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The Role of Interleukin-6 in the Induction of Hypercalcemia in Renal Cell Carcinoma Transplanted into Nude Mice

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ABSTRACT

Hypercalcemia is a well known complication of renal cell carcinoma (RCC). As RCCs can produce IL-6, and IL-6 may stimulate bone resorption and cause mild hypercalcemia, we examined whether IL-6 is involved in renal cancer-associated hypercalcemia in vivo. Three human renal cell carcinoma tumor lines (RC-8, RC-9, and NC-65) growing in nude mice were studied. Tumors were implanted s.c., and parameters of bone metabolism and serum human IL-6 levels were determined in relation to tumor volume (TV). All three tumor lines secreted human IL-6, although in different quantities. The maximum level of IL-6 in RC-8 was 434 pg/ml (TV, 200 mm³), that in RC-9 was 69 pg/ml (TV, 1800 mm³), and that in NC-65 was 2368 pg/ml (TV, 1800 mm³). Hypercalcemia developed in RC-8 and RC-9 tumor-bearing animals, but not in NC-65-bearing animals. The hypercalcemia in both RC-8 and RC-9 tumor lines was associated with elevated levels of PTH-related peptide (PTHrP) and loss of trabecular bone volume.

Serum calcium and phosphate concentrations showed an almost linear relationship with plasma PTHrP independently of the tumor line and serum IL-6 levels. No hypercalcemia occurred in the NC-65 animals, which had the highest levels of IL-6, but no detectable plasma PTHrP and PTHrP messenger RNA expression in the tumor. Administration of neutralizing antibodies to IL-6 to RC-8 animals normalized serum calcium concentrations and PTHrP values and induced a significant inhibition of tumor growth. No such effect on tumor growth of anti-IL-6 was seen in the other two tumor lines. We conclude that IL-6 secreted by RCC does not contribute directly to hypercalcemia, but may enhance hypercalcemia by stimulating the tumor growth of a subpopulation of PTHrP-secreting carcinomas. (Endocrinology 138: 1879–1885, 1997)

CANCER-ASSOCIATED hypercalcemia is primarily the result of increased bone resorption by humoral factors secreted by the tumors (1). Hypercalcemia is a well known paraneoplastic syndrome in renal cell carcinoma (RCC). PTH-related peptide (PTHrP) is recognized as the major humoral factor responsible for the induction of hypercalcemia, but other factors acting on bone may also contribute to the increase in serum calcium concentrations (2). One such factor is interleukin-6 (IL-6) (3–5). Renal cell carcinomas have been reported to produce IL-6 in vitro (6) and to express IL-6 messenger RNA (mRNA) and IL-6 receptor mRNA in vivo (7, 8). It is not clear, however, whether IL-6 secreted by the tumor has any biological effect. It is possible that IL-6 may act synergistically with PTHrP to cause hypercalcemia in RCC, as has been described in a patient with pheochromocytoma (9) and in nude mice inoculated with Chinese hamster ovarian cells transfected with complementary DNAs (cDNAs) for PTHrP and IL-6 (10). In an earlier study we reported cosecretion of IL-6 and PTHrP by a renal cell carcinoma that caused hypercalcemia after implantation into nude mice (11). After administration of neutralizing antibodies to IL-6, serum calcium concentrations fell to almost normal, suggesting a contributing role of IL-6 to the development of hypercalcemia in this tumor.

To examine further the role of IL-6 in hypercalcemia and its interaction with PTHrP, we studied tumor parameters, biochemical and histological indexes of calcium and bone metabolism, and secretion of human IL-6 (hIL-6) and PTHrP in nude mice inoculated with three different renal cell carcinoma tumor lines, two of which induced hypercalcemia.

Materials and Methods

Tumor lines

The three tumor lines, RC-8, RC-9, and NC-65, have all been derived from patients with renal cell carcinomas in the advanced stage and have been maintained in nude mice (12–15). During subsequent passages, no alterations occurred in the growth kinetics, histology, or immunohisto-chemistry of the tumors. The donor patient of tumor line RC-8 had hypercalcemia before operation; no information was available about the serum calcium concentrations of the other two patients.

