PATHOLOGICAL STUDIES ON THE ROLES OF BONE MORPHOGENETIC PROTEINS IN CANINE MAMMARY GLAND TUMORS

（イヌ乳腺腫瘍における骨形成因子の役割についての病理学的検討）

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BIOGRAPHY

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CONTENTS

PREFACE

Mammary gland tumor is one of the most common neoplasms in female dogs. Complex adenoma and benign mixed tumor are characterized by prominent proliferation of myoepithelial cells and of ectopic mesenchymal tissue, including cartilage and bone, which are the unique features of canine mammary gland tumor. In human breast tumors, proliferation of myoepithelial cells is rather rare phenomena, while human pleomorphic adenomas in the salivary gland have similar morphological natures to those of canine benign mixed tumors in the mammary gland. Although the mechanisms of the mesenchymal tissue formation in these tumors have been studied for many years, the conclusion has not been made. Several previous reports suggest that neoplastic or reactive myoepithelial cells may transform to the form these ectopic mesenchymal elements. Myoepithelial cells in both human salivary pleomorphic adenomas and canine mammary tumors are intensely positive for bone morphogenetic proteins (BMP)s. The findings indicate that the molecules and associating pathway may be involved in the ectopic cartilage or bone formation. In the meantime, Chondromodulin-I (ChM-I), which is involved in stimulating the proliferation of chondrocytes, is also suggested as an important factor, which is associated with the formation of ectopic cartilage and bone in human pleomorphic adenoma in thesalivary gland. However, there is little information concerning on the roles of ChM-I on the

mesenchymal tissue formation in canine mammary gland tumors.

Although the roles of BMPs on ossification have been studied for recent decades, other functions of BMPs including oncogenesis have been proposed. The expression of BMPs was detected in human melanoma, suggesting an important factor for the promotion of the tumor. In addition, BMPs are associated with the tumor growth in the lung carcinoma. In terms of the originally identified functions of BMPs, the oncology of skeletal tumor including osteosarcoma should be one of the important topics to investigate the malignant behavior. Osteosarcoma (OSA) is a malignant mesenchymal tumor with the proliferation of bone by the neoplastic osteoblastic cells. Several reports suggest that derivation of mammary gland OSA may be resulted from anaplastic transformations of myoepithelial cells. Mammary gland OSA is one of the common tumors of extraskeletal OSAs characterized by the proliferation of anaplastic cells with osteoid production without bone or perosteal involvement. OSA has the high potential for metastasis and poor clinical prognosis. Therefore, it is required to clarify the molecular mechanisms of the progression of OSA for more targeted therapies. In the malignant behaviors of OSA, invasiveness is an essential and important ability to form metastatic lesions. Several studies revealed that factors such as urokinase-type plasminogen activator, nuclear kappa B, Wnt-catenin pathway and BMP signaling

pathway might be associated with the malignant behavior of OSA. Among these factors, it was reported that BMP signaling pathway might be correlated to the invasiveness in human OSA. However, the molecular mechanisms of OSA metastasis have not been concluded. Especially, BMP-2 and BMPRII mRNA expression proved to be associated with a poorer prognosis for patients with OSA. Moreover, BMP-4 is suggested as an important factor, which may play roles in the malignant behavior of OSA.

The thesis is written as the final part of the education that leads to the professional title "Doctor of Philosophy" in the Veterinary field at the United Graduate School of Veterinary Sciences of Yamaguchi University. The main purposes are to accomplish better understanding about the tumor matrix formation of mammary gland tumor and about the role of BMP in canine OSA. Therefore, the chapter I described the association of ChM-I with ectopic mesenchymal tissue formation and compared the distribution patterns of ChM-I and BMP-6 among several types of canine mammary gland tumors. Then, because there are few cell lines of canine OSA with BMPs-expression and ability of ossification after transplantation into mice, the chapter II demonstrated the establishment of a cell line derived from a canine mammary gland OSA. The established cell line, MCO-Y4, was characterized whether these have the

ability for ossification and the expression of BMP-2/4 and BMPR II *in vitro* and *in vivo*. In addition, the effect of the administration of several extracellular matrix including fibronectin and noggin that is the inhibitor of the BMP signaling pathway on the proliferative activity of the established MCO-Y4 cells was investigated. Finally, to clarify the role of BMP signaling pathway in OSA, the chapter III described the biological functions of BMP-4 *in vitro* and *in vivo* in MCO-Y4. Then considering the results obtained from the series of the researches, the author proposed the roles of BMP signaling pathway in canine mammary gland tumors, especially in the mesenchymal tissue formation and associated malignancy.

CHAPTER I

Co-localization of Chondromodulin-I (ChM-I) and Bone Morphogenetic Protein-6 (BMP-6) in Myoepithelial Cells of Canine Mammary Tumors

ABSTRACT

To compare the roles of chondromodulin-I (ChM-I) and bone morphogenetic protein-6 (BMP-6) in ectopic mesenchymal tissue formation in canine mammary gland tumors, 33 tumors and 2 normal mammary glands were examined. Immunohistochemical analysis revealed co-expression of ChM-I and BMP-6 in canine mammary tumors. In mixed tumors, newly formed woven bone with ossified cartilage matrix was observed in 4/9 cases. The osteoblasts lining the woven bone showed moderate immunoreactivity to ChM-I and BMP-6. Almost all chondrocytes and proliferative myoepithelial cells within the basement membrane showed intense immunoreactivity to both, and the myoepithelial cells adjacent to the mature cartilage showed the most intense immunoreactivity. The immunoreactivity to ChM-I and BMP-6 of the interstitial myoepithelial cells in the myxomatous stroma varied in each focus of mixed tumors. Similar findings were found in complex adenomas. In simple adenomas, hyperplasic myoepithelial cells within the basement membrane showed moderate immunoreactivity to both markers. Western blot analysis detected a 25 kDa band of ChM-I in fresh tissue samples from three mixed tumors. Our results support the hypothesis that proliferating myoepithelial cells with ChM-I and BMP-6 expression play important roles in mesenchymal metaplasia in canine mammary tumors.

INTRODUCTION

Mammary gland tumors are the most common neoplasms in female dogs [15]. Their most unique morphological features are prominent proliferation of myoepithelial cells and formation of ectopic mesenchymal tissue, including cartilage and bone, especially in complex adenomas and benign mixed tumors. Although proliferation of myoepithelial cells is rare in human breast tumors, salivary pleomorphic adenoma is characterized by mixed proliferation of both glandular epithelial and myoepithelial cells with cartilage or bone formation. Several reports suggest that neoplastic myoepithelial cells contribute to the formation of these ectopic mesenchymal elements [4, 79].

Chondromodulin-I (ChM-I), a 25 kDa glycosylated protein composed of 335 amino acids, was first extracted and cloned from fetal bovine cartilage [9, 33]. The major biological functions of ChM-I are to stimulate the proliferation of chondrocytes and inhibit angiogenesis [30-32]. Several reports have indicated that, in human salivary pleomorphic adenomas, ChM-I might be expressed in the lacunar cells of the chondroid matrix and in the myoepithelial cells [48, 49]. These observations suggest that myoepithelial cells with ChM-I expression play major roles in the formation of ectopic cartilage and bone in these human tumors. However, there is little

information on the roles played by ChM-I in canine mammary gland tumors.

