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Short Communication

Parathyroid-Hormone-Related Protein in Sarcoidosis

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Parathyroid-hormone-related protein (PTHrP) is the main mediator of the humoral hypercalcemia of malignancy. It is also detected in many normal adult and fetal tissues. Altered calcium metabolism occurs in sarcoidosis, and two cases of sarcoidosis with hypercalcemia and elevated plasma PTHrP are described. An archival study of 20 lymph node biopsies with the pathological diagnosis of sarcoidosis was performed. Immunohistochemistry using a polyclonal antiserum to human PTHrP and *in situ* hybridization using a riboprobe to human PTHrP were performed on the lymph node biopsies. Immunohistochemistry for PTHrP was also performed on the biopsies from the two cases with elevated plasma levels. Immunohistochemical analysis detected PTHrP in macrophages within granulomata in 17 of the 20 (85%) biopsies. *In situ* hybridization detected a positive signal for messenger RNA in the granulomata of 11 of 19 (58%) biopsies. PTHrP immunoreactivity and PTHrP gene expression are present in sarcoid granulomata. PTHrP may contribute to the hypercalcemia of sarcoidosis. *(Am J Pathol 1998, 152:17–21)*

Sarcoidosis is a chronic granulomatous disorder of unknown etiology. Mild to severe hypercalcemia is detected in approximately 10% of patients, and up to 50% become hypercalciuric at some time in the course of their disease.[1] The discovery that a high proportion of patients with hypercalcemia of sarcoidosis had elevated 1,25-$(OH)_2D_3$ levels suggested the endogenous overproduction of 1,25-$(OH)_2D_3$ as the cause of the altered calcium metabolism in this condition.[1,2] The sarcoid macrophage, a prominent constituent of the sarcoid granuloma, is the source of the increased production of 1,25-$(OH)_2D_3$ in sarcoidosis,[3] and this results in increased intestinal absorption of calcium. Increased bone resorption is also an important contributor to the pathogenesis of hypercalcemia and hypercalciuria in this disease.[4]

Parathyroid-hormone-related protein (PTHrP) is a product of many cancers, causing hypercalcemia by its actions upon kidney and bone. PTHrP is also a product of normal fetal and adult tissues where it exerts autocrine and paracrine effects.[5] We have detected elevated plasma PTHrP levels in two hypercalcemic patients with sarcoidosis. This prompted an investigation of PTHrP expression in sarcoid inflammatory tissue by immunohistochemistry and by *in situ* hybridization. Biopsy material from these two patients as well as lymph node tissue from a consecutive retrospective series of 20 biopsies containing sarcoid granulomata were examined.

Case Reports

Case 1

A 43-year-old woman presented with renal colic due to a calcium apatite calculus. Investigations revealed hypercalcemia (ionized Ca^{2+} 1.41 mmol/L; reference range, 1.14 to 1.29) and hypercalciuria (24-hour urinary calcium excretion, 13 mmol; normal level, <2.8), with suppressed serum intact PTH (Nichols, San Juan Capistrano) at 0.2 pmol/L (reference range, 1.0 to 5.5). The hypercalcemia and hypercalciuria persisted for months. Physical examination was normal. Serum 1,25-$(OH)_2D_3$ was 110 nmol/L (reference range, 28 to 165). Plasma PTHrP measured by amino-terminal radioimmunoassay was 20.2 pmol/L (normal range, <2.0).[6] Chest x-ray, computerized tomography examination of neck, chest, abdomen, and pelvis and mammography were all normal. A gallium scan demonstrated marked tracer accumulation in thigh muscles bilaterally. Biopsy of the left vastus lateralis muscle demonstrated numerous small granulomata within the perimysial connective tissue, several of which contained Langerhans-type giant cells. The serum angiotensin converting enzyme (ACE) level was 23.7 IU/L (reference range, 8.3 to 21.4). A diagnosis of hypercalcemia due to sarcoidosis was made, and the patient was treated with glucocorticoids with rapid resolution of the hypercalcemia. Plasma

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PTHrP levels fell to 7.5 pmol/L after treatment, and the patient remained normocalcemic and normocalciuric on 5 mg of prednisolone daily 12 months after commencement of therapy.