Animals

Four- to 6-week-old Swiss nu/nu mice were housed in a laminar flow cage rack and were maintained at controlled temperature (25°C) and humidity (60%). Acidified water was given ad libitum, and (irradiated) standard rodent chow RMH-GS (Hope Farms, Bodegraven, The Netherlands) containing 0.84% calcium was provided.

Study protocol

Selected tumor pieces (2 × 2 × 2 mm³) were sc inserted unilaterally into the shoulder region of ether-anesthetized mice. Animals with a
tumor volume (TV) of 100 mm³ were selected and divided into groups of five mice. TV was assessed by measuring the two major diameters by the formula: TV = π/6(d₁ × d₂)². At different TVs, predefined on the basis of pilot experiments, animal weight was recorded, and blood was taken for the determination of biochemical parameters of calcium metabolism, PTHrP and IL-6. The blood of all animals per TV of each tumor line was pooled because that obtained from individual mice was not sufficient for the simultaneous measurement of all metabolic parameters; this was a problem, particularly in the hypercalcemic cachectic mice. Each group of animals was subsequently killed, femurs were excised for bone histomorphometry, and the tumor was removed for sample analysis. At the indicated tumor volumes, mice were killed, and the parameters were determined.

The data presented for Ca, P, PTHrP, and IL-6 indicate the values obtained from pooled blood of two to five animals (indicated by n). Values represent the mean ± SD; in groups of two animals, both values are reported.

a Two thirds of the bone samples examined.

b Two fifths of the bone samples examined.

Biochemical determinations

Serum calcium and phosphate concentrations were determined by automated analyzer techniques. Serum IL-6 was measured by an enzyme-linked immunosorbent assay specific for hIL-6 (Medgenix, Amersfoort, The Netherlands). Mouse IL-6 did not cross-react with the enzyme-linked immunosorbent assay specific for hIL-6 (Medgenix, Amsterdam, The Netherlands). As a positive control for PTHrP and IL-6, total RNAs from the squamous cell carcinoma cell line SCC-4 and from IL-6-stimulated human fibroblasts (18) were used. The blots were washed with 2 × SSC (0.30 M NaCl and 0.01 M sodium citrate) and 1% SDS for 60 min at 60°C.

RT-PCR

Semiquantitative RT-PCR was performed in a single reaction tube. In this experimental set-up, the RT reaction and the subsequent PCR were performed in a single 0.5-ml reaction tube. RNA was linearized by grinding (1 min; Polyclon, Kinematica, Kriens/Luzern, Switzerland) in ice-cold lithium chloride (3.3 M)-ureum (6.6 M). After overnight incubation at −20°C, the homogenate was centrifuged at 10,000 × g for 30 min at 4°C, and the pellet was resuspended in 10 mM Tris-0.5% SDS, pH 7.7. Total RNA was extracted with three cycles of phenol-chloroform-isooamyl alcohol (25:24:1) and subsequently precipitated at −20°C in 70% ethanol-0.1 M sodium acetate, pH 5.2. The pellet was resuspended in 10 mM Tris-1 mM EDTA, pH 7.6. Total RNA was quantified by spectrophotometry at 260 nm. RNA samples were analyzed using electrophoresis on a 1% denatured agarose gel containing 7.5% formaldehyde and transferred to a nylon membrane (Hybond N, Amersham, Aylesbury, UK). The membranes were hybridized with 32P-labeled probes specific for PTHrP (kindly provided by M. Karperien, Hubrecht Laboratories, Utrecht, The Netherlands) and hIL-6 cDNA (an 819-bp hIL-6 probe prepared using the reverse transcriptase-PCR (RT-PCR) technique according to the method of Kaasbock et al. (17), kindly provided by R. A. de Paus, Department of Hematology, University Hospital Leiden, The Netherlands) at 60°C in 7% SDS, 0.5 M NaHPO4 (pH 7.2), and 10 mM EDTA. 28S ribosomal RNA (rRNA) was used as an internal control (kindly provided by Dr. C. Backendorf, Gorlaeus Laboratory, Leiden, The Netherlands). As a positive control for PTHrP and IL-6, total RNAs from the squamous cell carcinoma cell line SCC-4 and from IL-6-stimulated human fibroblasts (18) were used. The blots were washed with 2 × SSC (0.30 M NaCl and 0.01 M sodium citrate) and 1% SDS for 60 min at 60°C.