Bone morphogenetic proteins (BMPs), belonging to a subgroup of the transforming growth factor-β (TGF-β) superfamily, and their receptors, have been detected in myoepithelial cells in canine mammary tumors [4, 79]. BMPs were originally identified as important factors for endochondral ossification [72, 82, 83]. BMP-6 is also involved in the development of the embryonic urinary system and in the differentiation of keratinocytes [12, 17]. Myoepithelial cells in human salivary pleomorphic adenomas are intensely positive for BMPs, suggesting that BMPs might be involved also in ectopic cartilage or bone formation in the tumor [28, 85].

Our aim was to examine the association of ChM-I with ectopic mesenchymal tissue formation and compare the distribution patterns of ChM-I and BMP-6 among several types of canine mammary gland tumors.

MATERIALS AND METHODS

Tissue samples: Surgical specimens from 33 mammary tumors and two normal mammary gland tissues were used. The tissues were fixed in methanol-Carnoy's solution. Paraffin sections 4 μm thick were made and stained with hematoxylin and eosin (HE) for routine histopathological examination. The diagnosis of each tumor

was based on the World Health Organization (WHO) classification [55]. The diagnoses are summarized in Table 1.

Antibodies: Immunohistochemistry was performed using an avidin-biotin-peroxidase complex (ABC) kit (PK4000, Vectastain, Burlingame, CA, USA). Goat antisera against human ChM-I (1:20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and BMP-6 (1:20, Santa Cruz Biotechnology) were used as primary antibodies. The secondary antibody was a biotinylated rabbit serum against goat immunoglobulin (1:20, Dako-Japan, Kyoto, Japan).

Immunohistochemistry: Sections were incubated with 3% hydrogen peroxide in methanol at room temperature for 10 min to block endogenous peroxidase activity. Sections were incubated at 37℃ for 40 min with phosphate-buffered saline (PBS) (pH 7.4) containing 3% bovine serum albumin (BSA) to avoid nonspecific binding. The sections were then incubated with primary antibodies at 37℃ for 45 min, followed by incubation with secondary antibody and ABC reagent at 37℃ for 45 min, respectively. The sections were exposed to 3, 3-diaminobenzidine-4HCl (DAB, Sigma, St.Louis, MO, USA) and then counterstained with Mayer's hematoxylin. In accordance with

the findings of previous reports [4, 17, 58, 72], mammary cells were classified into 4 types: (1) glandular epithelial cells, (2) resting and proliferating myoepithelial cells within the basement membrane, (3) proliferating myoepithelial cells at the interstitial myxomatous areas, and (4) chondrocytes in the ectopic cartilage. The intensity and distribution of immunoreactivity to the antibodies were quantified by assessing the labeled cells in 10 high power fields (×400) as follows: (−)=0%, (±)= 0%–5%, (+) = 5%–10%, (2+) = 10%–50% , and (3+) = >50%.

Western blot analysis: Fresh tissue samples from three benign mixed tumors were used for Western blot analysis. Western blot analysis was performed according to a previous report [58]. Briefly, samples were homogenized in extraction buffer and sonicated on ice for 2.5 min; this was repeated 5 times. Then the extracts were centrifuged at 10000×g for 10 min. The supernatants were dialyzed through a cellophane tube (Wako, Osaka, Japan) and freeze-dried. The samples obtained were separated by SDS-12% polyacrylamide gel electrophoresis and transferred to a polyvinylidiene difluoride (PVDF) membrane (Atto, Tokyo, Japan). After incubation with antibody against human ChM-1 (1:20, Santa Cruz Biotechnology) at 37°C for 1hr, the membrane was incubated with biotinylated rabbit anti-goat IgG (1:20, Dako-Japan)

at 37°C for 1 hr. The membrane was then reacted with ABC reagents at 37°C for 30 min. The attached antibodies were visualized using DAB (Sigma).

RESULTS

The distribution patterns of ChM-I- and BMP-6-positive myoepithelial cells were almost the same. The results of immunohistochemical analysis for ChM-I and BMP-6 are summarized in Tables 2 and 3.

Simple adenomas (n = 4): The tumors were composed of well-differentiated glandular or tubular epithelial cells with resting myoepithelial cells. The neoplastic epithelial cells showed two proliferating patterns, tubular (two cases) or papillary (two cases). In three cases, weak to moderate immunoreactivity to both ChM-I and BMP-6 was observed in the resting myoepithelial cells (Fig.1). A few neoplastic glandular epithelial cells were weakly positive for both. Myoepithelial cells in tubular adenomas tended to show more intense immunoreactivity to BMP-6 than those in papillary adenomas.

Complex adenomas (n = 8): The tumors were composed of glandular or tubular

epithelial cells and myoepithelial cells with various amounts of myxomatous stroma. Moderate to mild immunoreactivity to ChM-I was observed in the proliferative intraductal myoepithelial cells in all cases. Although immunoreactivity to ChM-I was weak in the interstitial myxomatous myoepithelial cells, the intraductal myoepithelial cells in most cases were intensely positive for ChM-I (Table 2 and Fig. 2). Most neoplastic glandular epithelial cells were negative for ChM-I. The distribution pattern of BMP-6 in 8 complex adenomas was consistent with that of ChM-I (Fig. 2). Immunoreactivity to BMP-6 in the proliferating myoepithelial cells in both intraductal and interstitial regions was mild compared with that to ChM-I (Table 2).

Benign mixed tumors (n = 9): In this type of tumor, ectopic cartilage formation was observed in addition to the proliferation of both myoepithelial and glandular epithelial cells. The most intense reaction to both ChM-I and BMP-6 was detected in the lacunar cells of the chondroid matrix in 6 of 9 cases (Fig. 3). Proliferating myoepithelial cells adjacent to the ectopic cartilage also showed intense immunoreactivity to both (Fig. 4). Woven bone with calcified cartilage matrix surrounded by osteoblasts was observed in 4 of 9 cases (Table 2). The osteoblasts lining the newly formed woven bone showed moderate immunoreactivity to both

markers (Fig. 5). Immunoreactivity to ChM-I in the proliferating intraductal myoepithelial cells varied among cases. Intense reaction to BMP-6 was observed in the intraductal and interstitial myxomatous myoepithelial cells of mixed tumors (Table 2). Proliferating myoepithelial cells in the interstitial myxomatous regions showed mild immunoreactivity to ChM-I and intense immunoreactivity to BMP-6 (Fig. 6). The immunoreactivity of the interstitial myoepithelial cells tended to mild compared with that of the intraductal myoepithelial cells. The neoplastic glandular epithelial cells were negative for both markers.

Simple adenocarcinomas (n = 8): This type of tumor was composed of poorly differentiated tubular and glandular epithelial cells. The proliferating patterns of these tumors were tubular (one case), papillary (three cases) and solid (4 cases). The distribution patterns of ChM-I and BMP-6 in the tumors were consistent with those of simple adenomas (Table 3). Proliferating myoepithelial cells were rare in solid adenocarcinomas. In tubular adenocarcinomas, resting and proliferating myoepithelial cells showed intense immunoreactivity for BMP-6 and ChM-I compared with the level of reactivity in tubular adenomas. The myoepithelial cells in papillary adenocarcinomas showed mild immunoreactivity to both markers compared with those

in papillary adenomas. In all types of adenocarcinomas, the neoplastic glandular epithelial cells were weakly positive for both markers.

Complex adenocarcinomas (n = 2): The morphological features of these tumors were consistent with those of complex adenoma, with the exception of apparent cellular atypia and invasive activity of neoplastic glandular epithelial and/or myoepithelial cells. The results of ChM-I- and BMP-6-immunostaining were consistent with those in complex adenomas (Table 3).