Case 2

A 65-year-old woman with a previous history of non-insulin-dependent diabetes presented with symptomatic hypercalcemia (total calcium, 3.66 mmol/L; reference range, 2.20 to 2.60). Serum intact PTH level was 1.7 pmol/L (reference range, 1.0 to 5.5; Nichols, San Juan Capistrano). Plasma PTHrP level measured by amino-terminal radioimmunoassay[6] was 3.6 pmol/L (normal range, <2), ACE activity was 236 U/L (reference range, 25 to 100), and 1,25-$(OH)_2D_3$ level was 72 pmol/L (reference range, 34 to 134; Incstar, Stillwater, MN). A computerized tomography scan of the chest and abdomen detected enlarged pretracheal lymph nodes and mild splenomegaly. A gallium scan demonstrated increased tracer uptake in the left hilum and spleen. A mediastinoscopy and lymph node biopsy were performed. Histological examination of the lymph node revealed extensive non-necrotizing chronic granulomatous inflammation consistent with sarcoidosis. Oral prednisolone therapy was commenced with subsequent normalization of plasma calcium levels occurring within 1 month. The patient remained well 2 years after diagnosis.

Materials and Methods

Samples

The muscle biopsy specimen from case 1 and lymph node biopsy specimen from case 2 were analyzed. Lymph node biopsy specimens from 20 consecutive patients with the pathological diagnosis of sarcoidosis were also obtained from the Anatomical Pathology archives of St. Vincent's Hospital, Melbourne, Australia. Tissue biopsies used for immunohistochemistry and *in situ* hybridization had been fixed in buffered 10% formalin and paraffin embedded, with the exception of the muscle biopsy specimen, which was snap frozen and stored at −70°C.

Immunohistochemistry

Polyclonal rabbit antiserum raised in rabbits against human amino-terminal PTHrP (1–14) was used. Its specificity was evaluated and confirmed by ELISA using PTHrP peptides of various amino acid sequences including amino-terminal, mid-molecule, and carboxyl-terminal fragments. This antiserum shows no cross-reactivity with PTH. The immunohistochemical procedure was a modification of the immunoperoxidase method of Sternberger et al[7] as previously described.[8]

Paraffin sections 3 μm thick were prepared on aminopropyltriethoxysilane (AES)-coated slides. The slides were dewaxed and rehydrated and then immersed in a methanol/0.3% hydrogen peroxide solution to block endogenous peroxidase activity. Sections were washed in phosphate-buffered saline (PBS) and incubated with the following reagents: 10% normal swine serum for 30 minutes, primary antibody diluted 1:500 and 1:750 at 4°C overnight, and swine anti-rabbit immunoglobulins (Dako Corp., Glostrup, Denmark) diluted 1:40 for 30 minutes. All of the above reagents were diluted with 5% newborn calf serum in PBS. Sections were then incubated with rabbit horseradish peroxidase-anti-peroxidase (PAP) complex (Dako) diluted 1:80 in PBS for 30 minutes. The immunological reaction was developed with 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, MO), and the sections were counterstained with Harris' hematoxylin. The sections were dehydrated through alcohols, mounted in DPX (Gurr, Poole, UK), and viewed and photographed using an Olympus Vanox microscope.

Controls included 1) alternating deletion of antibody layers (primary antiserum, secondary antiserum, PAP complex), 2) replacement of anti-PTHrP (1–14) antiserum with nonimmune rabbit serum, and 3) a positive control tissue consisting of normal human skin. Two normal lymph node sections from the mediastinum and pelvis were included in every experiment.

The stained sections were reviewed by two observers, who were blinded with respect to the controls, and the intensity of staining was graded independently using a four-step ordinal scale: 0, no staining; +, weak staining; ++, moderate staining; +++, strong staining.

In Situ *Hybridization*

A 423-bp riboprobe to exon VI of the human PTHrP gene was used. The method used for the synthesis of riboprobe, labeling of the probe with digoxigenin (DIG), and the *in situ* hybridization procedure have been described previously.[9] The specificity of the probe was confirmed by Northern blot analysis.

