Calcitonin Stimulation of Cyclic Adenosine 3':5'-Monophosphate Production with Growth Inhibition in Human Renal Adenocarcinoma Cell Lines

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ABSTRACT
Responsiveness of cyclic adenosine 3':5'-monophosphate (cAMP) to parathyroid hormone, calcitonin, and vasopressin was studied in six human renal adenocarcinoma cell lines. Four of six renal adenocarcinoma cell lines showed increased cAMP content in response to calcitonin while the other two did not. Neither parathyroid hormone nor vasopressin increased the concentration of cAMP in each of these cell lines. The growth rate of KU-2 cells, which responded to calcitonin with an increase of cAMP content, was inhibited by calcitonin. On the other hand the growth rate of calcitonin-nonsensitive KH-39 cells was unaltered. The growth inhibitory effect of the hormone on KU-2 cells could be considered to be mediated by the increased cAMP levels from the following results: (a) there was positive correlation between the cellular cAMP content and growth inhibition after various amounts of calcitonin addition; (b) KU-2 growth was also suppressed by N6-O2'-dibutyryl cAMP; and (c) a group of KU-2 cells which had become resistant to calcitonin-induced growth inhibition showed a diminished cAMP increase in response to calcitonin.

INTRODUCTION
A number of studies have been carried out on continuous cell lines from renal adenocarcinoma of human origin (1-5). Many of these cell lines have been well characterized with respect to their morphology, karyotype, and various enzyme activities. However, no evidence has been presented on the adenylate cyclase response of these cell lines to peptide hormones. This paper deals with studies on the effects of calcitonin, parathyroid hormone, and vasopressin upon the adenylate cyclase activity of six different renal adenocarcinoma cell lines, wherein a positive response to calcitonin was inhibited by CT

MATERIALS AND METHODS
Cells and Cell Culture. Six human renal adenocarcinoma cell lines, KU-2 (1), KO-RCC-1 (2), OUR-10 (3), NRC-12 (4), KH-39 (5), and KN-41 (5) were used. KU-2 was a gift from Dr. Y. Katsuda (Tokai University, Japan), KO-RCC-1 was from Dr. Y. Okada (Kobe University, Japan), OUR-10 from Dr. M. Matsuda (Osaka University, Japan), NRC-12 from Dr. S. Yamamoto (Niigata University, Japan), and KH-39 and KN-41 from Dr. K. Naito (Kanazawa University, Japan). These cell lines were cultured in the following culture medium at 37°C. The medium for KU-2 was MEM supplemented with 10% FCS (Grand Island Biological Co.), streptomycin (100 μg/ml), and penicillin (100 units/ml); for KO-RCC-1 it was MEM supplemented with 10% FCS; for OUR-10 and NRC-12 it was RPMI 1640 supplemented with 20% FCS; for KH-39 and KN-41 it was Ham's F12 with 20% FCS. Each medium was changed every 3 days and subculture was carried out every 1 or 2 weeks.

Hormones. Synthetic human PTH(1-34), (3130 units/mg), and ECT (6378 units/mg) were provided by Toyo Jozo Co. (Shizuoka, Japan) and dissolved in 0.01 N HCl containing 0.02% bovine serum albumin Fraction V (Sigma). Synthetic arginine VP (400 units/mg) was purchased from Protein Research Foundation and dissolved in 0.001 N HCl containing 0.02% bovine serum albumin.

RESULTS
Effect of ECT on KU-2 cell growth in the soft agar was also studied. According to the method described (6) cells were inoculated in 0.6 ml soft agar (MEM containing 0.33% agar) in a 60 mm plate (106 cells/ml) and ECT (50 ng/ml) was added to the medium. After incubation the medium was removed and 1 ml of 6% trichloroacetic acid was added to the cells. The cells were transferred to the tubes after being scraped with a rubber policeman and then were subjected to "freezing and thawing" several times. The tubes were centrifuged for 15 min at 3000 rpm and the supernatant was stored at -20°C before assay.

Expression of PTH, CT, and VP on cAMP Content of Cell Lines. In all experiments cells were inoculated in 24 multiwell culture plates. After reaching a confluent state the culture medium was removed and 1 ml of experimental medium (1:1 mixture of Dulbecco's modified MEM and Ham's F12 containing 1 mM isobutylmethylxanthine) was added to each well. The culture plates were continuously but gently shaken for 60 min in the Dubnoff metabolic shaker at 37°C. Then hormone solution (25 μl) was added to each well and incubated for 10 min. After incubation the medium was removed and 1 ml of 6% trichloroacetic acid was added to the wells immediately. The medium was stored at -20°C before assay.

The cells were harvested at different times for the determination of cell number and protein measurement.