Adenocarcinomas in benign mixed tumors (n = 2): The morphology of these tumors was similar to that of benign mixed tumors, except for the malignant features of neoplastic epithelial cells. Intraductal and interstitial myoepithelial cells were intensely positive for ChM-I. However, these myoepithelial cells were weakly positive for BMP-6 (Table 3).

Normal mammary gland tissues (n = 2): In intact mammary glands around the tumor mass, resting myoepithelial cells were weakly positive for both markers. The glandular epithelial cells were totally negative (Table 2).

Western blot analysis: Western blot analysis was performed to examine the presence of ChM-I protein in benign mixed tumors. Bands immunopositive for ChM-I were detected at 25kDa in all three mixed tumors examined; this corresponds to the molecular weight of human ChM-I (Fig. 7).

DISCUSSION

ChM-I was distributed in neoplastic myoepithelial cells in canine mammary tumors. Arai *et al.* [6] indicated that class II β-tubulin was associated with cartilaginous metaplasia of proliferative myoepithelial cells in canine benign mixed tumors. Gama *et al.* [20] also suggested p63 as an additional marker of myoepithelial histogenesis. Although previous studies have suggested that metaplasia of proliferative myoepithelial cells might be involved in the formation of cartilage or bone in canine benign mixed tumors, this hypothesis has not been completely supported. Our present results indicate that expression of ChM-I in myoepithelial neoplastic cells might be involved in the formation of cartilage in canine benign mixed tumors. Expression of ChM-I was observed in myoepithelial cells within several types of canine mammary tumors, especially in complex and mixed tumors. The basic distribution pattern of ChM-I-immunopositive cells was similar to that of BMP-6

positive cells, as previously reported [4, 79]. This suggests that ChM-I and BMPs play important roles in the metaplastic process.

Immunoreactivity to BMP-6 was observed in intraductal and interstitial myxomatous myoepithelial cells in complex adenomas and benign mixed tumors. Although intense immunoreactivity to ChM-I was observed in chondrocytes, interstitial myxomatous myoepithelial cells showed weak immunoreactivity to ChM-I. The most intense immunoreactivity to ChM-I was observed in the proliferative myoepithelial cells adjacent to ectopic cartilage and mature chondrocytes. The intense expression of ChM-I in these cells may be explained by the biological nature of this protein as an inducer of cartilage and bone formation [9, 31-33,78]. These observations suggest that both ChM-I and BMP-6 play roles in the metaplastic change of myoepithelial cells, and especially that ChM-I might contribute to metaplastic change of the cells into mature chondrocytes.

In addition, the osteoblasts lining the woven bone showed moderate immunoreactivity for ChM-I and BMP-6. Although ChM-I was first isolated as a growth-promoting factor from chondrocytes [34], Suzuki [77] suggested that ChM-I and - II might be associated with endochondral ossification. Nakamichi *et al.* [58] also reported that ChM-I knockout mice showed a significant increase in bone mineral

density and suggested that ChM-I might play a role in endochondral bone development. In agreement with these observations, our results suggested that ChM-I might be associated with not only chondral metaplasia but also osseous metaplasia of mixed tumors. Interestingly, intraductal myoepithelial cells showed distinct immunoreactivity to ChM-I and BMP-6, even in simple adenomas and in histologically intact mammary glands. This indicated that they might have certain roles in regulating the proliferation and/or transformation of myoepithelial cells. However, immunoreactivity to both markers was slightly decreased in the malignant counter parts of these tissues, suggesting that the intense expression of ChM-I and BMP-6 in myoepithelial cells might decrease with proliferation outside the basement membrane.

We observed the expression of ChM-I and BMP-6 in canine mammary gland tumors. Co-localization of ChM-I and BMP-6 in myoepithelial cells might be associated with mesenchymal metaplasia of canine mammary gland tumors. BMP-6 might be involved in all stages of metaplasia of myoepithelial cells to cartilages or bone, and ChM-I might especially participate in the latter process of cartilage formation, and also in endochondral ossification. To confirm some other additional roles of ChM-I, such as inhibition of the proliferation of neoplastic cells, further studies *in vivo* and *in*

vitro will be required.

Table. 1. Diagnosis of canine mammary tumors examined

Histological diagnosis	Number of cases
Normal	2
Simple adenoma	
Tubular	2
Papillary	2
Complex adenoma	8
Benign mixed tumor	9
Adenocarcinoma	
Tubular	1
Papillary	3
Solid	4
Complex adenocarcinoma	2
Carcinoma in mixed tumor	2
Total	35

Table. 2. Immunoreactivity to ChM- I and BMP-6 in canine benign mammary tumors

Diagnosis	Case No.	* Glandular ECs		Intraductual MCs		Interstitial MCs		Chondrocytes		Otesoblasts	
		ChM- I	BMP-6	ChM- I	BMP-6	ChM- I	BMP-6	ChM- I	BMP-6	ChM- I	BMP-6
Normal (n=2)	1	-	-	±	+						
	2	-	-	-	-						
Simple adenoma (n=4)											
Tubular	3	±	±	2+	+						
Papillary	4	-	±	±	2+						
	5	±	-	2+	2+						
	6	-	-	+	-						
Complex adenoma (n=8)	7	±	-	2+	2+	2+	±				
	8	-	-	2+	+	±	2+				
	9	±	±	2+	2+	±	2+				
	10	-	±	2+	2+	+	±				
	11	-	-	+	±	±	±				
	12	±	-	2+	+	±	±				
	13	±	-	+	±	±	-				
	14	±	±	2+	2+	±	±				
Benign mixed tumor (n=9)	15	-	-	+	2+	+	+	2+	2+	+	+
	16	±	±	2+	2+	±	±	3+	+		
	17	±	±	2+	2+	±	+	2+	3+	+	+
	18	±	±	2+	2+	+	2+	2+	2+	+	+
	19	-	-	+	±	±	+	+	+		
	20	±	±	2+	2+	2+	2+	+	2+	+	+
	21	±	±	3+	2+	2+	2+	2+	2+		
	22	±	±	2+	2+	2+	2+	2+	3+		
	23	±	±	±	2+	+	±	2+	+		

* EC=epithelial cells: MC=myoepithelial cells.

(-)= 0%, (±)=0-5%, (+)= 5-10%, (2+)= 10-50% and (3+)= >50% positive cells.

Table 3. Immunoreactivity to ChM- I and BMP-6 in canine malignant mammary tumors

	Case No.	* Glandular ECs		Intraductual MCs		Interstitial MCs		Chondrocytes	
		ChM- I	BMP-6	ChM- I	BMP-6	ChM- I	BMP-6	ChM- I	BMP-6
Adenocarcinoma (n=8)									
Tubular									
Papillary	24	±	-	2+	2+				
	25	-	-	+	+				
	26	-	-	-	-				
Solid	27	±	-	+	±				
	28	±	±						
	29	-	±						
	30	-	-						
Complex adenocarcinoma (n=2)	31	±	-						
	32	-	±	2+	2+	+	+		
Carcinoma in mixed tumor (n=2)	33	±	±	2+	+	2+	±		
	34	±	-	2+	+	±	±	2+	2+
	35	±	±	2+	2+	2+	+	3+	2+

* EC=epithelial cells: MC=myoepithelial cells.

(-)= 0%, (±)=0-5%, (+)= 5-10%, (2+)= 10-50% and (3+)= >50% positive cells.

