Modulation of the Production of a Parathyroid Hormone-like Protein in Human Squamous Carcinoma Cell Lines by Interaction with Fibroblasts

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ABSTRACT

Normal human keratinocytes as well as human squamous cell carcinomas produce a parathyroid hormone-like protein (PLP). However, PLP production by these cells is not a constant phenomenon. Since nothing is known about factors which regulate the production of PLP, in vitro studies were performed with normal keratinocytes and squamous carcinoma cell lines in order to establish conditions under which PLP production may vary.

PLP was measured as cyclic AMP production in parathyroid hormone target cells (osteoblasts) which could be inhibited by a parathyroid hormone antagonist. The presence of PLP was confirmed using a radio-immunoassay specific for PLP. Results from the bioassay correlated very well with the data obtained by radioimmunoassay for PLP.

The results confirm that human squamous carcinoma cells and normal keratinocytes produce PLP. PLP production appeared to be very sensitive to modulation of coculture of squamous carcinoma cells with fibroblasts. The effect of fibroblasts was not mediated by an effect on squamous carcinoma cell viability. Murine transformed fibroblasts (3T3 cells) as well as human normal foreskin fibroblasts were equally effective in inducing PLP production in these cells.

The fibroblastic factor was apparently present in a soluble form in the coculture system which prevented direct cell-cell contact but allowed communication through the medium. Nevertheless, conditioned medium from 3T3 cells failed to induce PLP production by squamous carcinoma cells. This suggests a more complicated interaction between the two cell types than a one way message from fibroblasts to keratinocytes.

Production of PLP by a number of squamous carcinoma cell lines was variable and not evidently correlated with the ability of these carcinoma cells to differentiate.

Production of parathyroid hormone-like protein not only is the expression of a disturbed metabolism of a specific cell type but also reflects the cell-cell interaction in tumor tissue.

INTRODUCTION

Humoral hypercalcemia of malignancy has been associated with the presence of a PLP which acts through the PTH receptor but is not recognized by PTH antisera (1, 2). PLP has been identified in squamous cell carcinomas, derived from various organs and in carcinomas of the kidney and bladder, and with much lower frequency in several other tumor types (1). Even normal epidermal keratinocytes have been shown to produce PLP (3). Analysis of the amino acid sequence of PLP from three different tumors associated with humoral hypercalcemia revealed that 8 from the first 13 amino acids in the amino terminal part of the protein were identical with PTH (4). Synthetic PLP[1–34] showed potent bioactivity parallel to that of PTH (5, 6). It could stimulate cAMP production and phosphate excretion in isolated rat kidneys (6), stimulate cAMP production in osteoblast-like cells, and induce bone resorption in vitro and in vivo (5, 6). PLP-induced cAMP generation in PTH target cells could be inhibited by PTH antagonists (1, 2). Neutralizing antisera to the PLP protein reduced the elevated serum calcium and urinary cAMP levels in two animal models of malignancy-associated hypercalcemia (7).

It is remarkable that only a fraction of squamous cell carcinomas causes hypercalcemia. Up to now nothing is known about conditions or factors that are involved in the initiation of PLP production by these tumor cells. The present study describes a mechanism controlling PLP production in human squamous carcinoma cell lines (SCC4 and SCC9) originating from carcinomas of the oral cavity (8). The results show that normal keratinocytes and SCCs produce PLP in significant amounts and that the production of PLP is modulated through interaction with fibroblasts. This may be important for the initiation of humoral hypercalcemia of malignancy and perhaps plays a role in physiological functions of PLP.

MATERIALS AND METHODS

Cell Culture. Normal keratinocytes and various squamous carcinoma cell lines (SCC4, SCC9, SCC13, SCC15, and SCC12F2), kindly provided by Dr. J. G. Rheinwald, were serially cultured using the Rheinwald-Green feeder layer technique (9). Epithelial cells (1.3 × 10^6/cm²) were grown together with lethally irradiated (3000 rads) 3T3 fibroblasts (1.9 × 10^5 cells/cm²) grown in a mixture of Dulbecco-Vogt modified Eagle’s and Ham’s F-12 medium (3:1), supplemented with 5% (v/v) FCS, penicillin (100 units/ml), streptomycin (100 µg/ml), and hydrocortisone (0.4 µg/ml) at 37°C in a humidified atmosphere containing 7.5% CO₂. Normal keratinocytes were cultured in medium of the same composition but enriched with epidermal growth factor (10 ng/ml). The cells were subcultured weekly.

