Antibodies to Parathyroid Hormone-related Protein Lower Serum Calcium in Athymic Mouse Models of Malignancy-associated Hypercalcemia Due to Human Tumors

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Abstract

A parathyroid hormone-related protein (PTHrP) has recently been isolated from tumors associated with hypercalcemia. In the present study, we tested the effects of neutralizing antisera to the PTHrP on serum calcium and urine cAMP in two animal models of malignancy-associated hypercalcemia. The animal models consisted of (*a*) a human squamous cell lung cancer and (*b*) a human laryngeal cancer, both serially carried in athymic mice. The antisera specifically reduced the elevated serum calcium and urinary cAMP levels in the tumor-bearing animals. We conclude that PTHrP plays a major role in the pathogenesis of malignancy-associated hypercalcemia.

Introduction

Hypercalcemia occurs in 10–20% of all patients with cancer. It has been postulated that humoral factors secreted by the tumors are responsible for the hypercalcemia in a large number of these patients (1, 2). Factors that have been implicated in the pathogenesis of this syndrome are PTH-like factors, transforming growth factor alpha (TGFα),[1] and cytokines (2). It has been suggested that PTH-like factors may be responsible for the renal manifestations (elevated urinary cAMP, phosphaturia, relative hypocalciuria) and TGFα-like factors or cytokines for the skeletal manifestations (increased bone resorption and decreased bone formation), or that the two might work in concert (2). Recently a PTH-related protein (PTHrP) has been isolated from several tumors associated with the hypercalcemia syndrome (3–6). Cloning of cDNA has allowed prediction of the sequence of this protein (7, 8) and biologically active amino-terminal peptides have been synthesized (9). The PTH-like biological activity of this protein resides in the first 34 amino acids (9, 10). In the assay of adenylate-cyclase response in clonal osteogenic sarcoma cells, purified PTHrP and PTHrP (1–34) were more potent than bovine or human PTH (1–34) (9, 11). In the bone resorption assay the peptide has been shown to be either less potent (9) or as potent as bovine PTH (12–14). Horiuchi et al. (11) demonstrated that when infused in vivo into thyroparathyroidectomized rats, PTHrP (1–34) caused hypercalcemia, hypophosphatemia, and increased urinary cAMP and serum 1,25-dihydroxyvitamin D_3 levels. Similar in vivo hypercalcemic effects have been observed in intact animals (13, 14). Although suggestive, these studies do not establish a role for endogenous PTHrP in the pathogenesis of malignancy-associated hypercalcemia. In the present work, we evaluated the effect of two neutralizing antisera against PTHrP on serum calcium and urinary cAMP and calcium levels in two animal models of malignancy-associated hypercalcemia.

Methods

Antisera. Two antisera against PTHrP were tested. One antiserum was directed against PTHrP (1–34), and the other against PTHrP (1–16). The peptides were synthesized by the previously described methods (9). The antisera were prepared by injecting New Zealand white rabbits with the peptides in Freund's adjuvant (5). PTHrP (1–16) was conjugated to soybean inhibitor before injections. The antisera were tested in an RIA and for their ability to neutralize the biological activity of PTHrP (1–34). For RIA the antiserum against PTHrP (1–34) was used optimally at a dilution of 1:8,000 and that against PTHrP (1–16) used at 1:6,000. ^{125}I-labeled PTHrP (1–34) was used as a tracer in both RIAs, and separation of bound from free was carried out using a solid phase second antibody (Sac Cell; Wellcome, Melbourne, Australia) (5). The ability of the antisera to neutralize biological activity was tested by incubating the antisera (1:50 dilution) for 18 h at 4°C with 2 ng/ml PTHrP (1–34) or 10 ng/ml human PTH (1–34). In addition to the two antisera against PTHrP, an antiserum developed in a goat against human PTH (1–34) was tested in vitro at a dilution of 1:100. The biological activity was assayed as the generation of cAMP in UMR 106-01 clonal osteogenic sarcoma cells in which the cellular ATP was labeled by preincubation with [^{3}H]adenine (15). Triplicate determinations were made for each point.