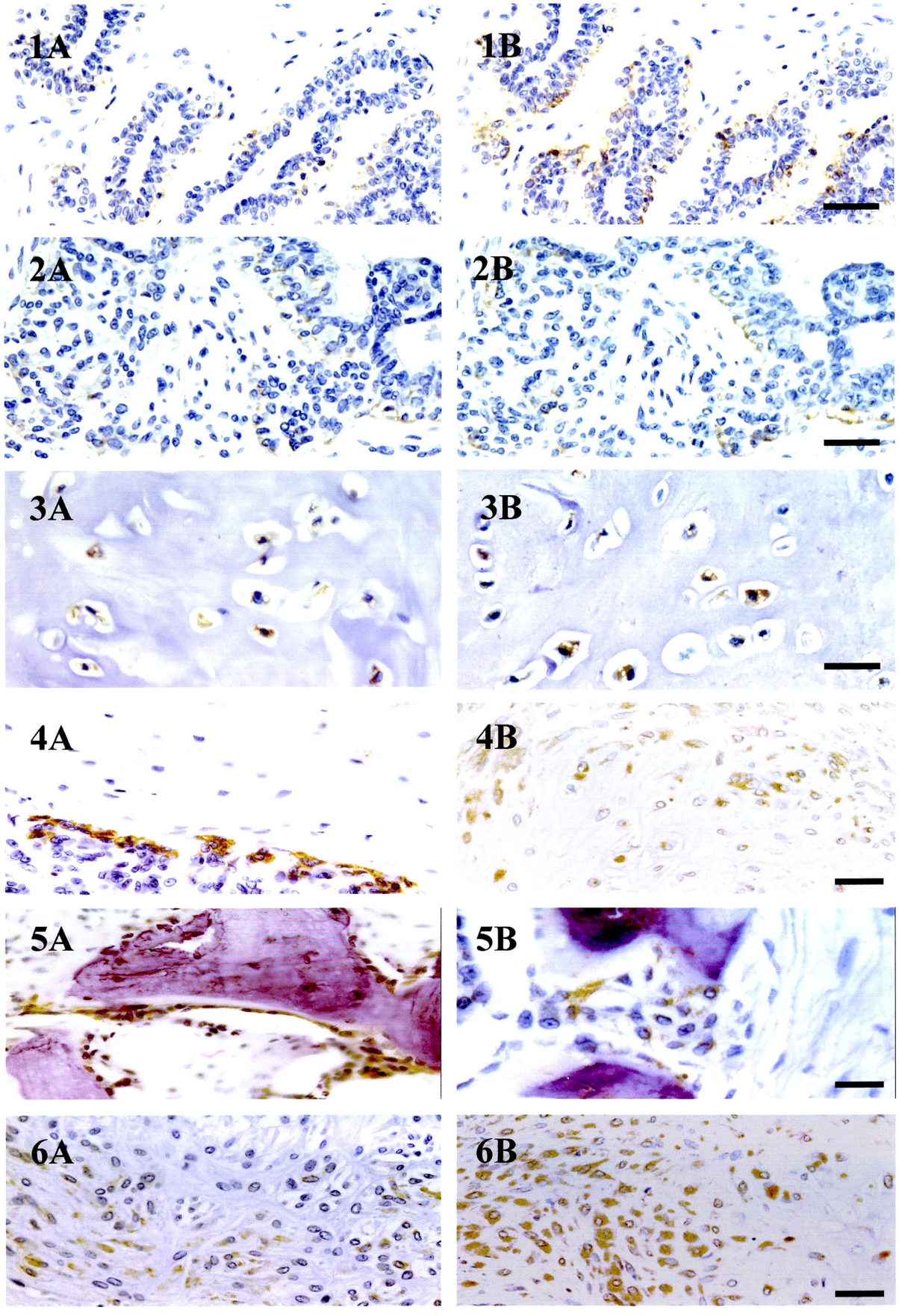

1A
1B
2A
2B
3A
3B
4A
4B
5A
5B
6A
6B

Fig. 1. Mammary gland; simple adenoma (case No. 3). Immunoreactivity of resting myoepithelial cells. ChM-I (A) and BMP-6 (B). Bar=60 μ m.

Fig. 2. Mammary gland; complex adenoma (case No. 14). Intense immunoreactivity of proliferating myoepithelial cells within the basement memabrane and weak reactivity of interstitial myoepithelial cells. ChM-I (A) and BMP-6 (B). Bar=60 μ m .

Fig. 3. Mammary gland; benign mixed tumor (case No. 22). Intense immunoreactivity of mature cartilage. ChM-I (A) and BMP-6 (B). Bar=70 μ m.

Fig. 4. Mammary gland; benign mixed tumor (case No. 21). Intense immunoreractivity of the proliferating myoepithelial cells adjacent to the ectopic cartilage. ChM-I (A) and BMP-6 (B). Bar=50 μ m.

Fig. 5. Mammary gland; benign mixed tumor (case No.18). Mild or moderate immunoreactivity of the osteoblastic cells. ChM-I (A) and BMP-6 (B). Bar=70 μ m.

Fig. 6. Mammary gland; benign mixed tumor (case No. 21). Moderate immunoreactivity of interstitial myxomatous myoepithelial cells. ChM-I (A) and BMP-6 (B). Bar=50 μ m.

Fig. 7

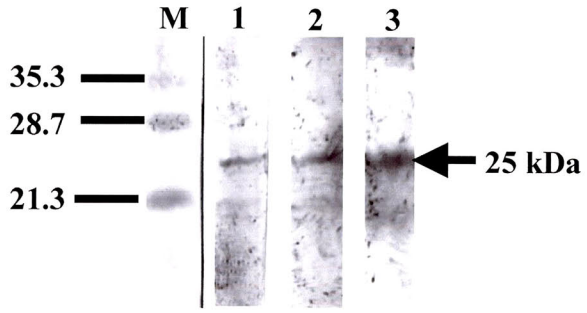


Fig. 7. Western blot analysis for a 25kDa protein of ChM-I from fresh tissue samples of 3 benign mixed tumors. M. Low molecular weight marker. Lanes 1-3. Fresh tissues samples from benign mixed tumors.

CHAPTER II

Establishment and Characterization of a Cell Line, MCO-Y4, Derived from Canine Mammary Gland Osteosarcoma

ABSTRACT

A cell line, MCO-Y4, was established from a mammary gland osteosarcoma of a 16-year-old female mongrel dog. Histopathologically the tumor was composed of osteoblastic cells with an osteoid meshwork and chondroid matrix. The mean doubling time of the cells at the 93rd passage was 32.39 ± 4.66 hr. Immunohistochemically, the osteoblastic and chondroblastic cells were positive for bone morphogenetic protein (BMP)-2/4 and BMP receptor (BMPR) II. The cultured cells were spindle in shape during the growth and the confluent phases. No tumor matrix was detected in the culture dish by alcian blue staining or von-Kossa silver impregnation. MCO-Y4 cells on the chamber slides showed intense immunoreactivity for BMP-2/4 and BMPR II. Noggin, an antagonist for BMP-2/4, showed the growth inhibition on MCO-Y4 cells. In addition, fibronectin might be potential for stimulating growth of MCO-Y4 cells. When transplanted into severe combined immunodeficiency mice, the cells formed tumors consisting of solid proliferation of osteoblastic and fibroblastic cells with woven-bone trabeculae. These tumor cells were intensely positive for BMP-2/4 and BMPR II. Our results suggest that the cell line might be useful for studying the role of BMPs in canine osteosarcoma and the mechanism of ossification.

INTRODUCTION

Extraskeletal osteosarcoma (EOS) is a rare malignant mesenchymal tumor characterized by osteoid production without bone or perosteal involvement. In dogs, EOS occurs mainly in older animals and is observed in several locations [52, 66], especially the mammary glands [66]. Mammary gland osteosarcoma (MGO) has aggressive biological behavior, and commonly shows pulmonary metastasis [52].

