant depletion of intracellular erythropoietin (Table 1), are consistent with secretion of preformed erythropoietin. The immediate release of an intracellular pool of preformed hormone in response to cAMP stimulation has been extensively reported in other endocrine systems. The present studies substantiate the utility of this human renal clear cell carcinoma cell line for investigation of cellular factors mediating the production of erythropoietin.

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REFERENCES

20. Hagiwara M, McNamara DB, Chen I-Li, Fisher JW: Role of endogenous prostaglandin E2 in erythropoietin production and dome
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