TABLE 1. Characteristics and biochemical parameters in nude mice implanted with three different renal cell carcinoma tumor lines

<table>
<thead>
<tr>
<th>Tumor vol (mm³)</th>
<th>BW (g)</th>
<th>Serum Ca (mmol/liter)</th>
<th>Serum phosphate (mmol/liter)</th>
<th>PTHrP (pmol/liter)</th>
<th>IL-6 (pg/ml)</th>
<th>BV/TV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC-8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 2</td>
<td>128/130</td>
<td>20.5/22.6</td>
<td>3.8</td>
<td>4.4</td>
<td>&lt;5</td>
<td>8.6/6.0</td>
</tr>
<tr>
<td>n = 3</td>
<td>215 ± 21.7</td>
<td>17.4 ± 3.1</td>
<td>4.8</td>
<td>11</td>
<td>434</td>
<td>3.8 ± 2.2</td>
</tr>
<tr>
<td>n = 4</td>
<td>469 ± 31.3</td>
<td>18.5 ± 1.0</td>
<td>6.2</td>
<td></td>
<td>415</td>
<td>3.0 ± 0.8</td>
</tr>
<tr>
<td>RC-9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 5</td>
<td>120 ± 26.8</td>
<td>21.5 ± 1.3</td>
<td>2.9</td>
<td>2.9</td>
<td>&lt;5</td>
<td>8.8 ± 2.2</td>
</tr>
<tr>
<td>n = 4</td>
<td>463 ± 55.5</td>
<td>20.5 ± 2.4</td>
<td>4.7</td>
<td>6.7</td>
<td>23</td>
<td>5.0 ± 1.9</td>
</tr>
<tr>
<td>n = 5</td>
<td>664 ± 44.5</td>
<td>21.1 ± 2.5</td>
<td>5.9</td>
<td></td>
<td>67</td>
<td>5.0 ± 0.7</td>
</tr>
<tr>
<td>n = 2</td>
<td>1827/1838</td>
<td>15.3/16.8</td>
<td>7.2</td>
<td></td>
<td>80</td>
<td>3.5/2.1</td>
</tr>
<tr>
<td>NC-65</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 5</td>
<td>122 ± 33.8</td>
<td>21.9 ± 1.5</td>
<td>2.8</td>
<td>3.0</td>
<td>&lt;1</td>
<td>421</td>
</tr>
<tr>
<td>n = 5</td>
<td>455 ± 54.5</td>
<td>22.5 ± 0.6</td>
<td>2.8</td>
<td>2.8</td>
<td>&lt;1</td>
<td>2060</td>
</tr>
<tr>
<td>n = 3</td>
<td>916 ± 50.2</td>
<td>22.6 ± 2.5</td>
<td>2.7</td>
<td>2.6</td>
<td>&lt;1</td>
<td>2368</td>
</tr>
<tr>
<td>n = 5</td>
<td>1855 ± 116.1</td>
<td>27.5 ± 3.8</td>
<td>2.6</td>
<td></td>
<td>2356</td>
<td>4.8/10.5</td>
</tr>
</tbody>
</table>

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Nikon microphot FXA microscope (Melville, NY) and an MX5 CCD camera (Adimex Image Systems BV, Eindhoven, The Netherlands). All measurements were made at a distance of approximately 0.5–1.0 mm from the epiphyseal plate.