Bone morphogenetic proteins (BMPs), a subgroup of the transforming growth factor family [45], were originally identified as an important factor involved in endochondral ossification [72, 73, 82, 83]. BMPs also play a role in intramembranous ossification [74], and signal by binding to serine-threonine kinase receptors [86] and BMP receptor (BMPR) types I and II. The heterodimer of BMPR II with BMPR IA or IB is critical for signal transduction through Smads. The activated BMPR phosphorylates Smad 1/5/8. The Smad complex was then translocated to the nucleus and binds specific sites in DNA and associated with other regulatory proteins. Noggin, originally cloned based on its dorsaling activity in *Zenopus* embryo, has been shown to be an antagonist which has high affinity to BMPs and decreases its bioactivites [76]. Several studies suggested that BMPs might be involved in the progression or metastasis of human osteosarcoma [24, 89].

Osteosarcoma cell lines have been established from human, dog and rat, and these cell lines have the ability to undergo ossification after xenotransplantation [36, 41, 50, 67, 69]. Thus the cell lines are useful for studies of the mechanism of ossification as well as the relationship between BMPs and metastasis of osteosarcoma. A cell line, D-17, which is derived from canine osteosarcoma and used widely, however, does not have the ability to form xenotransplanted tumor in mice.

In the present study, we established a cell line from a canine MGO that showed ossification following xenotransplantation, and examined the expression of BMP-2/4 and BMPR II in both the original and xenotransplanted tumors and the cultured cells. In addition, the effect of noggin on the proliferative activity of MCO-Y4 cells was investigated, and we demonstrated that fibronectin might be potential for growth stimulation.

MATERIALS AND METHODS

Case history: A 16-year-old female mongrel dog was admitted to the Teaching Animal Hospital at University of Miyazaki with a mass in the right mammary gland. Primary bone tumors were not observed by the gloss evaluation. The tumor, measuring approximately 4×5×2 cm, was removed surgically. Grossly, the white

mass was firm and divided into multiple lobules. Fresh tissue samples from the neoplastic mass were used for primary culture, and the remaining tissues were fixed for histopathology. The pathological diagnosis was established from these samples.

Histology: Most of the tissue samples were fixed in 10% formalin. For immunohistochemistry, small pieces of tissue were also fixed in methanol Carnoy's solution for 12-14 h. All of these fixed tissues were embedded in paraffin, then sections 4 □m thick were cut and stained with hematoxylin and eosin (HE). Some selected sections were also stained with alcian blue (pH 2.5) and von-Kossa silver impregnation. The neoplastic mass was diagnosed as MGO according to the World Health Organization (WHO) [55].

Establishment of the cell line: The tissue was dissected and digested at 37 ℃ for 2 h in a humidified atmosphere of 5% carbon dioxide in air with 4 mg/ml collagenase (232 U/mg Wako) in Dulbecco's Modified Eagle Medium (DMEM) and Ham's Mixture F-12 (Sigma) containing 10% fetal calf serum (FCS), 100 IU /ml penicillin and 100 □g/ml streptomycin. The tissue was cultured in 60-mm-diameter plastic dishes (Corning Coaster, Corning NY, U. S. A.) in DME/F-12 medium containing 10% FCS, 100 IU/ml penicillin and 100 μ g/ml streptomycin. The culture

dishes were maintained in 5% carbon dioxide in air at 37 ℃ and observed daily by a phase-contrast microscopy. The cells were subcultured by washing them with phosphate-buffered saline (PBS) containing 2 mM ethylendiaminetetraacetic acid (EDTA) and dispersed in 0.05% trypsin with PBS containing EDTA. Then the cells were placed in a new 60-mm dish at 60×10 cells/ml. The cells were stocked in DME/F-12 containing 10% FCS and 10% dimethylsulfoxide (DMSO).

Growth assay: To better understand, the effect of noggin on the proliferative activity in MCO-Y4 cells was examined. MCO-Y4 was incubated in DME and Ham's Mixture F-12 containing 5% FCS. After 12 hr for the adhesion of the cells, 1 or 10 μ g/ml of mouse recombinant noggin (R&D systems, Minneapolis, U.S.A.) were added to the media. The cells were detached and counted using hemotocytometer after 2 days. In addition, we examined the effect of fibronectin on the proliferation of the MCO-Y4 cells. MCO-Y4 were incubated in DME and Ham's Mixture F-12 containing 5% FCS on the non-coated chamber slides (LAB-TEK, Christchurch, New Zealand) or fibronectin-coated dishes (Wako, Osaka, Japan). The cells were detached and counted using hemotocytometer after 3 days.

Immunohistochemistry: Deparaffinized sections were incubated with 0.05% hydrogen peroxide in methanol for 20 min at room temperature to block endogenous

peroxidase activity, then incubated with 3% bovine serum albumin (BSA) at 37 ℃ for 20 min. The sections were incubated with primary antibodies against goat polyclonal human BMP-2/4 (1:20, Santa Cruz, CA, U. S. A.) and BMPR II (1:20, Santa Cruz) at 37 ℃ for 40 min, and then sections were incubated with biotinylated rabbit serum against goat immunogloblin (1:20, DAKO-Japan, Tokyo, Japan) at 37 ℃ for 40 min, followed by reaction with avidin-biotin-peroxidase complex (ABC) reagents (PK4000, Vectastain, Burlingame, CA, U. S. A.) at 37 ℃ for 30 min. The attached antibodies were visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, St.Louis, MO, U. S. A.) and counterstained with Mayer's hematoxylin. Immunocytochemistry for BMP-2/4 and BMPRII was performed on the cells plated on the non-coated chamber slides using 10% formalin fixation for 10 min at room temperature. In the growth assay, MCO-Y4 cells in the control and high dose groups were used for immnunostaing using Envision polymer reagents (Dako-Japan). The primary antibody was against mouse monoclonal proliferating cell nuclear antigen (PCNA, prediluted clone PC10, Dako-Japan). The results of immunohistochemistry for BMP-2/4 and BMPRII were quantified by assessing the labeled cells in 10 high-power fields (x200) using semi-quantitative analysis as follows: — = 0%, ± = 0-5%, + = 5-10%, 2+ = 10-50%, and 3+ = >50% positive cells.

Tumorigenicity: A suspension of 10^6 cells was inoculated subcutaneously into the back of three 6-week-old female severe combined immunodeficiency (SCID) mice. Two of the mice were killed 76 days after inoculation. The studies were approved by our institutional guidelines for animal care and use committee (admission number: 2004-062-3).

Chromosomal study: The cells were incubated with 0.01 μ g/ml of colcemid (Sigma) for 3 h. They were then trypsinized, washed and treated with a hypotonic solution of 0.075 M potassium chloride (Wako) and fixed in methanol-acetic acid. After drying, the cells were stained with Giemsa (Merck Japan, Tokyo, Japan) and photographed for counting.

Special staining of the cell line: At confluency, the cells were fixed with ethanol for 10 min at room temperature. Chondroid matrix and mineralization were visualized using alcian blue stain and von Kossa silver impregnation.

Statistical analysis: For statistical analysis of PCNA staining, analysis of variance (ANOVA) was carried out to evaluate the difference in positive cell number

between the control and low or high dose group. In addition, ANOVA was carried out to evaluate the difference in cell number between the fibronectin-coated dishes and non-coated chamber slides. The minimum level of significance was set at $P < 0.05$.

