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NOTE

Immunohistochemistry for parathyroid hormone-related protein (PTHrP) in benign and malignant mammary mixed tumors of dogs with and without hypercalcemia

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Abstract

We evaluated the expression of parathyroid hormone-related protein (PTHrP) by immunohistochemistry in eight benign and malignant mammary mixed tumors of dogs with (n = 4) and without (n = 4) hypercalcemia. Positive immunoreactive staining for PTHrP was observed in all four tumors from hypercalcemic dogs. The mammary tumors from 2 of the 4 normocalcemic dogs stained positively for PTHrP, but the numbers of immunoreactive cells and intensity of the immunoreaction were less than in the hypercalcemic dogs. In the other 2 tumors without hypercalcemia, the tissue samples were negative for PTHrP.

Key words: dog, mammary tumor, PTHrP.

Two types of cancer-associated hypercalcemia have been described: local osteolytic hypercalcemia (LOH) and humoral hypercalcemia of malignancy (HHM)[5]. LOH is due to direct bone destruction by primary or metastatic tumors. HHM is mediated by circulating factors secreted by malignant cells without evidence of bony disease. HHM is commonly found in certain human cancers, particularly squamous cell carcinoma and hematopoietic neoplasia[5,12]. In animals, spontaneous HHM has been reported in the adenocarcinoma derived from the apocrine gland of the anal sac of the dog[9,13]. It is now believed that hypercalcemia is due to the release of factors by malignant cells that ultimately cause calcium reabsorption from bone. One such factor is a parathyroid hormone-like protein known as parathyroid hormone-related protein or peptide (PTHrP). PTHrP has been identified in both human and animal cases[13,15]. PTHrP is composed of three isoforms containing 139, 141 or 173 amino acids[5]. Each of the three isoforms has common amino acids, residue positions 1 to

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139 (1-139). The N-terminus (1-36) and midregion (36-61) of the PTHrP are well conserved among species, suggesting that there is immunologic cross reactivity between species[14]. The N-terminus of PTHrP binds parathyroid hormone-receptors of osteoblasts and renal epithelial cells, resulting in symptoms similar to those of hyperparathyroidism[14].

One of the authors of the present paper, Okada[11], demonstrated PTHrP immunoreactivity in 58 canine mammary tumors, including benign and malignant mixed tumors. In that study, mammary epithelial cells and myoepithelial cells uniformly showed positive reactions against a chicken-derived polyclonal antibody to PTHrP (1-36) in all the tumors that they examined after antigen-retrieval with microwave heating. However, the study was not concerned with the status of the serum calcium level. Mammary tumor-associated HHM is very rare and has not been reported yet in dogs. We experienced 4 hypercalcemic dogs having mammary tumors that were clinically diagnosed as mammary tumor-associated HHM. PTHrP immunoreactivity in the neoplastic tissues taken from the hypercalcemic dogs was compared with other 4 cases of normocalcemic dogs using rabbit-derived polyclonal antibodies that recognize human PTHrP to investigate the contribution of PTHrP to the hypercalcemia of the cases.

Eight female dogs with mammary tumors were examined. The clinical and pathological findings are summarized in Table 1. Four dogs (cases 1-4) had hypercalcemia, and four dogs (cases 5-8) were normocalcemic. The serum calcium level was evaluated using KODAK ECTACHEM DT60 (Kodak, Tokyo, Japan). The hypercalcemic dogs had anorexia, depression and polydipsia. The hypercalcemia of the four dogs was treated with fluid therapy prior to surgical treatment. When the serum calcium level of these cases was decreased to the normal range, surgical operation for the tumor was performed. After the dissection of the tumors, the dogs maintained a normal serum calcium level. The radiographic examination revealed no osteolytic lesions or metastasis to the internal organs in any case. These cases were clinically diagnosed as HHM based on the findings of decreased serum calcium level after the surgery and no osteolytic lesions. The normocalcemic dogs had no clinical signs other than those concerning the tumor. In cases 1, 2 and 5, both sides of the whole mammary gland were surgically dissected. In cases 3, 4, 6 and 7, one side of the whole mammary gland including the tumor mass was dissected. In case 8, masses only were dissected. Two of the biggest masses in cases 1, 2, 4, 5 and 8 and one of the biggest masses in cases 3, 6 and 7 were fixed in 10% neutral-buffered formalin. Tissue samples taken from cases 1-7 contained overlying normal skin. The tissue samples were embedded in paraffin, and sections (5 micrometers in thickness) were prepared and stained with hematoxylin and eosin for histopathological examination. The histopathological diagnoses were made based on the classification of canine mammary tumors by Moulton[9]. Paraffin sections were also used for the immunostaining of PTHrP. A half batch of dewaxed sections for the immunohistochemistry was heated in a 10mM citric acid solution (pH 6.0) with a H2500 microwave processor (BIO-RAD, Tokyo, Japan) at 600W for 10 min according to Okada's protocol[11]. These preheated and non-heated sections were incubated with rabbit anti-human PTHrP polyclonal antibodies against three synthesized peptides (1-19, 1-34 or 15-34) (Yanaihara Institute Inc., Shizuoka, Japan) at a dilution of 1:6,000. The immunostaining procedure was performed by the peroxidase-conjugated avidin-biotin complex method using a commercial kit (Histofine SAB-PO(R), Nichirei, Tokyo, Japan). As a negative

