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 recently described the development of a 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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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 centrifuge.

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containing 5% fetal bovine serum (FBS), and 8-bromo cAMP or the

cells per plate) was reached. The cultures were then incubated at

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 × 10^7 viable cells in 5 mL of complete medium and incubated at 37°C in 5% CO_2-95% air until confluence (1 to 2 × 10^6 cells per plate) and incubated in standard culture medium with or without 10^{-4} mol/L of 8-bromo-cAMP, incubated without cells, contained nondetectable amounts of erythropoietin (<10 mU/mL). Erythropoietin appeared to have no effect on cell morphology, growth, or viability.

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 medium 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 lower (P < .02) 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). 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 mL/mU). Erythropoietin levels in

RESULTS

Effect of cAMP on intracellular and extracellular erythropoietin. Concentrations of 8-bromo-cAMP <10^{-5} 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

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Cell Passage No.</th>
<th>Effector</th>
<th>Sample Assayed</th>
<th>Incubation (hours)</th>
<th>Erythropoietin (mU per 10^6 Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37</td>
<td>cAMP</td>
<td>Supernatant</td>
<td>4</td>
<td>110 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cells</td>
<td></td>
<td>118 ± 39</td>
</tr>
<tr>
<td>2</td>
<td>86</td>
<td>cAMP (10^{-4} mol/L)</td>
<td>Supernatant</td>
<td>4</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cells</td>
<td></td>
<td>310 ± 38</td>
</tr>
<tr>
<td>3</td>
<td>87</td>
<td>cAMP (10^{-4} mol/L)</td>
<td>Supernatant</td>
<td>2</td>
<td>1143 ± 88</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cells</td>
<td></td>
<td>37 ± 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Supernatant</td>
<td>2</td>
<td>71 ± 18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cells</td>
<td></td>
<td>540 ± 40</td>
</tr>
</tbody>
</table>

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.
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 = .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 = .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. Induction 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^{-5}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 < .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 v 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 v 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 Hagihara and colleagues, as well as the human renal adenocarcinoma, 22 and the human renal carcinoma, 24 and mouse erythroleukemia cells. 25 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 the 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 cultures incubated with either 8-bromo-cAMP or with MIX were significantly higher than in medium from 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...
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 \( \pm 10^{-4} \text{ mol/L} \) to parathyroid gland explants resulted in significant stimulation of parathyroid hormone release 1 hour after incubation. Growth hormone release from bovine pituitary gland activated intracellular cAMP is similar to the response that has been observed for other hormones. Addition of dibutyryl cAMP resulted in significant stimulation of 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 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 hormone) was higher in the cAMP-treated culture \((P < .05)\). This effect occurred in the cultures of passages 86 and 87 after 1 and 2 hours of incubation. Passage 37 cultures had similar total erythropoietin levels at a 4-hour incubation period. After 24 hours, however, the cAMP-treated cultures had a significantly higher intracellular erythropoietin content, but cAMP and control cultures had similar levels in the medium. Although cAMP effects are short-range phenomena in endocrine systems, cAMP may have some long-term effects on erythropoietin production and on erythropoiesis.

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 1 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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