In some experiments the SCCs were cultured in the absence of 3T3 cells or in the presence of 1.9 × 10^5/cm² lethally irradiated (3000 rads) human juvenile foreskin fibroblasts or in the presence of conditioned medium from 3T3 cell cultures.

In experiments in which direct contact of SCC and feeder cells was prevented, the SCCs were plated onto a porous membrane (Nucleopore; pore size, 0.4 µm; Transwell Costar) and were transferred after 4 h into a 6-well cluster dish on the bottom of which 3T3 cells had been previously plated. Both cell types were subsequently cultured together in the same medium for the next 12 days.

The conditioned medium was collected at 3-day intervals, centrifuged (10 min at 200 × g) to remove cells shed into the medium, and kept at −20°C. Fresh medium that had not been in contact with cells was used as control.

PLP Bioassay. Osteoblast-enriched cell populations were prepared from 20-day-old fetal rat calvaria by sequential collagenase digestion (10). Sixty thousand cells/well were seeded on a 24-well cluster plate and cultured for 4 days to confluence in a minimal essential medium supplemented with 10% (v/v) FCS. Cells are then stimulated for 5 min at 37°C with control medium (unconditioned medium in which the cells are grown) or undiluted squamous cell-conditioned media, eventually with additives (Table 2). All experiments, except one (Fig. 1), were performed without phosphodiesterase inhibitors. The assay was terminated by medium aspiration and subsequent extraction of cAMP with 90% ice-cold propanol. After lyophilization the extracts were dissolved in 12.5 mM sodium acetate. The cAMP content is determined...
with the protein-binding assay of Lust et al. (11). Bovine PTH[1–84] was obtained from Sigma Chemical Company, St. Louis, MO. Both the PTH antagonist [Nle8,Nle18,Tyr34]bPTH[1–34]amide and synthetic human PLP[1–34] were purchased from Peninsula Laboratories.

PLP Radioimmunoassay. The reagents used are commercially available from Immuno Technology Service Productions BV, Wychen, The Netherlands. In short, the method is based on an extraction method prior to radioimmunoassay.

The antibody was raised in sheep against PLP[1–40] coupled to bovine serum albumin. Iodination of Tyr-PLP[1–34] was performed using ProtagI25 (Baker Chemicals, Deventer, The Netherlands) as oxidizing agent. The tracer was purified on a reverse phase column by high pressure liquid chromatography.

The following procedure was adopted for routine measurements of PLP immunoreactivity. C14 cartridges were subsequently pretreated with 3 ml of methanol, 3 ml of distilled water, and 3 ml of 4% acetic acid. Prior to applying the sample to the cartridge, 1 ml of cell culture medium was acidified with 1 ml of 4% acetic acid. The cartridge was washed with 3 ml of distilled water. The PLP is eluted with 2 ml of 4% acetic acid in 86% ethanol. The eluate is evaporated under a stream of nitrogen at 37°C. When the volume of the eluate was reduced to about 0.5 ml the tubes were rinsed with 1 ml of ethanol 100% and evaporated to complete dryness. Prior to the RIA, the residue was dissolved in 1 ml of borate buffer (pH 8.4). For measurement of the PLP concentration, 200 µl of diluted extract or standard PLP[1–34] were added to 100 µl of antiserum in a polystyrene tube, mixed, and covered up with paraffin. After incubation for 18 h at 4°C, 100 µl of tracer were added to all tubes. A further incubation was carried out for 24 h at 4°C. Free and bound hormone were separated by pipetting 100 µl of second antibody solid phase suspension and incubated for 30 min at 20°C. After addition of 1 ml of distilled water, tubes were centrifuged and the supernatants were decanted. Pellets were counted for radioactivity. The lower limit of detection was 1 pg/tube. The 50% intercept was 23 pg/tube. When different amounts of PLP[1–34] were added to the culture medium the recovery was 95%. Intraassay and interassay coefficients of variation were 7.6 and 11.1%, respectively.