control, non-immunized rabbit serum (Cederlane, Ontario, Canada) was used, replacing the primary antiserum at a dilution of 1 : 200.

Histopathological examination of the tissues obtained from the four hypercalcemic dogs revealed two malignant (cases 1 and 4) and two benign mammary mixed tumors (cases 2 and 3). Neoplastic epithelial components of the two malignant mixed tumors were diagnosed as papillary carcinoma. The two benign cases had lobular adenoma. Stromal proliferation of all four dogs was characterized by myxomatous and fibrous growths of myoepithelial cells (MECs) without malignant features. Formation of hyaline cartilage derived from metaplastic MEC was present in cases 1-3. Formation of bone tissue also was present in case 3 (Table 1).

All four of the normocalcemic dogs had benign mixed mammary tumors. All of the tumors had lobular adenoma and fibrous and myxomatous growths of MECs. Only one dog (case 7) had formation of hyaline cartilage (Table 1).

The immunoreactivity for PTHrP in the lesions of mammary tumors without microwave pretreatment is summarized in Table 2. With microwave pretreatment, PTHrP immunoreactivity was not enhanced in the lesions of the tumors, but normal epidermal cells showed enhanced immunoreactivity. In case 1, the dog had mild hypercalcemia, and a weak positive reaction to only one antibody, anti-PTHrP (1-19) antibody (among the three antibodies tested in this study) was detected. The positive cells for PTHrP were MECs of fibrous and myxomatous areas, but the neoplastic epithelial component was negative for PTHrP. Intense positive staining for PTHrP was demonstrated in cases 2-4 with severe hypercalcemia. In case 2, MECs in fibrous and myxomatous areas were positive for PTHrP (1-19) , (1-34) and (15-34). This case showed no immunoreactivity for PTHrP in the epithelial component. In case 3, intense positive staining in response to all antibodies was present in MECs surrounding neoplastic alveoli (Figs. 1a and b) and proliferating in the fibrous and myxomatous areas (Fig. 1b). In addition, PTHrP immunoreactivity was demonstrated in chondrocytes of hyaline cartilage and in osteoblasts (Fig. 1c) and osteoclasts (Fig. 1d) of the bone tissue. In cases 2 and 3 as well as in case 1, epithelial components were negative for PTHrP. In case 4, the cytoplasm of the neoplastic epithelial cells in the alveoli had a moderate, diffusely positive immunoreaction with anti-PTHrP (1-19) and PTHrP (15-34) antibodies (Fig. 2). MECs surrounding the alveoli and proliferating in the interstitial area did not have immunoreactivity for PTHrP in case 4.

In the mammary tumors from two of the four normocalcemic dogs (cases 5 and 6), MECs of fibrous and myxomatous areas had positive reactions for PTHrP (Fig. 3); however, the numbers of immunoreactive cells and intensity of the immunoreaction were less than in the hypercalcemic dogs. No immunoreactivity for PTHrP was shown in the epithelial components from these cases. In the other two cases (7 and 8) without hypercalcemia, the tissue samples, including adenomatous alveoli, were negative for PTHrP (Fig. 4). Omission of the primary antibody, as well as the substitution of the primary antibody by a negative control, non-immune rabbit serum, resulted in negative staining. Both the epithelial cells and MECs of the normal mammary and sweat glands and epithelial cells of the sebaceous gland that were included in the biopsies failed to show a positive reaction. The normal skin overlying the tumors was weakly positive for the antibodies in the epidermal keratinocytes.