**Statistical analysis**

Tumor growth rates, analyzed per tumor, were calculated from the slopes obtained by linear regression analysis. In tests for differences between the various groups, series of one-way ANOVAs with equal and unequal sizes were used. When significant ($P < 0.05$) differences were found by ANOVA, Duncan’s multiple range test (20) or, in the case of an unequal number of replications, Duncan’s multiple range test adjusted by Kramer (21) was performed ($P = 0.05$). In cases of two conditions, differences were analyzed by two-sided Student’s $t$ test.

**Results**

**Growth characteristics**

Tumors were developed in nude mice after sc implantation of all three tumor lines. The RC-8 tumors displayed the lowest growth rate and reached a much lower TV due to death of the animals at TVs between 400–500 mm$^3$ associated with extreme cachexia. In animals implanted with RC-9 tumors, there was also a significant loss of weight, which, however, occurred at a much later stage and at higher TVs (1000 mm$^3$). NC-65 tumors could be maintained in the animals without any weight loss until a very high tumor load was achieved (2000 mm$^3$; Table 1).

**Hypercalcemia**

After transplantation, two of the three tumor lines, RC-8 and RC-9, induced severe hypercalcemia (to a maximum of 6.24 and 7.22 mmol/liter, respectively) and hypophosphatemia (to a minimum of 1.55 and 1.37 mmol/liter, respectively; Table 1). The degree of hypercalcemia and hypophosphatemia was similar in animals bearing these two tumors; there was, however, a pronounced difference in the TV associated with these levels (469 ± 31.3 mm$^3$ in RC-8 versus 1827 and 1838 mm$^3$ in RC-9). Generally, the RC-8 tumor induced hypercalcemia of a degree comparable to that of RC-9 at approximately half the TV. Hypercalcemia and hypophosphatemia did not occur in NC-65 tumor-bearing animals up to TVs of 1855 ± 116 mm$^3$.

**Serum levels of IL-6**

After transplantation into nude mice, all three tumor lines produced hIL-6 (Table 1). Serum IL-6 levels increased initially with tumor growth in RC-8 and NC-65 tumor-bearing animals, reaching a plateau, whereas there was a small increase in RC-9-bearing animals. There were wide variations among the different tumor lines. The NC-65 tumor line produced the highest amounts of IL-6 (maximum, 2368 pg/ml), and RC-9 produced the lowest amounts (maximum, 80 pg/ml), whereas the IL-6 levels in RC-8 were intermediate (maximum, 434 pg/ml). There was no relation between serum IL-6 levels and serum calcium concentrations.

**Plasma PTHrP levels**

Both RC-8 and RC-9 induced comparable rises in plasma PTHrP to maximums of 20.1 and 22.1 pmol/liter, respectively. In contrast, plasma PTHrP remained undetectable in...
No such relation was found for serum IL-6 values. However, plasma PTHrP concentrations were significantly decreased serum calcium concentrations. These differences were observed with shorter exposure times of the films. The decrease in serum calcium concentrations induced by anti-IL-6 treatment was associated with undetectable levels of PTHrP; this was 3.4 pmol/liter in the vehicle-treated animals. There was no change in expression of IL-6 mRNA or PTHrP mRNA with treatment, but anti-IL-6 treatment significantly decreased the rate of growth of RC-8 tumors from 27 ± 2 to 15 ± 2 mm³/day (P < 0.001; Table 2). In this set of experiments, no hypercalcemia developed in mice implanted with RC-9 tumors with or without anti-IL-6 despite TV up to 500 mm³. Also, no circulating plasma PTHrP was detectable. Treatment had no effect on the rate of growth of RC-9 tumors. Anti-IL-6 treatment did not influence the growth of NC-65 tumors.