This RIA is specific for PLP, since no cross-reactivity was found for human PTH[1–38] (Bissendorf Peptide, West Germany) and bovine PTH[1–84] (Sigma), adrenocorticotropic hormone, calcitonin gene-related peptide I and II, and a panel of other hormones (all from Immuno Technology Service). Cross-reactivity with PLP[1–34] and PLP[1–40] was 100 and 105%, respectively.

In Vitro Bone Resorption Assay. Bone resorption was measured as the release of previously incorporated 45Ca from the radium and ulnae of 17-days-old fetal mice in cocultures of these bone explants with squamous carcinoma cells.

One day before explantation 30 µCi 45Ca was injected s.c. into the dam. The explants were isolated and subsequently precultured for 24 h for 45Ca exchange with the medium.

One day prior to coculture with the bones, the SCC4 cells were seeded in a 24-well cluster dish (Transwell; Costar), together with irradiated 3T3 cells.

Repeat experiments were performed in Dubelcco's modified Eagle's medium/Ham's F-12 medium supplemented with 10% heat-inactivated FCS, 20% O2, and 7.5% CO2. From day 1 onward the SCC4 cells and radii/ulnae were cocultured for 7 days in the same medium with medium refreshments at day 3 and 5.

Killing bone explants serves as a control for the physicochemical effects of changes in the culture medium (pH, e.g.) on 45Ca release. Killing is expected by 3 cycles of freeze-thawing. Osteoclastic resorption of the calcified matrix is measured as percentage of 45Ca released into the medium (12).

Statistics. The data given in Tables 1, 5, 6, and 7, and Fig. 1 are results of single experiments. In these experiments the conditioned medium of one culture dish or control medium was used to stimulate duplicate osteoblast cultures. cAMP content in each osteoblast culture was always measured in triplicate according to the binding assay of Lust et al. (11). The internal variation in the mean values of the cAMP production in the two osteoblast cultures was never larger than 10%. In Table 4 the data of several independent experiments are summarized and statistically evaluated using a paired Student's t test.

RESULTS

Kinetics of the Production of a cAMP-inducing Factor by SCC4 Cells. Medium from SCC4 cultures appears to contain a factor that stimulates the production of cAMP in osteoblast-enriched cell populations (Table 1). The degree of stimulation, expressed as treatment/control ratio, varied from 5 to 58.5 in various experiments. This variation can be ascribed to heterogeneity of the primary cultures of osteoblasts from fetal rat calvaria.

The degree to which this medium can stimulate cAMP production depended on the presence of fibroblasts and was maximal between day 3 and 6 of culture. The ability to induce adenylate cyclase activity did not disappear or reappear immediately after withdrawal or readdition of 3T3 cells to SCC4 cultures. For complete loss or regaining of this property the SCC4 cells had to be subcultured for at least two subsequent passages. This observation was reproducible in repeat experiments.

The presence of 3T3 cells did not influence the growth capacity of SCC4 cells, since the number of SCC4 cells at the end of the culture period (12 days) was 3.63 × 10^6/dish in the presence of 3T3 cells and 3.76 × 10^6/dish when SCC4 cells were cultured for four subsequent subcultures in their absence. This cAMP-inducing factor originated in the SCC4 cells since conditioned medium of 3T3 cells alone fails to stimulate cAMP production in osteoblasts. Concurrent changes in cell culture characteristics must be taken into account in the interpretation of the effect of duration of cell culture on the rate of production of this cAMP-inducing factor. The first 3 culture periods in this coculture coincide more or less with the lag, the exponential, and the plateau phase of the proliferation of SCC4 cells. During the course of the culture the number of irradiated 3T3 cells decreases gradually. In the last two periods 3T3 cells are virtually absent due to the expansion of SCC4 cells that leads to detachment of the feeder cells in their vicinity; the fibroblasts that are shed into the culture medium are discarded during the medium renewal. The second culture period (days 3–6) is characterized by an intermediate cell number, exponential growth of SCC4 cells, subconfluency, and close contact of SCC4 cells with 3T3.