PTHrP was originally isolated from tumors associated with hypercalcemia, and it is thought to be responsible for many causes of

Table 1. Cinical and pathological findings of the canine subjects with mammary tumours

Case no.	Age (years)/ Sex	Breed	Serum-Ca level (mg/dl) Pre/Postoperation	Hypercalcemia	Gross Morphology	Histopathology Diagnosis	Myxomatos growth	Cartilage/bone formation
1	18/F	Dachshund	13.5/10.8	Yes	Two masses 9cm in diameter each, and 2 other masses 3 cm in diameter each	Malignant MMT (Carcinomatous)	Yes	Yes/No
2	9/F	Yorkshire Terrier	15.4/9.2	Yes	Multiple growths of masses 0.5-3cm in diameter	MMT (Adenomatous)	Yes	Yes/No
3	5/F	Shih Tzu	17.6/10.5	Yes	One mass 2.5cm in diameter and multiple growths of masses about 0.5cm in diameter	MMT (Adenomatous)	Yes	Yes/Yes
4	10/F	Pomeranian	14.1/8.6	Yes	Six masses 2.5cm in diameter and multiple growths of masses about 0.5cm in diameter	Malignant MMT (Carcinomatous)	Yes	No/No
5	8/F	Maltese	10.0/NE	No	Two masses 3cm in diajeter each	MMT (Adenomatous)	Yes	No/No
6	9/F	Shih Tzu	10.2/9.7	No	One mass 1.5cm in diamer	MMT (Adenomatous)	Yes	No/No
7	10/F	Poodle	9.8/9.9	No	One mass 1cm in diameter and multiple growths of masses, about 0.3cm in diameter	MMT (Adenomatous)	Yes	No/No
8	10/F	Mongrel	10.4/NE	No	Two masses 0.7cm in diameter each	MMT (Adenomatous)	Yes	No/No

F=female, MMT=mammary mixed tumor, NE=not examined

Table 2. PTHrP-immunoreactive cells in the canine mammary tumours

Case No.	Tumor cells	Reactivity for PTHrP (1-19)	(1-34)	(15-34)	Case No.	Tumor cells	Reactivity for PTHrP (1-19)	(1-34)	(15-34)
Hypercalcemic dogs					Normocalcemic dogs				
1	Alveolar epithelial cells	−	−	−	5	Alveolar epithelial cells	−	−	−
	MECs surrounding the alveoli	−	−	−		MECs surrounding the alveoli	−	−	−
	MECs in the fibrous and myxomatous areas	+	−	−		MECs in the fibrous and myxomatous areas	±	±	±
2	Alveolar epithelial cells	−	−	−	6	Alveolar epithelial cells	−	−	−
	MECs surrounding the alveoli	++	++	++		MECs surrounding the alveoli	−	−	−
	MECs in the fibrous and myxomatous areas	++	++	++		MECs in the fibrous and myxomatous areas	±	±	±
3	Alveolar epithelial cells	−	−	−	7	Alveolar epithelial cells	−	−	−
	MECs surrounding the alveoli	++	++	++		MECs surrounding the alveoli	−	−	−
	MECs in the fibrous and myxomatous areas	++	++	++		MECs in the fibrous and myxomatous areas	−	−	−
	Chondrocytes, Osteoblasts, Osteoclasts	++	++	++					
4	Alveolar epithelial cells	++	++	++	8	Alveolar epithelial cells	−	−	−
	MECs surrounding the alveoli	−	−	−		MECs surrounding the alveoli	−	−	−
	MECs in the fibrous and myxomatous areas	−	−	−		MECs in the fibrous and myxomatous areas	−	−	−

++ : Most cells were strongly positive, +: Most cells were weakly positive, ±: Some or a few cells were weakly positive, −: Most cells were negative.
MEC=myoepithelial cell

