NOTE *Internal Medicine*

Parathyroid Hormone-Related Protein (PTHrP) Produced by Dog Lymphoma Cells

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ABSTRACT. Parathyroid hormone-related protein (PTHrP) was investigated in a canine lymphoma case with hypercalcemia by means of immunoradiomentric assay (IRMA) and molecular analysis. The plasma calcium level of the patient dog was 13.7 mg/d*l*. The PTHrP concentration examined by IRMA was 6.1 pmol/L in the plasma sample from the dog, but it was undetectable (< 1.1 pmol/L) in plasma samples from 4 lymphoma cases without hypercalcemia or 5 normal dogs. The PTHrP concentration examined in the culture supernatant of the lymphoma cells from this case was 1.3 pmol/L, whereas those of the lymphoma cells from a lymphoma case without hypercalcemia was undetectable. PTHrP mRNA was clearly detected not only in the lymphoma cells from this dog with hypercalcemia but also in lymphoma cells from 4 lymphoma cases without hypercalcemia and 2 canine lymphoma cell lines.

KEY WORDS: hypercalcemia, lymphoma cell, mRNA, parathyroid hormone-related protein, RT-PCR.

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Hypercalcemia is one of the most common paraneoplastic syndromes in dogs with lymphoma, occurring in approximately 10% to 40% of the clinical cases [8]. This symptom can be debilitating and associated with various degrees of polyuria, thirst, anorexia, nausea, vomiting, dehydration, and coma [4]. Therefore, hypercalcemia, as a paraneoplastic syndrome, is one of the major clinical problems in malignancies in dogs [4].

The molecular mechanism which develops hypercalcemia in dogs with lymphoma has not been fully understood. It has been suggested that hypercalcemia can be induced by a parathormone-like protein, called parathyroid hormone-related protein (PTHrP), produced in neoplastic cells [8]. High levels of PTHrP in plasma samples from human lymphoma patients have been detected by immunoradiometric assay (IRMA) [2–4, 6, 11]. The cDNA clones encoding dog PTHrP have been isolated and sequenced, and the predicted amino acid sequence of dog PTHrP was shown to have high sequence homology to that of human PTHrP, especially in its biologically active regions [10]. Therefore, the detection system for human PTHrP might be useful for the measurement of PTHrP in dog lymphoma cases with hypercalcemia.

In the present case, we carried out the measurement of dog PTHrP by IRMA for detection of human PTHrP and reverse transcription-polymerase chain reaction (RT-PCR) in dog lymphoma cases.

A 7-year-old male shetland sheepdog weighing 13.25 kg was referred to an animal hospital with chief complaints of anorexia, polydipsia and incontinence.

On physical examination, massive generalized subcutaneous lymphoadenopathy with splenomegaly presented in the dog. Microscopic examination of the specimen by fine-needle aspiration from the left superficial cervical lympho node disclosed a monomorphic population of round cells with large nuclei, prominent nuclei, and no cytoplasmic granules or vacuoles. Initial laboratory values in the dog were: hemoglobin, 14.0 g/d*l*; white blood cell count, 12, 400 μl; platelets, 7,100 μl; and the plasma calcium level was 13.7 mg/d*l*.

The treatment of this case was started with chemotherapy with vincristin, L-asparaginase and prednisone. Three days later, the dog died and could not put be autopsied.

Monoclonal antibodies directed to canine CD3, CD4, CD8α, CD11b, CD18, CD45RA, MHC class II and Thy-1 [10] were used to examine the cell surface phenotype of the lymphoma cells. The staining procedure was the same as described previously [5]. In brief, 1×10^6 lymph node cells were incubated with each of the antibodies followed by incubation with FITC-labeled anti-mouse or anti-rat IgG (Cappel Research Products, Aurora OH, U.S.A), and then analyzed with a flow cytometer, CYTOACE-150 (Japan Spectroscopic, Tokyo, Japan). For the detection of surface immunoglobulin, the cells were directly stained with FITC-labeled anti-canine IgM antibody (Cappel Research Products).

Flowcytometric analysis showed that the tumor cells obtained from the lymphoma case with hypercalcemia were positive for CD3, CD18 and Thy-1, but negative for CD4, CD8α, CD11b, CD45RA, MHC class II and surface immunoglobulin. These results indicated that the tumor cells originated in the T-cell lineage.

The concentrations of PTHrP in the plasma and culture supernatant samples were measured with a commercial IRMA kit for human PTHrP, PTHrP IRMA Mitsubishi (Mitsubishi-Kagaku Bio-Clinical Laboratories, Ibaraki,

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Japan) with a monoclonal antibody directed at the N-terminal region (1–34) of human PTHrP and a polyclonal antibody directed at its C-terminal region (50–83) [2]. These regions were seen in amino acid sequences in canine PTHrP [10].

The plasma PTHrP concentration in the dog lymphoma case with hypercalcemia was 6.1 pmol/L, whereas those in 4 dog lymphoma cases without hypercalcemia and in 5 normal dogs were undetectable (< 1.1 pmol/L).

Primary lymphoma cells from the biopsy specimens of dog with lymphoma were washed three times with PBS, and the cells (1×10^5 cells) were cultured in RPMI-1640 medium (GIBCO, New York, NY, U.S.A.) supplemented with 20% fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2. After cultivation for five days, the culture supernatants were collected to examine the PTHrP concentration by IRMA.

A canine T-cell lymphoma cell line (CL-1) [5], a canine B-cell leukemia cell line (GL-1) [7] and a canine kidney cell line (MDCK) (ATCC, CCL-34) were also used to examine the production of PTHrP.