Sections 5 μm in thickness were cut and placed onto AES-treated slides. Sections were dewaxed with xylene and rehydrated through graded ethanol to diethylpyrocarbonate-treated distilled water. Sections were deproteinized with 0.2 mol/L hydrochloric acid and proteinase K (2 μg/ml) and then post-fixed in 4% paraformaldehyde/PBS before prehybridization for 1 hour with buffer containing 50% formamide, 2% blocking reagent (Boehringer Mannheim, Mannheim, Germany), 5× SSC (1× SSC contains 0.15 mol/L sodium citrate and 0.15 mol/L sodium chloride, pH 7.0), 0.1% *N*-lauroyl sarcosine and 0.02% sodium dodecyl sulfate (SDS). Sections were hybridized with fresh buffer and labeled probe (4 ng/μl) at 42°C overnight. The sections were then treated with RNAse to remove nonhybridized riboprobe and then washed at a different stringency with SSC. Alkaline-phosphatase-coupled anti-DIG antibody was applied to detect the hybridized probe.

Controls included 1) sections treated with RNAse before the application of the antisense probe, 2) deletion of probe only, 3) deletion of probe and anti-DIG antibody to detect endogenous alkaline phosphatase, and 4) positive control tissue comprising normal human skin included in every experiment. One normal lymph node section from the mediastinum was included in every experiment.