In order to verify whether the increased PLP activity measured in conditioned medium of SCC4 cells grown in the presence of 3T3 cells corresponded to the production of a cAMP-inducing factor, dilution experiments were carried out in which the conditioned medium of SCC4 cells was added to 3T3 cells. The conditioned medium of SCC4 cells was diluted with conditioned medium of 3T3 cells to a ratio of 1:1. The coculture system was exposed to dilutions of conditioned medium of SCC4 cells that corresponded to 3:1 to 1:3. The addition of conditioned medium to 3T3 cells led to a decrease in cAMP production by 37% when a 1:1 dilution was used. When the same experiments were carried out using conditioned medium of 3T3 cells, cAMP production by 3T3 cells was not influenced by the addition of conditioned medium of SCC4 cells.
ence of 3T3 cells is due to increased PLP production, the intracellular PLP bioactivity (in cell homogenates) was determined as well. We found parallelism between the increase in extracellular (SCC4 - 3T3 = 35 pmol cAMP/well; SCC4 + 3T3 = 97 pmol cAMP/well) and intracellular (SCC4 - 3T3 = 15 pmol cAMP/well; SCC4 + 3T3 = 25 pmol cAMP/well) PLP bioactivity. This suggests that the enhanced PLP bioactivity in the presence of fibroblasts is most probably due to enhanced PLP production.

Influence of a PTH Antagonist on cAMP Induction. The competitive PTH antagonist [Nle8,Nle18,Tyr34]bPTH[3-34] amide markedly reduced the cAMP response to SCC4 conditioned medium and this suppression was dose related (Table 2).

cAMP Production in Rat Osteoblast-like Cells Induced by Synthetic hPLP[1-34]. Fig. 1A shows the dose-response curve of synthetic hPLP[1-34] on cAMP production in rat osteoblast-like cells in the presence or absence of the cAMP phosphodiesterase inhibitor IBMX. The values obtained with IBMX are as bioassayable PLP was about 4-5 times higher than in the absence of IBMX.

Fig. 1B shows the dose-response curves of serial dilutions of conditioned medium of SCC4 cells (day 6 of culture), in the absence and presence of 3T3 cells, on cAMP production in rat osteoblast-like cells in the presence of 0.5 mM IBMX. It is clear that both lines show parallelism with the hPLP[1-34] standard, also measured in the presence of 0.5 mM IBMX. The hPLP[1-34] standard curve was used to calculate the equivalent concentration of PLP activity present in the conditioned medium of SCC4 cells in the absence (1.2 × 10^{-9} M) and presence (5.0 × 10^{-9} M) of 3T3 cells.

Measurement of PLP by RIA and Bioassay. Since a RIA for PLP became available in the final stage of this investigation, it was used to confirm that the observed cAMP-inducing factor was indeed PLP.

Using this newly developed RIA significant amounts of PLP could be detected in the conditioned media of SCC4 cells (Table 3). To compare these two methods we measured PLP in SCC4-conditioned media co-cultured with and without irradiated fibroblasts for three culture periods. As shown in Table 3, the PLP values obtained by the bioassay, inferred from the dose-response curve of Fig. 1B, are 5-8 times higher than the values obtained by RIA. An explanation for this discrepancy might be that part of the molecular fragments of PLP bigger than 1-34 or even 34 can be detected in the conditioned media of SCC4 cells (Table 3). To compare these two methods we measured PLP in SCC4-conditioned media in the absence or presence of 0.5 mM IBMX and increasing concentrations of synthetic human PLP[1-34]. cAMP assay was performed as described in “Materials and Methods.” Fig. 1B, dose-response relationship between serial dilutions of conditioned medium of SCC4 cells, cultured for 6 days in the absence or presence of 3T3 cells, and cAMP production in rat osteoblast-like cells (in the presence of 0.5 mM IBMX). Conditioned medium was tested undiluted and in dilutions of, respectively, 1:1, 1:10, 1:50.

Table 2 Effect of a PTH antagonist (PTH-ant) on the stimulation of cAMP production in osteoblast-like cells by bovine PTH[1-84], synthetic human PLP[1-34], and conditioned media from SCC4 cells

<table>
<thead>
<tr>
<th>Media</th>
<th>Additives</th>
<th>cAMP (pmol/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean SEM</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>1.5 0.5</td>
</tr>
<tr>
<td>Bovine PTH[1-84]</td>
<td>4×10^{-9} M</td>
<td>14.4 0.3</td>
</tr>
<tr>
<td>Human PLP[1-34]</td>
<td>4×10^{-9} M</td>
<td>11.5 0.2</td>
</tr>
<tr>
<td>Human PLP[1-34]</td>
<td>3×10^{-10} M</td>
<td>30.7 3.1</td>
</tr>
<tr>
<td>cm-SCC4</td>
<td></td>
<td>20.0 0.4</td>
</tr>
<tr>
<td>cm-SCC4</td>
<td>4×10^{-9} M</td>
<td>10.6 0.5</td>
</tr>
<tr>
<td>cm-SCC4</td>
<td>2×10^{-10} M</td>
<td>7.7 0.4</td>
</tr>
<tr>
<td>cm-SCC4</td>
<td>1×10^{-11} M</td>
<td>4.0 0.2</td>
</tr>
</tbody>
</table>

* Significantly different from control condition (P < 0.01).