PTHrP concentration in the culture supernatant of the lymphoma cells obtained from a lymphoma case with hypercalcemia was 1.3 pmol/L, after being cultivated for 5 days, but those of the lymphoma cells from a non-hypercalcemic lymphoma case, CL-1 cells, GL-1 cells, and MDCK cells were undetectable (< 1.1 pmol/L).

Primary lymphoma cells obtained from one canine lymphoma case with hypercalcemia and from another canine lymphoma case without hypercalcemia, and peripheral blood mononuclear cells from a normal dog were kept at –80°C until use for RNA extraction. CL-1, GL-1 and MDCK cells were also used for RT-PCR. Total RNAs were extracted from the cells (1×10^5 cells) with RNeasy total RNA kit (QIAGEN, Hilden Germany). Reverse transcription of the poly(A)$^+$ RNA was performed with an Omniscript^{TM} Reverse Transcriptase kit (QIAGEN). Reverse transcription of the poly(A)$^+$ RNA was performed with a Omniscript^{TM} Reverse Transcriptase kit (QIAGEN).

PCR primer sequences used for amplification of dog PTHrP mRNA were 5'-GCTCGGTGGAGGAGCTCGGC-3' (primer PTHrP1S, nt. 241–260 in canine PTHrP, GenBank accession No. U15593) and 5'-AGCAG-GAAAAAAAGAAACGT-3' (primer PTHrP1R, nt. 578–589) [10]. With these primers, an approximately 300-bp fragment containing the coding sequence of canine PTHrP gene was expected to be amplified. The cDNAs from sample cells were amplified by PCR in a reaction mixture (30 μl) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 mM each deoxynucleotide triphosphate, 1.0 unit of *Taq* polymerase (Takara, Kyoto, Japan) and 0.5 μg of a pair of primers. The PCR amplification was carried out for 35 cycles consisting of template denaturation (1 min, at 94°C), primer annealing (2 min, at 62°C) and polymerization (2 min, at 72°C). The PCR products were analyzed 2% agarose gel electrophoresis and then directly cloned into pCRII vector (Invitrogen, Carlsbad, CA, U.S.A.). The plasmid DNAs were extracted with a QIAGEN plasmid kit (QIAGEN) and sequenced by dideoxy chain termination with ABI PRISM 310 Genetic Analyzer (Perkin-Elmer, Foster City, CA, U.S.A.).

In the RT-PCR analysis for the expression of PTHrP mRNA, a distinct band of about 300-bp was generated from the primary lymphoma cell sample derived from the lymphoma case with hypercalcemia. DNA fragments of the same size with similar band intensity were also generated from the lymphoma cell sample derived from non-hypercalcemic case and the cells of CL-1 and GL-1 cell lines, MDCK cells and peripheral blood mononuclear cells of a normal dog (Fig. 1). The results indicated the presence of PTHrP mRNA not only in the primary lymphoma cells from the lymphoma case with hypercalcemia but also in those from the non-hypercalcemic lymphoma case and the lymphoid tumor cell lines examined in this study. The nucleotide sequence of the PCR fragments generated by RT-PCR for dog PTHrP mRNA in this study was 100% identical to that of the canine PTHrP gene reported previously [10].

The lymphoma cells from the hypercalcemic lymphoma case were considered to release PTHrP which induced hypercalcemia. On the other hand, PTHrP was not detected in the culture supernatants of lymphoma cells from a non-hypercalcemic lymphoma case and CL-1 and GL-1 lymphoid tumor cell lines, but PTHrP mRNA was detected by RT-PCR in these lymphoid tumor cells. The canine patients from which these cell lines were derived did not show signs of hypercalcemia. There are several reasons to be considered to understand the disagreement between the results of the IRMA for PTHrP in the plasma and culture supernatant samples and RT-PCR for PTHrP in lymphoid tumor cells. Rosol and Capen described how PTHrP is produced by normal tissues, including epidermis, endocrine glands, lymphocytes, bone, brain, cardiac and smooth muscle, and epitherial cells in many organs where it functions as a paracrine factor [9]. Moreover, because the RT-PCR used in this study was not a quantitative assay, the results might not be able to show the difference in the amount of PTHrP mRNA in those lymphoid tumor cell samples. As another possibil-

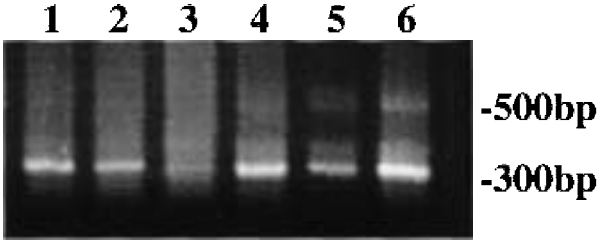

Fig. 1. Dog PTHrP mRNA expression in the tumor cells. The dog PTHrP mRNA levels in the tumor cells, CL-1 cell lines, GL-1 cell lines and normal peripheral white blood cells were determined by RT-RCR. Lanes: 1, tumor cells with hypercalcemia; 2, tumor cells without hypercalcemia; 3, CL-1 cells; 4, GL-1 cells; 5, MDCK cells; 6, normal peripheral white blood cells. Reaction products were run on 2% agarose gels.

ity, differences in the translation step, processing of the protein, and/or its release from the cells may be responsible for the difference in the production of PTHrP form lymphoma cells. It will be necessary to investigate factors which regulate PTHrP production from lymphoid tumor cells.

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