Affinity purification. The antiserum against PTHrP (1–16) was affinity purified. The peptide PTHrP (1–16) was reduced in 50 mM Tris HCl (pH 8.0) and 10 mM mercaptoethanol for 30 min. The reduced peptide was isolated using a Sep Pak C_{18} cartridge eluted with 0.1% trifluoroacetic acid and 60% acetonitrile and reacted with thiopropyl

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1. *Abbreviations used in this paper:* PTHrP, parathyroid hormone-related protein; TGF, transforming growth factors.

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Sepharose 6B as recommended by manufacturer's instructions (Pharmacia Pty. Ltd., North Ryde, Australia). The antiserum was then purified by affinity chromatography using the PTHrP (1–16) peptide Sepharose. The antibody was eluted with 50 mM glycine buffer, pH 2.0. Antibody-free serum (the column flow-through before elution) and the eluted fractions were tested in an enzyme-linked immunoassay. Immunoreactivity was absent in the antibody-free serum. This serum and the column fractions with the maximal activity in the enzyme-linked immunoassay were then tested in vivo as described later.

Animal models. The two animal tumor models consisted of human tumors obtained from hypercalcemic patients and serially transplanted into athymic mice. One was a squamous cell carcinoma of the lung and the other a squamous cell carcinoma of the larynx. Both tumors cause hypercalcemia, hypophosphatemia, and elevated urine cAMP levels in athymic mice, manifestations similar to those seen in the human syndrome of malignancy-associated hypercalcemia (16–18). One of these tumor models has been previously described in detail (19). 2–3 wk after subcutaneous transplantation of 1–2-mm^3 pieces of tumor, the animals became hypercalcemic, at which time the tumors were removed for immunohistochemical staining.

Immunohistochemical staining. The tumors were either frozen or fixed in 10% buffered formalin overnight and embedded in paraffin blocks. 5-μm-thick sections were obtained and stained immunohistochemically. The peroxidase-antiperoxidase method used was a modification of that described by Sternberger et al. (20). For the purposes of immunostaining, the antiserum against PTHrP (1–16) was used. This antiserum showed very low cross-reactivity (< 0.001%) with human PTH (1–34) and a very low background in the immunoperoxidase studies (21).

Animal protocols. 2–3 wk after implantation, tumor volumes were generally > 1 cm^3. At this time the animals were bled via tail vein and 50–75 μl of blood was obtained in a capillary hematocrit tube. The serum was separated and analyzed for calcium. If the serum calcium levels were > 11.5 mg/dl, the animals were used for injection of various sera. The sera tested were (*a*) normal rabbit serum, (*b*) antiserum against PTHrP (1–34) (this serum was tested whole), (*c*) affinity-purified fraction of the antiserum against PTHrP (1–16), and (*d*) the antibody-free serum obtained during the affinity-purification procedures. All sera or serum fractions were injected in a final volume of 0.1 ml. The lyophilized form of affinity-purified antibodies and the antibody-free serum were reconstituted in 0.9% NaCl and sterile water, respectively. The amount of these materials injected into each animal represented ~ 0.1 ml of the original serum. Under ether anesthesia, the injections were made into the tail vein using a 28-gauge needle. The animals were then bled at 3, 6, 24, 48, and, in some cases, 72 h after injections. Spontaneously voided spot urines were collected by putting the animals on a cold clean metal platform. The urine (30–130 μl) was then aspirated into a syringe containing 10 μl glacial acetic acid and frozen for subsequent analysis for calcium, cAMP, and creatinine. Attempts at urine collections were made before the injections of various sera and then before each bleeding. Not all animals voided at each time point. Six to seven animals were studied in each group. Urine values were available for four to seven animals for each time point.

Analytical methods. Serum calcium was analyzed by the EGTA titration method using a Calcette ultramicro calcium analyzer (Precision Systems, Inc., Sudbury, MA). Urine calcium and creatinine were determined using a Gem Star chemistry analyzer (Electro-Nucleonics Inc., Fairfield, NJ). Urine cAMP was determined by a competitive protein-binding assay using a commercial kit (Diagnostic Products Corp., Los Angeles, CA).