Fig. 1A. dose-response relationship between synthetic human PLP[1-34] [hPLP[1-34]] concentration and cAMP production in rat osteoblast-like cells in the absence or presence of 0.5 mM IBMX. For determination of a cAMP levels, confluent cell cultures were stimulated for 5 min at 37°C with control medium (medium to culture SCC4 cells) with or without 0.5 mM IBMX and increasing concentrations of synthetic human PLP[1-34]. cAMP assay was performed as described in “Materials and Methods.” Fig. 1B, dose-response relationship between serial dilutions of conditioned medium of SCC4 cells, cultured for 6 days in the absence or presence of 3T3 cells, and cAMP production in rat osteoblast-like cells (in the presence of 0.5 mM IBMX). Conditioned medium was tested undiluted and in dilutions of, respectively, 1:1, 1:10, 1:50.

Table 2 Effect of a PTH antagonist (PTH-ant) on the stimulation of cAMP production in osteoblast-like cells by bovine PTH[1-84], synthetic human PLP[1-34], and conditioned media from SCC4 cells

The squamous (carcinoma) cells produced no PTH, as was verified by a radioimmunoassay for PTH (data not shown).

Production of PLP by Normal Keratinocytes and a Number of SSC Cell Lines with Varying Differentiation Potential. To examine if fibroblast-inducible PLP production is a feature common to all epithelial cells, PLP production was measured in normal keratinocytes and in a number of other squamous carcinoma cells. Cell lines were chosen with a different ability to differentiate. This ability decreases in the following order: normal keratinocytes > SCC12F2 > SCC13 > SCC15 > SCC9 > SCC4 (8). Normal keratinocytes and the majority of the squamous carcinoma cells appeared to be able to produce PLP, but a lower extent than SCC4 cells (Table 4). The kinetics of PLP production by the other SCC cells, however, was quite different.
similar to that of the SCC4 cells. Under the culture conditions used here, the growth of normal keratinocytes, in contrast to that of the SCC cells, depends on the presence of a feeder layer; this makes it impossible to measure PLP production by normal keratinocytes in the absence of feeder cells.

Coculture of SCC4 Cells with Human Fibroblasts. Since 3T3 fibroblasts are transformed cells and originate from mice, we examined the effect of nontransformed human fibroblasts on PLP production by SCC4 cells. Human juvenile foreskin fibroblasts appear as capable to stimulate PLP production by SCC4 cells as are 3T3 cells (Table 5). The kinetics of PLP production was also comparable.

Table 4 Production of PLP by normal keratinocytes and a number of different squamous carcinoma cell lines in the presence or absence of 3T3 cells