Effect of ECT on KU-2 cell growth in the soft agar was also studied. According to the method described (6) cells were inoculated in the 0.6 ml soft agar (MEM containing 0.33% agar and 10% FCS) (106 cells/ml) and ECT (50 ng/ml) was added to the medium. After incubation the medium was changed every 3 days. Cells were harvested at different times for the determination of cell number and protein measurement.

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Cyclic AMP Assay and Protein Measurement. Concentrations of cAMP were measured by radioimmunoassay using the antiserum kindly provided by K. Martin (7). Protein was measured by the method of Lowry et al. (8).
Effect of HPTH(1-34), ECT, and AVP on the cellular cAMP content of 6 renal adenocarcinoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cellular cAMP (pmol/10^6 cells) for each cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation conditions</td>
<td>KU-2</td>
</tr>
<tr>
<td>Control</td>
<td>9.0 ± 1.3</td>
</tr>
<tr>
<td>HPTH(1-34), 10 ng/ml</td>
<td>9.1 ± 0.9</td>
</tr>
<tr>
<td>HPTH(1-34), 100 ng/ml</td>
<td>7.2 ± 0.4</td>
</tr>
<tr>
<td>ECT, 10 ng/ml</td>
<td>132.6 ± 6.8</td>
</tr>
<tr>
<td>ECT, 100 ng/ml</td>
<td>191.8 ± 28.3</td>
</tr>
<tr>
<td>AVP, 10 ng/ml</td>
<td>1.6 ± 0.6</td>
</tr>
<tr>
<td>AVP, 100 ng/ml</td>
<td>0.7 ± 0.1</td>
</tr>
</tbody>
</table>

* HPTH(1-34), synthetic human PTH(1-34); AVP, arginine vasopressin.

bated with peptide hormones, and cAMP levels in the cells were measured (Table 1). In 4 cell lines (KU-2, KO-RCC-1, OUR-10, NRC-12) the cAMP content increased dose dependently when incubated with ECT for 10 min. An increase of cAMP levels was most prominent in the KU-2 cell line (191.8 ± 28.3 (SE) versus 9.0 ± 1.3 pmol/10^6 cells as a control; n = 4, P < 0.001). In 2 other cell lines (KH-39, KN-41), however, cAMP levels did not increase in response to ECT. In none of the 6 cell lines did cAMP levels increase in response to either synthetic human PTH(1-34) or arginine VP.

**Experiment 2, Effect of ECT on Cell Growth of KU-2 and KH-39.** KU-2 (a cell line in which cAMP increased markedly in response to ECT) and KH-39 (a cell line in which cAMP did not increase in response to ECT) were cultured in the serum-free defined medium containing ECT (10 ng/ml) or control solvent alone (Chart 1). A daily addition of ECT did not inhibit the cell growth of KH-39 whereas that of KU-2 was inhibited completely for 8 days. After an 8-day culture even in the presence of ECT KU-2 had begun to grow at the same rate (cell doubling time, 31 h) as that of the cells cultured in the medium without ECT (cell doubling time, 30 h). The growth of KU-2 cells was significantly inhibited dose dependently in the presence of ECT (from 0.01 ng/ml to 10 ng/ml) at the ninth culture day as assessed by cell number and protein content (from 10^5 x 4.92 ± 0.22 cells and 40.1 ± 1.8 µg/20 cm2 plate in the absence of ECT to 10^5 x 1.52 ± 0.21 cells and 30.1 ± 1.2 µg/20 cm2 plate in the presence of ECT, 10 ng/ml, respectively). Addition of DBcAMP (10^-7 M) also inhibited KU-2 growth (Chart 2). The cellular and medium cAMP contents were also measured at the ninth culture day, 24 h after...
the last medium change (Chart 3). ECT increased the cAMP levels of KU-2 dose dependently also at the concentrations of ECT that ranged from 0.01 to 10 ng/ml. As shown in Chart 4 ECT also inhibited the growth of KU-2 cells even when the addition of ECT was begun at the logarithmic phase. Cessation of ECT addition at the fifth culture day did not change the growth rate of KU-2 as compared to that of the cells continuously exposed to ECT (Chart 5). This result shows that ECT could only inhibit the proliferation of the cells during the first 3 days of the culture period and that the cells cultured in a medium containing ECT lose their sensitivity to ECT.

To eliminate the possibility of cell detachment due to ECT in the monolayer culture the soft agar technique was used to count the colonies of KU-2 cells. Colony formation in the soft agar was clearly inhibited dose dependently by ECT (from 0.03 to 30 ng/ml). Addition of DBcAMP (3 x 10^{-6} M) also inhibited the colony formation significantly as shown in Chart 6.