Statistical comparisons were based on one-way analysis of variance. Data are reported as mean±SE.

Results

In the RIA the antiserum against PTHrP (1–34) was used optimally at a dilution of 1:8,000 and the one against PTHrP (1–16) at a dilution of 1:6,000. At these dilutions a K_d value of 10^{-11} liter/mol was obtained for each antiserum. Human PTH (1–34) and several other unrelated peptide hormones failed to compete significantly for binding at the tested concentrations of up to 10^{-7} M.

As shown in Table I, both PTHrP (1–34) (2 ng/ml) and human PTH (1–34) (10 ng/ml) in the presence of nonimmune rabbit serum caused a significant stimulation of the adenylate cyclase activity. The antiserum against PTHrP (1–34) at a dilution of 1:50 completely blocked the biological activity of PTHrP (1–34) without having a significant effect on human PTH-stimulated activity. Similar results were obtained with the antiserum against PTHrP (1–16) and with the affinity-purified antibodies (data not shown). In contrast, the goat antiserum developed against human PTH (1–34) completely blocked the human PTH (1–34) effect without a significant effect on PTHrP (1–34)-stimulated activity. The antibody-free serum did not have any significant blocking activity (data not shown).

On immunohistology, sections from both tumors stained positive for PTHrP, similar to the observations reported with other squamous cell carcinomas (21). The staining was blocked when the antiserum was preabsorbed with an excess of PTHrP (1–16). Preabsorption with bovine PTH (1–34) did not influence the level of staining.

As shown in Fig. 1, *A* and *B*, in both squamous cell lung and squamous cell larynx models the antiserum reduced serum Ca levels significantly at 3, 6, and 24 h. In the squamous cell lung model, serum calcium levels remained significantly decreased for up to 48 h. Normal serum calcium levels (< 10.5 mg/dl) were reached in four of seven mice with squamous lung tumor and two of seven mice with squamous cell laryngeal tumor. Urine cAMP levels, which were elevated in the tumor-bearing mice, were also significantly decreased by the antiserum treatment, while these levels were unaffected by the normal rabbit serum treatment (Fig. 1, *C* and *D*). Urine calcium levels were also significantly lower in the antiserum-injected animals as compared with the normal rabbit serum-injected animals at 24, 48, and 72 h in the squamous cell lung model. These levels were 2.3±1.1, 2.3±0.6, and 3.2±1.0 mg/mg creatinine in the normal rabbit serum–treated mice,

Table I. Effect of Antibody Incubation on Biological ([^{3}H]cAMP Generation) Activity of Peptides

	Nonimmune (rabbit serum dilution 1:50)	Anti-PTHrP (1–34) (rabbit, dilution 1:50)	Anti-PTH (goat, dilution 1:100)
Control	3,811±37	4,353±225	2,667±65
hPTH (1–34) (10 ng/ml)	34,232±501	32,705±3,825	3,530±43
PTHrP (1–34) (2 ng/ml)	30,971±591	5,372±494	38,002±2,205

hPTH, human parathyroid hormone. hPTH (1–34) and PTHrP (1–34) were incubated for 18 h at 4°C with the antisera or nonimmune rabbit serum, and the activity then determined in the UMR 106-01 intact cell adenylate cyclase assay (15). The cells were labeled by incubation with [^{3}H]adenine. Results are expressed as cpm [^{3}H]cAMP generated and are means±SEM of triplicate determinations.