<table>
<thead>
<tr>
<th>Cell lines (conditioned media)</th>
<th>3</th>
<th>6</th>
<th>9</th>
<th>12</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keratinocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCC4 +3T3</td>
<td>1.3 ± 0.2</td>
<td>3.9 ± 2.0</td>
<td>6.9 ± 1.3</td>
<td>4.3 ± 1.2</td>
<td>6.3 ± 3.2</td>
</tr>
<tr>
<td>+3T3</td>
<td>1.2 ± 0.2</td>
<td>4.2 ± 0.8</td>
<td>20.1 ± 4.6</td>
<td>14.1 ± 4.1</td>
<td>7.7 ± 1.9</td>
</tr>
<tr>
<td>-3T3</td>
<td>1.8 ± 0.5</td>
<td>1.1 ± 0.3</td>
<td>3.7 ± 2.0</td>
<td>4.0 ± 1.7</td>
<td>ND</td>
</tr>
<tr>
<td>SCC9 +3T3</td>
<td>1.4 ± 0.3</td>
<td>1.4 ± 0.4</td>
<td>5.9 ± 2.4</td>
<td>3.1 ± 1.0</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>-3T3</td>
<td>1.8 ± 0.5</td>
<td>1.1 ± 0.3</td>
<td>3.7 ± 2.0</td>
<td>4.0 ± 1.7</td>
<td>ND</td>
</tr>
<tr>
<td>SCC15 +3T3</td>
<td>1.3 ± 0.9</td>
<td>2.4 ± 0.9</td>
<td>3.3 ± 0.4</td>
<td>2.8 ± 0.5</td>
<td>4.0 ± 1.4</td>
</tr>
<tr>
<td>-3T3</td>
<td>1.6 ± 0.6</td>
<td>2.6 ± 1.6</td>
<td>3.0 ± 1.0</td>
<td>4.1 ± 3.5</td>
<td>28 ± 1.8</td>
</tr>
<tr>
<td>SCC12F2 +3T3</td>
<td>1.3 ± 0.2</td>
<td>2.2 ± 0.8</td>
<td>3.3 ± 0.5</td>
<td>2.9 ± 0.6</td>
<td>2.6 ± 1.2</td>
</tr>
</tbody>
</table>

* Values of CAMP production in osteoblasts, expressed as pmol CAMP/well ± SEM, from multiple experiments. n = number of experiments.

CAMP induction by SCC4 cells cocultured with human foreskin fibroblasts (HFF)

<table>
<thead>
<tr>
<th>SCC4 previous cultures*</th>
<th>SCC4 coculture</th>
<th>cAMP productiona on following day of medium harvest</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 6 9 12</td>
<td></td>
</tr>
<tr>
<td>+3T3</td>
<td>1.2 ± 0.2</td>
<td>15.5 ± 8.9</td>
</tr>
<tr>
<td>-3T3</td>
<td>1.1 ± 0.0</td>
<td>11.0 ± 8.6</td>
</tr>
<tr>
<td>+HFF</td>
<td>1.4 ± 0.2</td>
<td>1.3 ± 2.3</td>
</tr>
<tr>
<td>-HFF</td>
<td>1.1 ± 0.2</td>
<td>11.0 ± 8.6</td>
</tr>
</tbody>
</table>

* Prior to the experiments the SCC4 cells were serially cultured either in the presence (+3T3) of absence (-3T3) of the feeder layer.

** a CAMP production in osteoblasts is expressed as the mean ratio (treatment/control ratio) of cAMP production obtained with SCC4 conditioned medium and control medium as described in "Materials and Methods."
well as disappearance of fibroblasts from the culture system due to crowding out by the expanding SCC4 cells. Under these culture conditions the two different cell types can communicate only through the culture medium. This type of interaction appears sufficient for induction of PLP production (Table 7). The kinetics of PLP production in the present experiment is different from all the others (cf. Table 1). PLP production reaches a maximum at later stages of cell culture. This can be explained by the much larger physical distance between the two cell types in this culture. The constant presence of fibroblasts, on the other hand, may explain why the rate of production remains constant after reaching the maximum.

Coculturing these cells while avoiding cell-cell contact has an additional advantage. It allows experiments with nonirradiated instead of irradiated fibroblasts. Previous irradiation of fibroblasts did not influence PLP production by SCC4 cells (data not shown).

Ability of SCC4 Cells to Stimulate Bone Resorption. SCC4 cells appear to be able to stimulate 45Ca release in PTH-sensitive long bone explants (Table 8). Comparison with killed controls showed that this enhanced 45Ca release was due to a cellular mechanism and not caused by physicochemical effects. 3T3 cells were not able to affect 45Ca release. Although SCC4 cells were able to stimulate 45Ca release significantly, this effect was not as large as one would expect from its potency to induce cAMP when both actions are compared with those of PTH. A similar discrepancy has been found in studies with synthetic human PLP[1–34].