Experiment 3, Decrease of Hormone Responsiveness of KU-2 Cells after 2 Weeks' Culture in ECT-containing Medium. KU-2 cells were inoculated in 20 cm² culture plates (10^5 x 1.5 cells/plate) in a volume of 3 ml serum-free defined medium. A solution of ECT (in 100 µl serum-free medium; final concentration, 10 ng/ml) was added daily (ECT pretreatment) and the medium was changed every 3 days. As a control solvent alone was added daily to the other plates. After 14 days' culture the ECT-pretreated and control cells were dispersed, washed three times with MEM, and inoculated in 24 multiwell plates in a volume of 1 ml MEM supplemented with 10% FCS (10^4 x 5 cells/well). Then both the ECT-pretreated and control cells were cultured for another 5 days without ECT. The medium was changed every 2 days. Thereafter the increase of cAMP content in these cells in response to ECT was investigated following the procedure used in Experiment 1. As shown in Chart 7 the increase of cAMP in the pretreated cells was significantly diminished in comparison with that of the control cells.

DISCUSSION

Although there are several cell lines originating from human renal adenocarcinomas the effect of peptide hormones on these cell lines has not been reported. The aim of our experiments was to investigate the effects of PTH, CT, and VP, which were known to increase the cAMP contents in the renal tubules (9-12), on these renal adenocarcinoma cells by measuring the cAMP contents and cellular proliferation. Our experiments disclosed that cAMP contents increased in 4 of 6 adenocarcinoma cell lines in response to calcitonin. In contrast PTH and AVP did not increase the cAMP production significantly in any of these cell lines. Calcitonin is known to stimulate the adenylate cyclase activity only in the medullary and cortical ascending limbs of Henle and in the early distal convoluted tubules along the human nephron (9). It may be speculated that some of these renal adenocarcinomas have originated from the cells of these segments of the nephrons.

We also showed that the proliferation of KU-2, which increased cAMP contents in response to ECT, was inhibited significantly by ECT. On the other hand the proliferation of KH-39, which did not increase cAMP contents in response to ECT, was not...
affected by ECT. ECT caused a dose-related increase of cAMP in KU-2 cells along with a dose-related inhibition of KU-2 growth. Moreover the addition of DBcAMP produced a significant inhibition of KU-2 growth. These results strongly suggest that in renal adenocarcinoma cells the growth inhibition by calcitonin is mediated by cellular cAMP increases.

The inhibitory effect of ECT on KU-2 growth was observed in both the lag and logarithmic phase. However, even when ECT was added to the medium daily the effect disappeared on the eighth culture day and the cell doubling time after the eighth day was the same as that of the cells cultured in a medium without ECT. After 2 weeks’ culture in ECT-supplemented medium KU-2 cells showed a significantly diminished cAMP increase in response to ECT stimulation even after subculture to multiwell plates and 5 days' culture. These phenomena support the speculation that the calcitonin-induced growth suppression of KU-2 cells are mediated by the increased cAMP production and might be explained as follows. As KU-2 is not a cloned cell line (1) it might be a mixture of high and low responders to ECT stimulation. The cAMP content may increase markedly in the high responders, however, and only slightly in the low responders. The increased intracellular cAMP is thought to inhibit cell proliferation. Therefore the growth of high responders may be strongly inhibited in the presence of ECT and the growth of low responders may also be inhibited by the large amount of cAMP produced by the high responders. Low responders may be able to proliferate even in a medium containing ECT if they are not mixed with high responders. After several days’ culture in the presence of ECT the population of high responders of KU-2 might have been remarkably diminished because the intracellular cAMP of high responders would have been extremely high and their growth strongly inhibited accordingly. If the population of high responders had decreased the cAMP production might have decreased and even in the ECT containing medium, low responders might have begun to grow at the same rate as in the medium without ECT.

However, these phenomena could also be explained as follows even if KU-2 were composed of homogeneous cells. If KU-2 cells alter in character from high to low responders in the presence of ECT growth suppression of KU-2 cells by ECT may be diminished. To investigate the precise developmental mechanism of resistance to calcitonin-induced growth suppression after 8 days' culture single cell cloning of KU-2 cells might be necessary. It is well known that cAMP inhibits the growth of cultured breast cancer cells (T47D, MCF7) (13, 14), adrenal cancer cells (15), lymphoma cells (16), and luteal cells (17). Calcitonin has also been reported to inhibit the proliferation of cultured cells. However, this inhibition was restricted to the cells which had specific calcitonin receptors and increased cAMP contents in response to calcitonin stimulation (14, 18, 19). The growth of T47D, a human breast cancer cell line, was definitely inhibited
by calcitonin and this effect was reported to be mediated by increased cAMP and the cAMP-dependent protein kinase, type II (18). Taken together our data strongly suggest that many human renal adenocarcinoma cells respond to calcitonin by increasing cAMP contents and that their growth could be suppressed by calcitonin-induced cAMP increase. It will be worthwhile to explore the possibilities of calcitonin action as a potent anticancer agent against renal adenocarcinomas.

REFERENCES

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