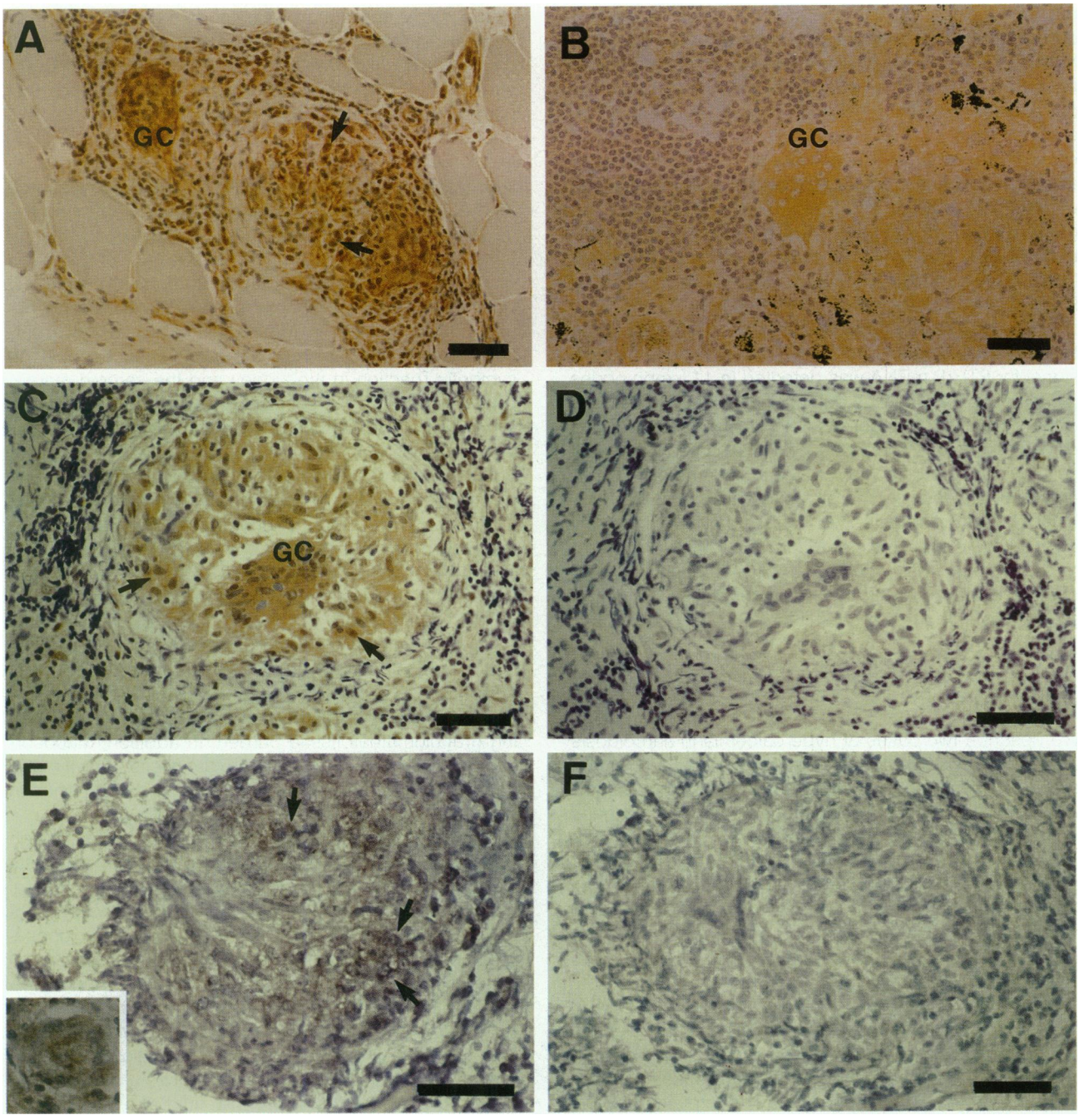

Figure 1. Immunohistochemistry (IHC) and *in situ* hybridization (ISH) of PTHrP in sarcoid tissue. **A** and **B:** IHC of two case report biopsies. **C to F:** IHC and ISH analysis of one archival lymph node. All sections were counterstained with hematoxylin. IHC using a polyclonal antiserum against PTHrP (1–14) detected positive brown reaction product within macrophages (**arrows**) and a multinucleated giant cell (GC) in **A**, **B**, and **C**. **A:** PTHrP antiserum (1:250) treated skeletal muscle from case 1. **B:** PTHrP antiserum (1:250) treated lymph node from case 2. **C** and **D:** PTHrP antiserum (1:500) treated lymph node and nonimmune serum treated negative control, respectively. **E** and **F:** PTHrP antisense treated and RNAse pretreated lymph node, respectively. Positive purple signal for PTHrP was located within macrophages (**arrows**) within granuloma and multinucleated cells (**E, inset**). Scale bar, 50 μm.

Results

All lymph node biopsy sections showed sarcoid granulomata, some with multinucleated giant cells. There was distortion and replacement of the normal lymph node architecture to varying degrees by the chronic inflammatory infiltrate. Immunohistochemical analysis of the muscle biopsy in case 1 and the lymph node biopsy in case 2 localized the PTHrP antigen within the macrophages and multinucleated giant cells in the sarcoid granulomata (Figure 1, A and B). No staining was observed when primary antiserum was replaced by nonimmune rabbit serum.

Immunohistochemical examination of tissue sections from 20 patients revealed positive cytoplasmic staining for PTHrP in 17 cases (85%), as illustrated by the brown

reaction product (Figure 1, C and D). Plasma calcium concentrations were available in only 12 of the 20 cases for which lymph node biopsies were examined. Only one patient had an elevated plasma calcium; all others were normocalcemic. Staining was strong in two cases, moderate in seven, weak in eight, and negative in three. PTHrP was localized in the cytoplasm of macrophages and multinucleated giant cells within granulomata and within macrophages and plasma cells in surrounding granulomatous inflammatory tissue. No staining was detected when the nonimmune rabbit serum was substituted for the primary PTHrP antibody. There was no PTHrP staining detected in the normal lymph nodes.

In situ hybridization could be performed on lymph node tissue biopsy sections from 19 patients only. A positive signal for PTHrP mRNA was detected in 11 lymph nodes (58%). Strong signal was detected in three samples and weak signal only in eight. The signal was located in the cytoplasm of macrophages and multinucleated giant cells within granulomata or within inflammatory cells around or between the margins of granulomata (Figure 1, E and F). Sections treated with RNAse did not display any cytoplasmic signal. No signal for PTHrP mRNA was detected in the normal lymph node. All 11 sections that had positive signal by *in situ* hybridization also had positive staining by immunohistochemistry.

Discussion

We describe two cases of hypercalcemia in sarcoidosis associated with elevated circulating levels of PTHrP measured by an amino-terminal radioimmunoassay.[6] PTHrP was identified in the cytoplasm of sarcoid macrophages in the granulomata in both cases by immunohistochemistry. No alternative source of PTHrP was found after extensive investigations, and both patients remained well 2 years after treatment with prednisolone was commenced, which is consistent with the diagnosis of sarcoidosis. In the two patients, plasma 1,25-$(OH)_2D_3$ levels were within the normal range.