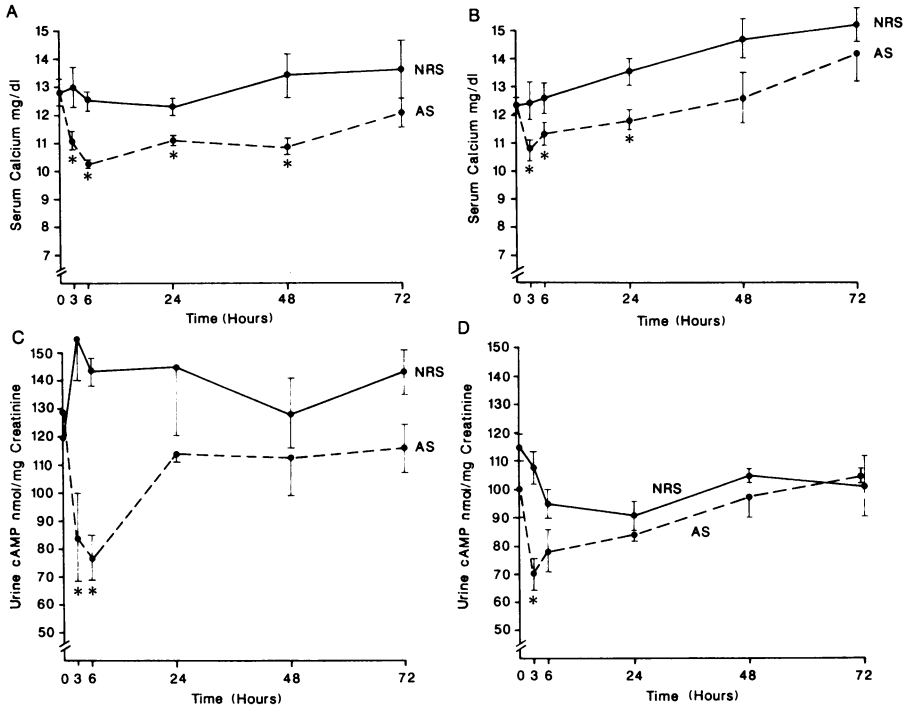

Figure 1. Effect of PTHrP (1–34) antiserum (AS) or normal rabbit serum (NRS) on serum calcium or urinary cAMP in the squamous cell lung cancer model (*A*, *C*) and in the squamous cell laryngeal cancer model (*B*, *D*). At time 0, the animals were injected intravenously with 0.1 ml AS or NRS via tail vein injection. The animals were bled (tail vein) and spontaneously voided urines collected at baseline and at 3, 6, 24, 48, and 72 h after injection. Each point for serum calcium measurement represents the mean±SE of seven observations. Urine cAMP values are based on four to seven observations since not all animals provided samples at each of the time periods. *Significant differences ($P < 0.05$) between the groups by analysis of variance.

and 0.3±0.1, 0.3±0.1, and 0.6±0.2 mg/mg creatinine in the antiserum-treated animals at 24, 48, and 72 h, respectively. In the squamous cell larynx model also, urine calcium levels were lowered by the antiserum treatment. However, due to large variations within groups, the results did not reach a statistical significance. In separate experiments, PTHrP (1–34) antiserum did not have any significant effect on serum calcium in normal athymic mice without tumors. Serum Ca levels after the antiserum injection were 8.9±0.2, 8.7±0.2, 8.2±0.3, 8.8±0.1, and 8.3±0.2 mg/dl at 0, 3, 6, 24, and 48 h, respectively.

As shown in Fig. 2, the affinity-purified antibodies against PTHrP (1–16) lowered serum calcium and urine cAMP in the mice with the squamous cell lung tumor. Serum calcium levels were normalized in five out of six mice. In contrast, the antibody-free serum did not have significant effect on these parameters in the tumor-bearing animals. Urine calcium levels were significantly lower in the antibody-injected mice as compared with antibody-free serum-injected animals at 24 and 48 h after injection. These levels were 0.3±0.1 and 0.4±0.1 mg/mg creatinine and 0.8±0.2 and 0.9±0.2 mg/mg creatinine at 24 and 48 h in the two groups, respectively.