RESULTS

Histopathology of the original tumor: The nonencapsulated mammary mass was divided into multiple lobules by thin fibrous septa. Each neoplastic lobule was composed of a proliferation of chondroblastic cells and osteoblastic cells in the central and peripheral areas, respectively. Some lobules had large necrotic foci. The osteoblastic cells were surrounded by an extensive osteoid meshwork or chondroid matrix (Fig.1a), and multinucleated osteoclast-like giant cells were scattered among them. The osteoblastic cells each had an irregularly shaped nucleus of varying size with several conspicuous nucleoli, and eosinophilic cytoplasm. There were approximately 2-3 mitotic figures per high-power field. No calcification was observed by von Kossa silver impregnation. The results of immunohistochemistry are summarized in Table 1. The osteoblastic and chondroblastic cells showed intense immunoreactivity for BMP-2/4 (Fig.1b) and BMPR II (Fig.1c).

Characterization of the established cell line: When plated on the dish, the cells

had a spindle-shaped morphology in both the growth and confluent phases (Fig. 2a). Nuclear abnormalities characterized by large pleomorphic nuclei with prominent nucleoli were observed. Formation of neither extracellular matrix nor mineralization was observed by alcian blue staining and von Kossa silver impregnation. The mean doubling time of the cells at the 93rd passage was 32.39±4.66 h, and the cells reached a plateau on day 6 (Fig. 3). The mean number of chromosomes was 66 per cell, ranging from 46 to 99. In the chamber slides at 93rd passage, the neoplastic cells showed intense immunoreactivity for BMP-2/4 (Fig. 2b) and BMPR II (Fig. 2c).

Growth assay: Growth of MCO-Y4 cells was significantly decreased in 26.2 % and 46.3% in low and high dose group, respectively (Fig. 4a). The mean number of PCNA-positive cells decreased to 11% in the high dose group compared to the control group. (Fig. 4b). The mean number of MCO-Y4 cells on the non-coated dish and fibronectin-coated dish was 11 ± 0.84 and 14 ± 0.84, respectively. A significant difference between both dishes was observed (Fig. 5).

Tumorigenicity: Tumors were formed at the sites of injection of the cell line 8 weeks after inoculation in two SCID mice. These tumors measured 2×1×1.5 cm and 2×1.5×1 cm, respectively. Histologically, each tumor was composed of centrally

located woven-bone trabeculae with proliferation of osteoblastic or fibroblastic cells. The osteoblastic cells were irregularly round in shape with multiple nucleoli and had scanty eosinophilic cytoplasm. The fibroblastic cells showed a fascicular and/or interlacing growth pattern and were characterized by irregular nuclei with abundant eosinophilic cytoplasm. In the central area of the tumor, the intratrabecular stroma consisted of osteoblastic cells and collagen products (Fig. 6a). The results of immunohistochemistry are summarized in Table 2. The osteoblastic cells lining the trabeculae were positive for BMP-2/4 (Fig. 6b) and BMPR II (Fig. 6c). The fibroblastic cells showed mild immunoreactivity for BMP-2/4 (Fig. 6d) and weak immunoreactivity for BMPR II (Fig. 6e).

DISCUSSION

Histologically the original tumor was composed of osteoblastic cells with osteoid and a chondroid matrix, arranged in a fascicular and/or interlacing pattern. The origin of osteosarcoma in mammary gland is still unclear. In human osteosarcoma in breast, several researches revealed that the origin might be associated with connective tissue elements of pre-existing benign tumors such as fibroadenoma and papilloma [11, 22, 43, 60]. In the meantime, several reports suggested that derivation of

osteosarocoma in mammary gland or salivary gland which has embryological similarities with mammary gland might be an anaplastic myoepithelial cells or pluripotential cells [8, 29]. Although immunohistochemical features of the xenotransplanted tumors were similar to those of the original tumor, the morphology in SCID mice was not representative of the original tumor. The results suggested that MCO-Y4 could form a well-differentiated tumor with bone formation after xenotransplantation. Some human osteosarcoma cells have no ability to form bone in xenotransplanted tumor [5, 56]. Mechanism of bone formation is still unclear. This unique feature of the MCO-Y4 cells could be a useful tool for the study of ossification as well.

Interactions between integrin receptors and fibronectin have been said to be important for osteoblast differentiation [56, 57]. Some studies suggested that integrin might mediate signal transduction associated with differentiation, proliferation and matrix remodeling [2, 15, 38]. In humans, Nissinen *et al.* [61] have suggested that α 2 integrin might be associated with progression of osteosarcoma. In the present study, fibronectin might be potential for stimulating growth of MCO-Y4 cells, suggesting that MCO-Y4 cells could be used for study on interaction between proliferative activity of osteosarcoma and fibronectin.

No metastasis of the inoculated cells was observed in the SCID mice in the present study. Many factors are reported to be involved in the metastasis or progression of tumors. In human osteosarcoma, some proteases are suggested to be important for invasion or metastasis of osteosarcoma cells [19, 47]. Yoshikawa *et al.* [89] have reported that BMPs are the potential factors involved in the metastasis or progression of osteosarcoma. In the present study, the xenotransplanted tumor showed mild immunoreactivity for BMP-2/4 and intense immunoreactivity for BMPR II. Moreover in human melanoma, Rothhammer *et al.* [71] suggested that BMP-2, -4, and -7 might be important factors for tumor invasion or migration. Although we were unable to obtain any evidence for a link between the expression of BMP-2/4 or BMPR II and the lack of metastatic lesions observed in the present study, MCO-Y4 might be useful for further investigations of the association between BMPs and invasion or migration of osteosarcoma.

BMP-6 has been shown to induce apoptosis in the epidermis of murine skin [84]. Hamdy *et al.* [25] suggested that high levels of BMP-6 expression might be associated with bone formation in bone metastases from prostate cancer. Therefore, BMPs, BMPRs and Smads have been considered to be important factors for suppression, rather than progression or metastasis, of prostate and cutaneous tumors [37, 44, 51].

However, expressions of BMPs and their receptors have been confirmed in a human osteosarcoma cell line, suggesting a mechanism involving the simultaneous activation of BMPs and BMPRs in osteosarcoma [21]. In addition, expression of BMP-2 and BMPR II in osteosarcoma has been shown to be associated with poor prognosis [24, 88]. The present study demonstrated that the fibroblastic cells in the xenotransplanted tumor and the cultured cells were positive for BMP-2/4 and BMPR II and that no tumor matrix was present in the culture dishes. These observations suggested that the intense immunoreactivity for BMPs and BMPR II in the neoplastic cells might be associated with not only bone formation and calcification but also some other role in the neoplastic cells. The growth assay revealed that noggin might be potential for inhibiting the cell proliferative activity in MCO-Y4 cells. This result supported the hypothesis that BMP-4 might be an important factor for the malignant behavior of osteosarcomas [7]. Moreover, our results suggested that fibronectin might cause growth stimulation in MCO-Y4 cells. Further experiments will be needed to clarify the role of BMPs and BMPR II in canine MGO.

In conclusion, our MCO-Y4 that has been newly established from a canine MGO is characterized by proliferation of cells and ossification ability in the xenotransplanted tumor. Moreover our results suggested that BMP signal and

fibronectin might play roles in proliferative activity in MCO-Y4 cells. Hence, MCO-Y4 cells might be useful for the further study of progression and malignant behavior of canine osteosarcomas.

Table 1 Results of immunohistochemistry of original tumor and cultured cells

	Original		Cultured cells	
Antibodies	Osteoblastic cells	Chondroblastic cells	Non-coated chamber slide	Fibronectin-coated dish
BMP-2/4	3+	±	3+	3+
BMPR II	3+	±	3+	3+

— = 0%, ± = 0-5%, + = 5-10%, 2+ = 10-50%, and 3+ = >50% positive cells.