PTHrP immunoreactivity was also identified in the cytoplasm of sarcoid macrophages and multinucleated giant cells in 85% of a series of lymph node biopsies. PTHrP mRNA was detectable in 58% of all cases by *in situ* hybridization. This discrepancy may be explained by the lack of stability of the PTHrP mRNA. PTHrP mRNA has a short half-life due to the presence of nucleotide motifs in the PTHrP gene that allow proteins to bind to mRNA and facilitate its degradation.[10] The processing and storage of the tissue biopsies may also result in loss of PTHrP mRNA.[11]

PTHrP immunoreactivity was also detected in the cytoplasm of plasma cells. We have previously measured elevated plasma PTHrP levels in patients with multiple myeloma and lymphoma of B cell lineage. Positive immunohistochemical staining for PTHrP in lymphocytes was demonstrated in one of the cases, indicating that the hormone can be a product of B lymphocytes.[12]

The association of disordered calcium metabolism and sarcoidosis has been long recognized.[1] Vitamin D was implicated in the abnormal calcium metabolism of sarcoidosis after it was discovered that patients with sarcoidosis who had hypercalciuria or hypercalcemia absorbed high fractions of dietary calcium and that normocalcemic patients were prone to hypercalcemia after exposure to ultraviolet light.[1] Elevated 1,25-$(OH)_2D_3$ levels were eventually detected in a high proportion of hypercalcemic sarcoid patients.[1,2] An extrarenal source for 1,25-$(OH)_2D_3$ was established when an anephric patient with hypercalcemia and sarcoidosis was reported to have an elevated serum 1,25-$(OH)_2D_3$ level.[13] Synthesis of 1,25-$(OH)_2D_3$ from 25-OHD_3 has been demonstrated *in vitro* from lymph node homogenates[14] and from pulmonary macrophages from hypercalcemic patients with sarcoidosis.[3] Factors that exert a regulatory influence on the synthetic reaction in the kidney and the sarcoid macrophage may differ. For example, the sarcoid macrophage 1-α hydroxylation is very sensitive to stimulation by certain cytokines.[15,16]

Previous studies have not detected PTHrP in the plasma of patients with sarcoidosis.[17,18] This may reflect PTHrP production in an amount that is only detectable in the local inflammatory tissue environment. Only when endogenous PTHrP production is excessive may it enter the systemic circulation, as occurred in the two patients in the present report.

The mechanism by which glucocorticoids were effective in reducing the serum calcium in the two cases reported could be attributed to multiple effects. They promote the resolution of inflammatory processes reducing granulomatous tissue burden. In addition, glucocorticoids inhibit extrarenal 1-α hydroxylase activity and have been shown to reduce PTHrP gene expression *in vitro*.[1,19]

The presence of PTHrP protein and mRNA in a high percentage of sarcoid lymph node biopsies suggests that PTHrP may play an important role in the abnormal calcium metabolism in this disease. Increased production of PTHrP in sarcoidosis could arise as a consequence of cytokine production within the sarcoid granulomatous tissue. Increased cytokine synthesis and mRNA expression has been demonstrated in the bronchoalveolar lavage (BAL) fluid of sarcoid patients with interstitial lung disease. Increased protein production and gene expression of tumor necrosis factor (TNF)-α and interleukin (IL)-6 are detected in the BAL fluid and BAL macrophages in sarcoidosis but not in normal controls.[20,21] Both TNF-α and IL-6 stimulate human lung squamous cell carcinoma line (BEN) cells to produce increasing amounts of PTHrP.[19] It is possible that TNF-α and IL-6 could stimulate increased PTHrP production in sarcoid macrophages.

As is the case with PTH, the amino-terminal region of PTHrP promotes renal 1-α hydroxylation of vitamin D *in vitro* and *in vivo*.[22–24] Serum 1,25-$(OH)_2D_3$ concentrations in the hypercalcemia associated with malignancy may be low, normal, or elevated, but in the absence of bone metastases there is a significant correlation between PTHrP and 1,25-$(OH)_2D_3$ concentrations, which is consistent with a stimulating action of PTHrP on the renal 1-α hydroxylase occurring in the clinical setting.[24] It is pos-

sible that PTHrP expressed in the sarcoid macrophage may exert an autocrine action on 1-α hydroxylase activity resulting in the increased production of 1,25-$(OH)_2D_3$, as do γ interferon-γ and IL-2.[15,16]

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