**DISCUSSION**

Some tumors, and in particular squamous carcinomas, produce a protein having partial homology with PTH and acting through the PTH receptor of PTH target cells (1). The present study confirmed that human squamous carcinoma cell lines and normal keratinocytes produce a PTH-like protein (1, 3). Conditioned medium from SCC and normal keratinocyte cultures contained a factor that induced cAMP production in PTH target cells (osteoblasts). The cAMP production induced by SCC4-conditioned media was inhibited by a PTH antagonist in a dose-related manner. The dose-response curves of serial dilutions of conditioned medium of SCC4 cells in the absence and presence of 3T3 on cAMP production in osteoblasts was exactly parallel with the synthetic human PLP[1–34] standard curve indicating that indeed only PLP is measured. SCC4 cell products stimulated 45Ca release in long bone explants. Finally, we could confirm the presence of PLP in the SCC media by a radioimmunoassay for PLP.

The key finding of our investigation is that the PLP production by squamous carcinoma cells is modulated by fibroblasts. The finding that there is a parallelism between the increase in intracellular and extracellular PLP bioactivity suggests that the enhanced PLP bioactivity in the conditioned media is a result of fibroblast-induced increase of PLP production. However, to confirm this, the modulation at mRNA level should be investigated. Since the epithelial cells used in this study are routinely cultured in the presence of a feeder cell layer, one might suggest that suboptimal growth of SCC cells in the absence of fibroblasts might be responsible for the reduced PLP production in this condition. This is, however, not true. SCC4 cells do not require fibroblasts for clonal growth (14). This is again confirmed in the present study by the absence of any effect of feeder cells on the SCC4 cell number at the end of the culture period. The only reason for routine use of a feeder layer for SCC4 cells is to make culture conditions comparable to those of other SCC cells and normal keratinocytes which occasionally do require feeder cells (14). 3T3 cells have no effect on the response of SCC4 cells to a number of extracellular stimuli (15). In those cell lines which require a feeder layer for optimal growth, optimal growth is also obtained when one uses 3T3-conditioned medium instead of a feeder layer (14). However, 3T3-conditioned medium does not induce enhanced PLP production in SCC4 cells. The effect of fibroblasts on PLP production is therefore genuine.

In addition to the observation that fibroblasts have a large influence on PLP production, there is also evidence that this action is achieved through a soluble factor since direct cell-cell contact is not required for the induction of PLP. It is remarkable, however, that direct addition of conditioned medium from 3T3 cells fails to affect PLP generation in SCC4 cells. Apparently, it involves more than a one way message from fibroblasts to keratinocytes and is an expression of cell-cell interaction. A message of SCC cells to the fibroblasts, which is absent when one uses conditioned medium from 3T3 cells only, is probably also involved. The existence of a labile factor secreted into the medium by fibroblasts, however, cannot be excluded entirely.

PLP production by SCC cells did not occur only in the presence of murine transformed fibroblasts (3T) but also with nonirradiated, not transformed fibroblasts of human origin. This emphasizes the general nature of this interaction and may turn out to be relevant for some until now unknown physiological functions of PLP.

The evidence suggests that PLP production in some SCC cells can be induced by until now unidentified paracrine factors originating from fibroblasts, implying the existence of a specific relation between mesenchymal and epithelial cells. The observation that PLP production, during subsequent passages of SCC4 cells in the absence or presence of fibroblasts, was lost.
or regained only gradually suggests that the fibroblastic factor(s) do(es) not primarily modulate PLP secretion but rather that it may promote synthesis of PLP. Mesenchymal-epithelial interactions are well known phenomena during embryonic development, when soluble factors of mesenchymal origin regulate epithelial proliferation and differentiation and organogenesis (16). The nature of such mesenchymal factors is still unknown. It has been suggested that growth factors are involved (17).

Many epithelial tumors are characterized by aggressive and invasive behavior toward their environment. This is associated with interaction with neighbor cells and with extracellular proteolysis, including interaction of squamous carcinoma cells with fibroblasts, which might deliver the stimulus for enhanced production of PLP. It is at present not clear why some SCCs are associated with humoral hypercalcemia and others not; neither can one predict what event triggers hypercalcemia. Hypercalcemia will often appear suddenly after the prolonged existence of a malignancy. In the present study, the ability to produce PLP was not uniform in all SCC lines, nor was PLP production associated with differentiation characteristics. Whatever the details of the mechanism, production of PLP by squamous cell carcinomas is probably not merely the result of a disturbed cell metabolism but expresses also the disturbance of cell-cell interaction in the tumor tissue.

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Modulation of the Production of a Parathyroid Hormone-like Protein in Human Squamous Carcinoma Cell Lines by Interaction with Fibroblasts


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