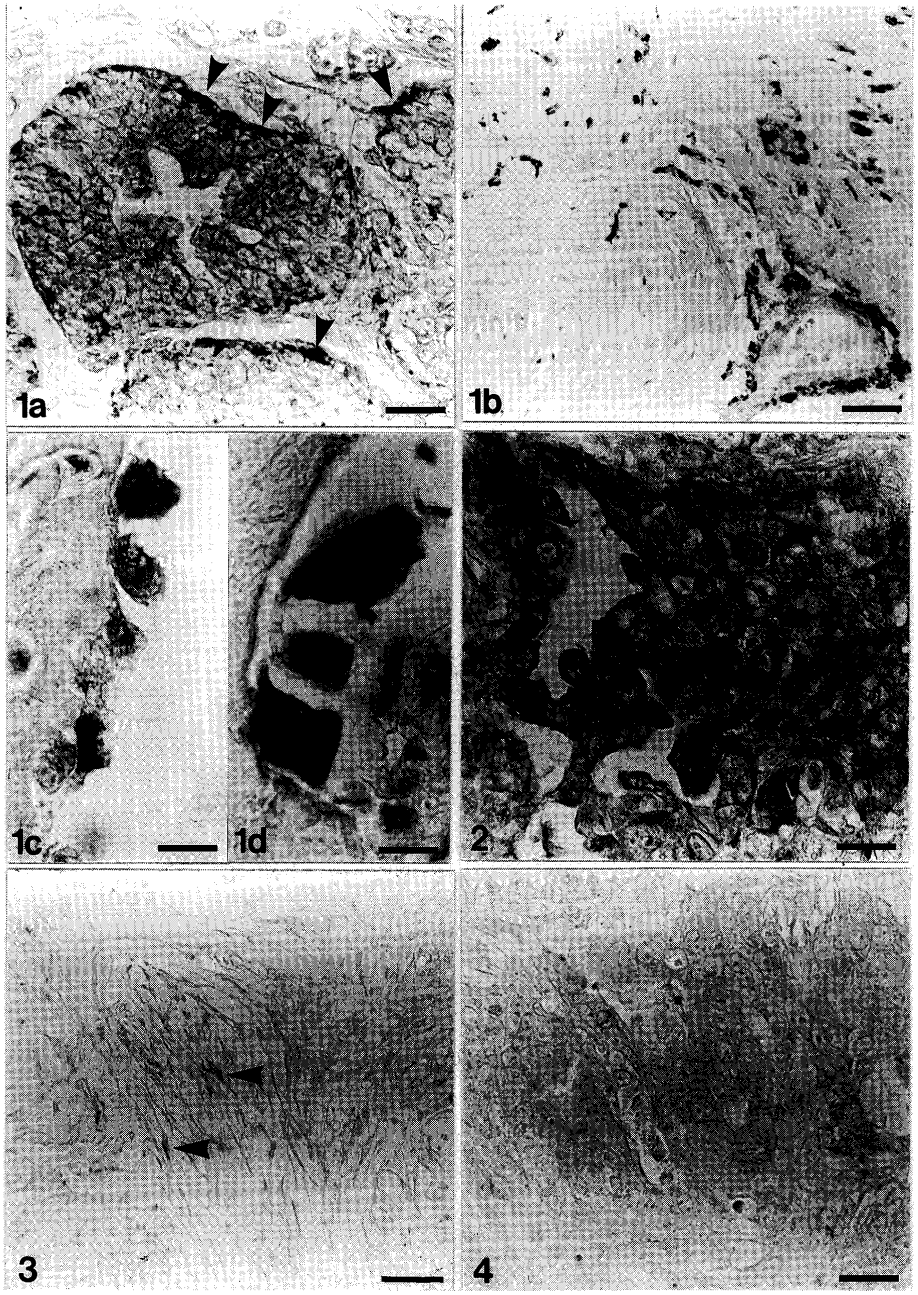

Fig. 1a: Case 3 with hypercalcemia. Myoepithelial cells surrounding the neoplastic lobules in the mixed mammary tumor have strong immunoreactivity for PTHrP (arrowheads). PTHrP (15-34)-immunoperoxidase staining. Bar=23.5μm

Fig. 1b: Case 3. Myoepithelial cells surrounding the neoplastic mammary gland (lower right of the figure) and myoepithelial cell-derived chondrocytes in the cartilage (Upper left of the figure) have positive staining for PTHrP. PTHrP (1-34)-immunoperoxidase staining. Bar=29.4μm

Fig. 1c: Case 3. Osteoblasts in the bone tissue express PTHrP immunoreactivity in their cytoplasm. PTHrP (1-34)-immunoperoxidase staining. Bar=11.7μm

Fig. 1d: Case 3. Intense cytoplasmic PTHrP immunoreactivity in the osteoclasts of bone tissue. Bar=14.7μm