Table 2 Results of immunohistochemistry of xenotransplanted tumor

	Xenotransplantation No.1		Xenotransplantation No.2	
Antibodies	Osteoblastic cells	Fibroblastic cells	Osteoblastic cells	Fibroblastic cells
BMP-2/4	3+	+	3+	+
BMPR II	3+	±	3+	±

— = 0%, ± = 0-5%, + = 5-10%, 2+ = 10-50%, and 3+ = >50% positive cells.

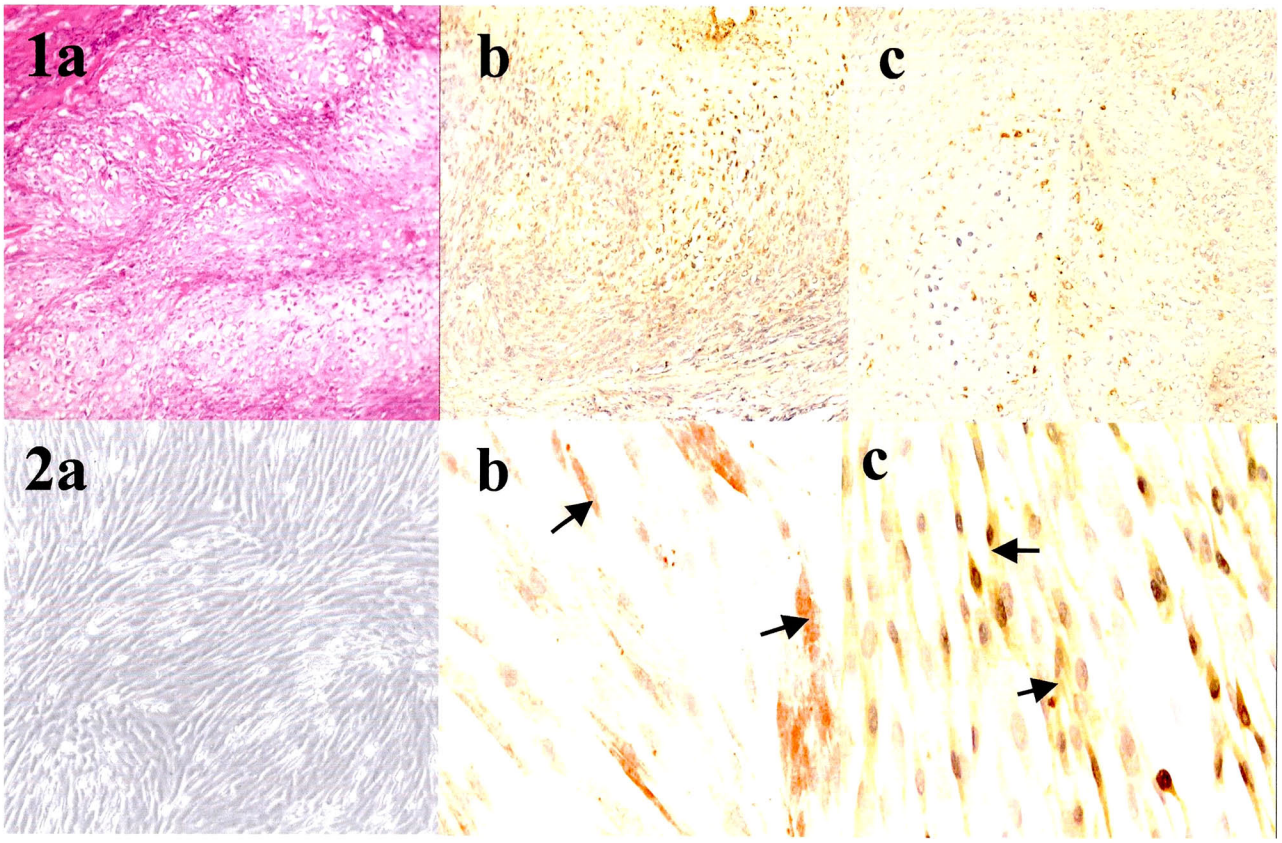


Fig. 1. Original tumor. Fig. 1a. The osteoblastic cells are surrounded by an extensive osteoid meshwork or chondroid matrix. HE stain. Original magnification, ×100. Fig. 1b. Immunostaining for BMP-2/4. The osteoblastic and chondroblastic cells are intensely positive for BMP-2/4. Original magnification, ×300. Fig. 1c. Immunostaining for BMPRII. The osteoblastic and chondroblastic cells are intensely positive for BMPRII. Original magnification, ×300.

Fig 2. Cultured cells on the non-coated chamber slide. Fig. 2a. The cultured cells exhibit mixed cell type consisting of spindle and round cells. Original magnification, ×100. Fig. 2b. Immunostaining for BMP-2/4. The cultured cells are intensely positive for BMP-2/4 (arrows). Cultured cells on the non-coated chamber slide. Original magnification, ×200. Fig. 2c. Immunostaining for BMPRⅡ. The cultured cells are intensely positive for BMPRⅡ (arrows). Cultured cells on the non-coated chamber slide. Original magnification, ×200.

Fig. 3

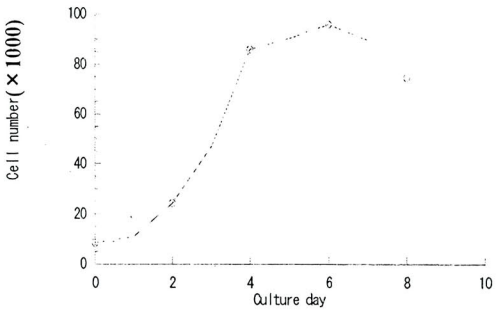


Fig. 4a

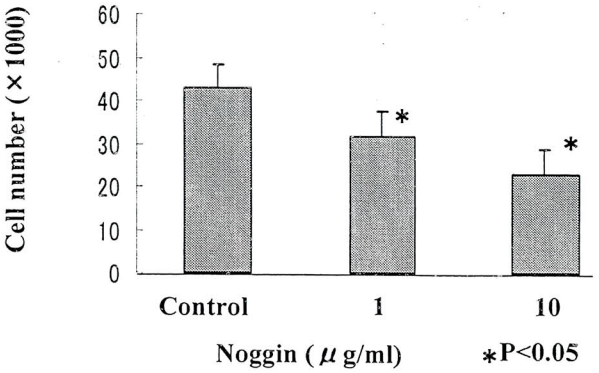


Fig. 4b

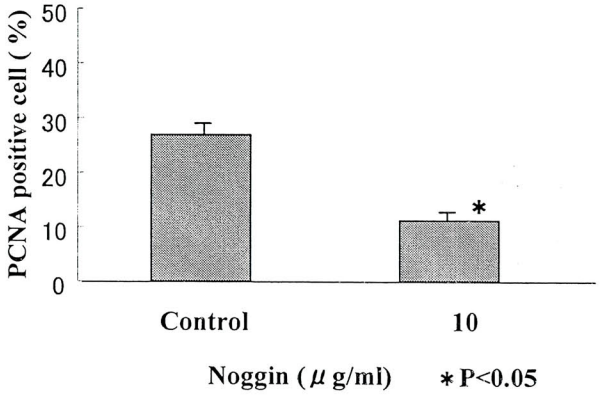


Fig. 5

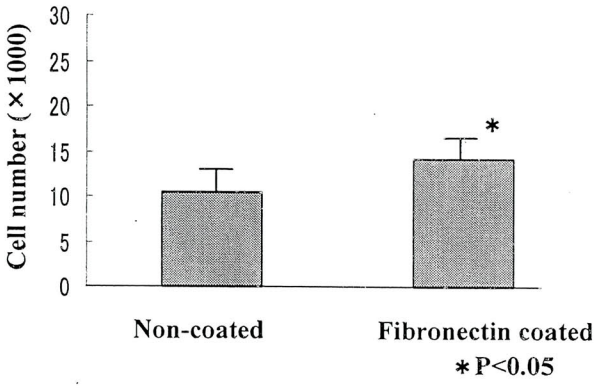


Fig. 3. Growth curve of MCO-Y4 at 93 rd passage.