Discussion

Previous studies have suggested that IL-6 may contribute to the development of malignancy-associated hypercalcemia, but its precise role has not been determined (23). Because hypercalcemia is a known complication of renal cell carcinomas that have also been shown to produce IL-6 (6–8), in the present study we examined the role of IL-6 in the induction of hypercalcemia in nude mice implanted with different human renal cell carcinoma tumor lines.

After implantation, all three tumors investigated produced significant amounts of IL-6. However, hypercalcemia developed only in the mice implanted with the RC-8 and RC-9 tumors, and it was associated with marked decreases in BV/TV. These findings suggest that under these conditions, increased bone resorption was the primary mechanism for the increase in serum calcium concentrations. In contrast, in the NC-65 tumor-bearing animals with the highest levels of circulating IL-6, both serum calcium concentrations and BV/TV did not change. These results strongly suggest that in renal cell carcinomas, IL-6 alone is not a hypercalcemic factor and does not stimulate bone resorption. This is in agreement with a recent study showing that IL-6 is not a powerful bone-resorbing factor in mice in vivo, but may act synergistically with other humoral factors in the induction of hypercalcemia (10). PTHrP has been established as a major humoral factor in malignancy-associated hypercalcemia, particularly that accompanied by solid tumors (1). Increased levels of IL-6 and PTHrP have been found in hypercalcemic animals and humans, and De La Mata et al. recently suggested that IL-6 may potentiate the action of PTHrP on bone resorption (10). These investigators reported in studies with nude mice inoculated with Chinese hamster ovarian cells transfected with cDNAs for IL-6 and PTHrP that IL-6 in-
creases the pool of early osteoclast precursors that can further differentiate to mature resorbing osteoclasts by the action of PTHrP. This is in line with earlier observations of our group of the decreased effectiveness of PTHrP on osteoclastic resorption in fetal bone explants from IL-6-deficient mice, which could be restored with IL-6 treatment (24). In the present study the animals that developed hypercalcemia also produced high amounts of PTHrP, and there was an almost linear relationship between serum calcium and phosphate concentrations and plasma PTHrP levels. This relationship was independent of the prevailing IL-6 levels, and for a given PTHrP value, mice with relatively low or high IL-6 levels showed similar increments in serum calcium concentrations.

This suggests that in renal cell carcinomas, PTHrP is the major hypercalcemic factor, and IL-6 does not appear to have any modulatory action, a conclusion in line with a recent study of the expression of IL-6 and PTHrP mRNAs in tumors from patients with renal cell carcinomas (25). In this human study we found that all renal cell carcinomas expressed IL-6 mRNA, but hypercalcemia developed only in a patient who showed simultaneous expression of IL-6 and PTHrP mRNA. This contrasts with the finding in squamous cell carcinomas, where all tumors expressed PTHrP mRNA, but not every patient became hypercalcemic. We obtained similar results in nude mice implanted with squamous cell carcinomas (unpublished observations). The different behavior of renal cell carcinomas was thus confirmed here by showing a close relation between PTHrP mRNA expression and circulating levels of PTHrP, which were independent of the expression or the circulating values of IL-6. A synergistic effect of the two factors cannot, however, be totally ruled out, as the concentration of IL-6 required to trigger an increase in the osteoclast precursor pool in vivo is not yet known. If this is low, then the present results could be consistent with a synergistic effect of IL-6 and PTHrP in the induction of bone resorption and consequently of hypercalcemia. We did not have a renal carcinoma cell line producing only PTHrP to test
this possibility, but the studies of treatment of mice with an antibody to IL-6 have been helpful in this respect.

In the present and in a previous study (11) we found that treatment of mice implanted with the RC-8 tumor with an antibody against hIL-6 significantly reduced the rate of growth of the tumor and prevented the increase in serum calcium concentrations. In addition, this treatment prevented any rise in circulating PTHrP, which remained undetectable. We have no good explanation for the lack of an antitumor effect of anti-IL-6 in the other tumors. The dose of antibody used has been found to effectively neutralize values of circulating hIL-6 similar to those obtained in our experiment (Dr. L. Aarden, personal communication). Furthermore, the immunoassayable IL-6 in the mice of the present study was active, as confirmed by measurement of some samples in the mouse hydridoma B9 bioassy for IL-6 (own unpublished observations).