Discussion

The data, therefore, demonstrate that neutralizing antisera to PTHrP decreases serum calcium, urine calcium, and urine cAMP in the tumor-bearing hypercalcemic animals. The decrease in urine calcium paralleled the decrease in serum calcium. The calcium-lowering effects of the antisera were specific since (*a*) normal rabbit serum did not have any significant effect, and (*b*) with the PTHrP (1–16) antiserum, the calcium-lowering activity was contained only in the affinity-purified antibody fraction. These findings indicate that endogenous

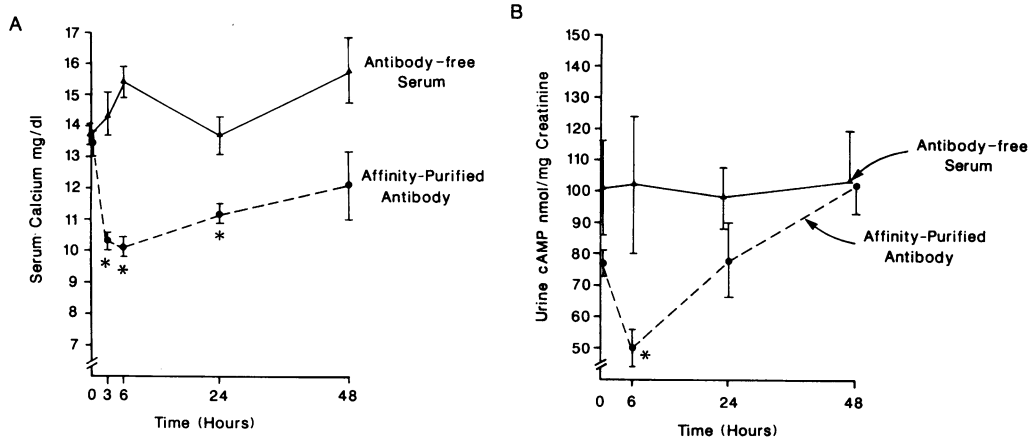

Figure 2. Effect of affinity-purified antibodies (PTHrP 1–16 antiserum) or antibody-free serum on serum calcium (*A*) or urinary cAMP (*B*) in the squamous cell lung cancer model. Animal protocol and data presentation are as in Fig. 1. Each point for serum calcium measurement represents mean±SE of six observations. Urine cAMP values are based on four to six observations.

PTHrP secreted by the tumor was indeed responsible for at least a major component of the hypercalcemia. However, the fact that serum calcium levels did not completely normalize in all animals suggests that either the amount of antiserum was insufficient to neutralize all of the PTHrP activity, or that other cofactors also contributed to the development of hypercalcemia.

The clinical manifestations of malignancy-associated hypercalcemia and primary hyperparathyroidism are similar. One major difference, however, is that bone formation is decreased in malignancy-associated hypercalcemia and normal to increased in primary hyperparathyroidism (22–24). The effects of PTHrP on bone formation may be similar to those of PTH (25). On the other hand, TGFα has been shown to decrease bone formation (26). The present study did not directly assess the role of TGFα or cytokines in the pathogenesis of malignancy-associated hypercalcemia. TGFα, tumor necrosis factors α and β, and interleukins all stimulate bone resorption in vitro (26–28), but they do not stimulate adenylate cyclase activity in renal cortical membranes or in the osteoblast-like cells (unpublished observations). In one of the tumors (squamous lung), we have previously demonstrated the presence of mRNA for TGFα and have shown that the in vitro bone resorption activity derived from this tumor is neutralized by an antiepidermal growth factor receptor antiserum (29). However, the relationship between the in vitro osteolytic activity and in vivo hypercalcemia-producing capacity is not always clearly established. For example, epidermal growth factor has significant in vitro bone resorption activity comparable to that of bovine PTH on a molar basis (30). However, when infused in vivo it either causes no increase (31) or only a minimal increase (32) in serum calcium. The 24–48-h duration of hypocalcemic effect is consistent with the half-life of IgG in the rodents (33). On the weight of current evidence, it is likely that PTHrP alone is sufficient to cause the hypercalcemia, although the possibility of a contribution by another substance has not yet been totally excluded. Further studies with higher doses of antibodies to PTHrP and/or studies with antisera to TGFα or cytokines may establish whether the latter two have any role in the pathogenesis of malignancy-associated hypercalcemia. The possibility of using PTHrP monoclonal antibodies in the acute management of humoral hypercalcemia of malignancy should be explored.

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