Fig. 2: Case 4. The neoplastic epithelial cells that are papillary proliferating in the alveolus express PTHrP immunoreactivity. PTHrP (1-19)-immunoperoxidase staining. Bar=18.4μm

Fig. 3: Case 5 with normocalcemia. A few myoepithelial cells in the myxomatous areas have PTHrP immunoreactivity (arrowhead). PTHrP (1-34)-immunoperoxidase staining. Bar=23.5μm

Fig. 4: Case 6 with normocalcemia. There is no PTHrP-immunoreactivity in the neoplastic alveolar epithelial cells or the surrounding myoepithelial cells. PTHrP (1-34)-immunoperoxidase staining. Bar=23.5μm

malignancy-associated hypercalcemia[5]. However, widespread localization of PTHrP in normal canine and human tissues, including the epidermis and mammary gland, has been demonstrated in non-association with hypercalcemia by immunohistochemistry. In the normal canine mammary gland, alveolar epithelial cells, duct epithelial cells and myoepithelial cells are positive for the protein[2,11]. Expression of PTHrP mRNA has been also reported in chondrocytes and osteoblasts of the cartilage and bone tissues, respectively, of mice by *in situ* hybridization[1]. It has been considered that autocrine and/or paracrine PTHrP modulates the proliferation and differentiation of its target cells in the normal tissues[1,16]. In canine mammary tumors, although clinical signs, including the serum calcium level, were not investigated, the immunohistological expression of the protein was demonstrated in alveolar epithelial cells, myoepithelial cells and metaplastic chondrocytes of all 58 canine mammary tumors tested, including benign and malignant mixed tumors[11]. PTHrP positive cells were also found in ovarian small cell carcinomas of both hypercalcemic and normocalcemic human patients[7]. The precise function of PTHrP in tumors, including mammary cancer, is currently unknown, but it may play a role in the growth and differentiation of tumor cells as well as normal cells[3,6]. Thus, PTHrP is detectable in many normal cells and tumors in non-association with hypercalcemia by immunohistochemistry. In fact, we detected the immunoreactivity in proliferating MECs in 2 of the 4 normocalcemic dogs.

In our present study, there was an increased number of PTHrP-immunoreactive cells in the neoplastic mammary tissues with hypercalcemia compared with normocalcemia, and the positive staining intensity was greater in the hypercalcemic dogs than in the normocalcemic dogs. In addition, the alveolar epithelial cells and MECs of normal mammary gland adjacent to the tumor tissues showed no immunoreactivity. This study reports for the first time the relationship between the serum calcium level and PTHrP-immunoreactivity of mammary tumor tissue. Although PTHrP presents ubiquitously in tissues, circulating (serum) PTHrP is thought to be a useful predictive marker of HHM[10]. In fact, the serum PTHrP levels in human patients with HHM are significantly higher than those in normal subjects. The time course in two hypercalcemic patients with esophageal carcinoma revealed that serum PTHrP levels were elevated before hypercalcemia developed and that changes in PTHrP and corrected serum calcium levels were significantly correlated[10]. Thus, the possibility arises that the increased number and staining intensity of immunoreactive cells in the hypercalcemic dogs as compared with the normocalcemic dogs is due to circulating PTHrP secreted from the tissues at a level sufficient to elevate the serum calcium level. Quantitative studies of PTHrP in serum samples by ELISA and its mRNA in tissues by *in situ* hybridization and northern blotting are needed to prove this hypothesis.

One of the authors of the present paper, Okada[11], showed that microwave heating enhances the immunoreactivity for PTHrP in both tumor cells and adjacent normal mammary tissue. However, that pretreatment had no effect on the tumors of the present cases in spite of same heating conditions. Chicken polyclonal antibody against human PTHrP (1-36) was used as primary antibodies in his study. Antigen-retrieval with microwave heating might depend on the property of the primary antibody. The effect of microwave heating on antigen-retrieval also depends on fluid media and heating time[4]. To detect a small amount of PTHrP, optimum conditions, including those of fluid media and heating time have to be ev-

aluated.

References.