IL-6 has been previously recognized as an autocrine growth factor in some renal cell carcinomas (26). It may, therefore, be that in these carcinomas a synergism between IL-6 and PTHrP in the induction of hypercalcemia does not occur at the level of osteoclastogenesis, but is, rather, the result of the stimulating action of IL-6 on tumor growth. In cancer cells that have the capacity to produce PTHrP, IL-6, by stimulating their growth, enhances PTHrP production, which, in turn, stimulates bone resorption and induces hypercalcemia. Treatment with an antibody against IL-6 disrupts this sequence of events and prevents the increase in serum calcium concentrations. These observations in mice may also have practical clinical implications. Patients with renal cell carcinomas and PTHrP-induced hypercalcemia initially respond favorably to treatment with antiresorptive agents, such as the bisphosphonates. Hypercalcemia, however, recurs and then it is difficult to control, probably because of the increased renal tubular reabsorption of calcium induced by PTHrP, an action that cannot be blocked by antiresorptive agents. In this setting, anti-IL-6 antibodies may theoretically offer a better therapeutic alternative. However, this possibility needs to be tested in humans in vivo.

An interesting additional observation in our studies was that the mice implanted with NC-65 tumors and having the highest levels of IL-6 did not develop cachexia. In contrast, in the other mice there was a significant weight loss. IL-6 has been implicated in the cachexia of malignant tumors, either alone or in association with other cytokines, such as IL-1 and tumor necrosis factor (27). The results of the present study strongly suggest that at least in renal cell carcinomas, IL-6 alone cannot be considered as a factor inducing cachexia, and further studies in this direction are warranted.

Our findings, therefore, demonstrate that IL-6 is not directly involved in the induction of hypercalcemia in renal cell carcinomas and that PTHrP is the main humoral factor responsible for this effect, primarily through stimulation of bone resorption. IL-6 may, however, play an important role by stimulating the growth of renal tumors that have the capacity to produce PTHrP.

### References


### Table 2.

Nude mice implanted with RC-8, RC-9, and NC-65 tumor received saline solution (controls) or anti-IL-6 as a single ip injection at a tumor volume of 100 mm³

<table>
<thead>
<tr>
<th>n</th>
<th>Tumor growth rate (mm³/day)</th>
<th>Serum calcium (mmol/liter)</th>
<th>Serum phosphate (mmol/liter)</th>
<th>PTHrP (pmol/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC-8</td>
<td>7</td>
<td>27 ± 2</td>
<td>4.2 ± 0.3</td>
<td>3.4</td>
</tr>
<tr>
<td>RC-8, anti-IL-6</td>
<td>7</td>
<td>15 ± 2*</td>
<td>2.8 ± 0.4*</td>
<td>&lt;1</td>
</tr>
<tr>
<td>RC-9</td>
<td>6</td>
<td>50 ± 3</td>
<td>2.9 ± 0.5</td>
<td>&lt;1</td>
</tr>
<tr>
<td>RC-9, anti-IL-6</td>
<td>9</td>
<td>44 ± 3</td>
<td>3.0 ± 0.7</td>
<td>&lt;1</td>
</tr>
<tr>
<td>NC-65</td>
<td>5</td>
<td>55 ± 9</td>
<td>2.5 ± 0.3</td>
<td>&lt;1</td>
</tr>
<tr>
<td>NC-65, anti-IL-6</td>
<td>5</td>
<td>66 ± 12</td>
<td>2.7 ± 0.2</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

* Significantly different from controls (P < 0.001).

After a period of 11 days, mice were killed, tumor volume was measured, and biochemical parameters were assessed. n, Number of animals per group. Values represent the mean ± sd, with the exception of PTHrP that was measured in pooled plasma.