1) Amizuka, H., Karaplis, A. C., Henderson, J. E., Washawsky, H., Lipman, M. L., Matsuki, Y., Ejiri, S., Tanaka, M., Izumi, N., Ozawa, H. and Goltzman, D. 1996. Haploinsufficiency of parathyroid hormone-related peptide (PTHrP) results in abnormal postnatal bone development. *Develop. Biol.,* 175: 166-176.
2) Gröne, A., Wekmeister, J. A., Steinmeyer, C. L., Caper, C. C. and Rosol, T. J. 1994. Parathyroid hormone-related protein in normal and neoplastic canine tissues: immunohistochemical localization and biochemical extraction. *Vet. Pathol.,* 31: 308-315.
3) Hastings, R. H., Summers-Torres, D., Cheung, T. C., Ditmer, L. S., Petrin, E. M., Burton, D. W., Spragg, R. G., Li, J. and Deftos, L. J. 1996. Parathyroid hormone-related protein, an autocrine regulatory factor in alveolar epithelial cells. *Am. J. Physiol.,* 270: 353-61.
4) Imam, S. A., Young, L., Chaiwun, B. and Taylor, C. R. 1995. Comparison of two microwave based antigen-retrieval solutions in unmasking epitopes in formalin-fixed tissue for immunostaining. *Anticancer Research,* 15: 1153-1158.
5) Kaiser, S. M. and Goltzman, D. 1993. Parathyroid hormone-related peptide. *Clin. Invest. Med.,* 16: 395-406.
6) Malakouti, S., Asadi, F. K., Kukreja, S. C., Abcarian, H. A. and Cintron, J. R. 1996. Parathyroid hormone-related protein expression in the human colon: immunohistochemical evaluation. *American Surgeon,* 62: 540-545.
7) Maties-Guiu, X., Prat, J., Young, R. H., Capen, C. C., Rosol, T. J., Delellis, R. A. and Scully, R. E. 1994. Human parathyroid hormone-related protein in ovarian small cell carcinoma. *Cancer,* 73: 1878-81.
8) Meuten, D. J., Cooper, B. J., Capen, C. C., Chew, D. J. and Kociba, G. J. 1981. Hypercalcemia in dogs with adenocarcinoma derived from apocrine glands of the anal sac. *Vet. Pathol.,* 18: 454-471.
9) Moulton, J. E. 1990. Tumors in Domestic Animals, 3rd ed., pp.518-552. University of California Press, Berkeley.
10) Nagasaki, K., Otsubo, K., Kajimura, N., Tanaka, R., Watanabe, H., Tachimori, Y., Kato, H., Yamaguchi, H., Saito, D., Watanabe, T., Adachi, I. and Yamaguchi, K. 1996. Circulating parathyroid hormone-related protein (109-141) in malignancy-associated hypercalcemia. *Jpn. J. Clin. Oncol.,* 26: 6-11.
11) Okada, H., Nishijima, Y., Yoshino, T., Gröne, A., Capen, C. C. and Rosol, T. J. 1997. Immunohistochemical localization of parathyroid hormone-related protein in canine mammary tumors. *Vet. Pathol.,* 34: 356-359.
12) Ralston, S. H., Gallache, S. S. and Patel, U. 1990. Cancer-associated hypercalcemia: morbility and mortality. *Ann. Intern. Med.,* 112 : 499-504.
13) Rosol, T. J., Capen, C. C., Danks, J. A., Suva, L. J., Steinmeyer, C. L., Hayman, J., Ebeling, P. R. and Martin, T. J. 1990. Identification of parathyroid hormone-related protein in canine apocrine adenocarcinoma of the anal sac. *Vet. Pathol.,* 27: 89-95.
14) Rosol, T. J., Capen, C. C., Danks, J. A., Suva, L. J., Steinmeyer, C. L., Hayman, J., Ebeling, P. R. and Martin, T. J. 1992. Biology of disease. Mechanisms of cancer-induced hypercalcemia. *Lab. Invest.,* 67 : 680-702.
15) Stewart, A. F., Wu, T., Goumas, D., Burtis, W. J. and Broadas, A. E. 1987. N-terminal amino-acid sequence of two novel tumor-derived adenylate cyclase-stimulating proteins: Identification of parathyroid hormone-like and parathyroid hormone-unlike domains. *Biochem. Biophys. Res. Commun.,* 146: 672-682.
16) Suda, N., Shibata, S., Yamazaki, K., Kuroda, T., Senior, P. V., Beck, F. and Hammond, V. E. 1999. Parathyroid hormone-related protein regulates proliferation of condylar hypertrophic condrocytes. *J. Bone Miner. Res.,* 14: 1838-1847.