Fig. 4. The effect of noggin on MCO-Y4 cells. Fig. 4a. Noggin inhibits growth of MCM-K1 cells. Fig. 4b. PCNA-positive cells are significantly decreased in 10 μ g/ml group.

Fig. 5. The effect of fibronectin on MCO-Y4 cells. Fibronectin induces a slight growth stimulation in MCO-Y4 cells.

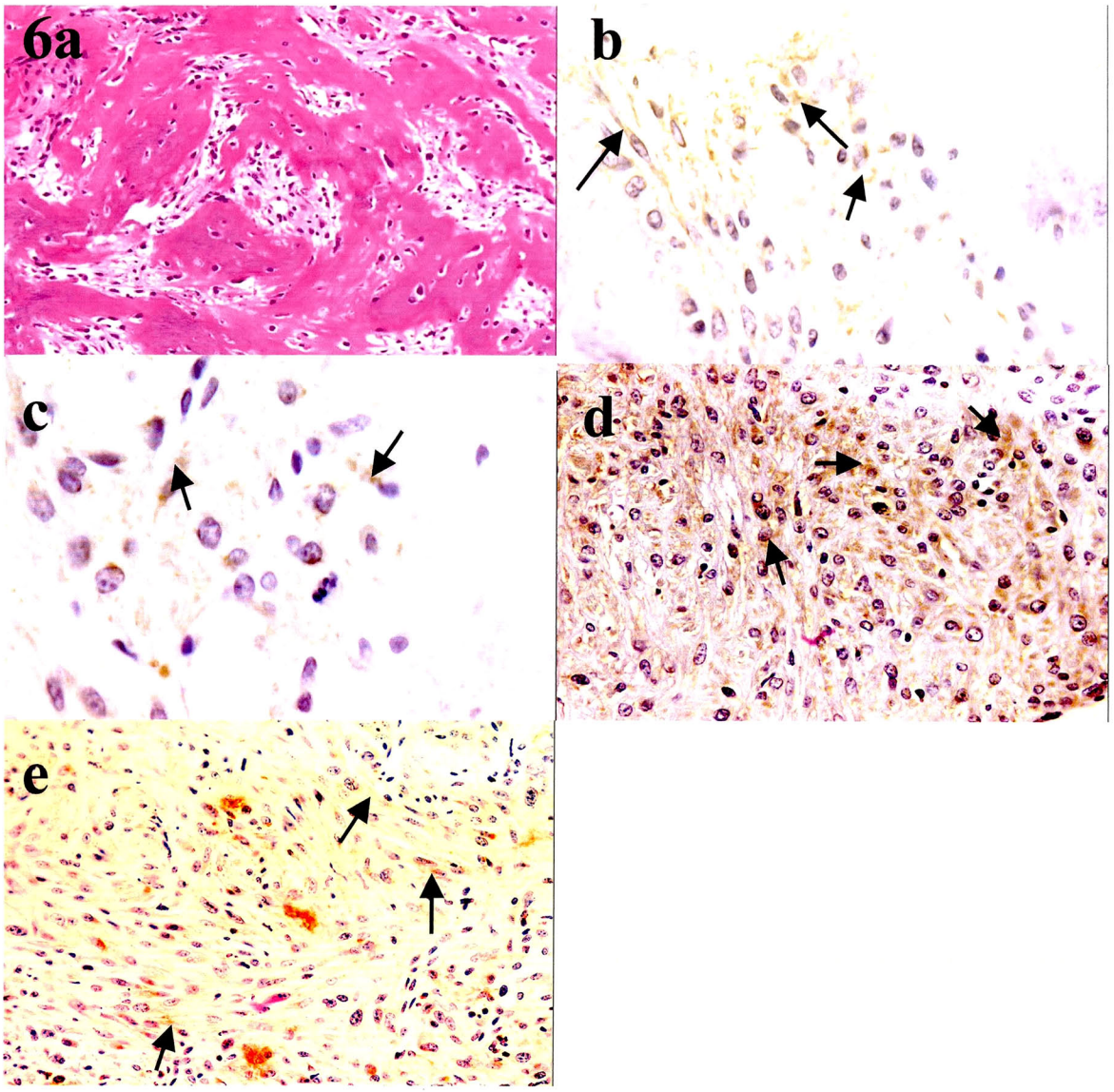


Fig. 6. Transplanted tumor. Fig. 6a. The formation of woven-bone trabeculae is observed. HE stains. Original magnification, ×100. Fig. 6b. Immunostaining for BMP-2/4. The osteoblastic cells are intensely positive for BMP-2/4 (arrows). Original magnification, ×400. Fig. 6c. Immunostaining for BMPRII. The osteoblastic cells are intensely positive for BMPR Ⅱ (arrows). Original magnification, ×400. Fig. 6d. Immunostaining for BMP-2/4. The fibroblastic cells show mild immnunoreactivity for BMP-2/4(arrows). Original magnification, ×200.Fig. 6e. Immunostaining for BMPRII. The fibroblastic cells show weak immunoreactivity for BMPRII (arrows). Original magnification, ×200.

CHAPTERIII

Bone Morphogenetic Protein-4 Signaling Stimulates the Growth, but not Metastasis, of Xenotransplanted Osteosarcoma in Severe Combined Immunodeficiency Mice.

ABSTRACT

The roles of bone morphogenetic proteins (BMP)s in progression of canine osteosarocoma (OSA) was examined using spontaneous OSA and MCO-Y4 from a canine mammary gland OSA. Thirty-one spontaneous OSAs were collected to examine the relationship between BMPs and the amount of extracellular matrix. The dominant neoplastic cell type of the tumors was osteoblastic, osteoblastic plus fibroblastic, osteoblastic plus chondroblastic and osteoblastic plus fibroblastic plus chondroblastic, and these tumors contained various amount of the extracellular matrix (mean 46.6 ± 2.7 %). The expression of BMP2/4 and BMPRII was detected independently upon the degree of ossification and the dominant cell types of the tumors. Immunocytochemical analysis revealed the expression of BMP-4 in MCO-Y4. The expression of BMP-4 and BMPRII in MCO-Y4 was confirmed by reverse transcription polymerase chain reaction. Incubation of MCO-Y4 with recombinant noggin resulted in the marked decrease in phospho-Smad 1/5/8 protein level, and co-cultured with recombinant BMP-4 did not promote the expression of phospho-Smad 1/5/8 compared with that in non-treated control. Recombinant BMP-4 had no stimulating effect on MCO-Y4 to invade into the collagen membrane. The administration of antibodies for BMP2/4, BMPRII or recombinant noggin resulted in significant decrease in the sizes of the xenotransplanted tumors formed by the inoculation of MCO-Y4. Although the treatment of recombinant BMP-4 increased the sizes of the xenotransplanted tumors, no metastatic lesions were observed in all groups. All findings indicated that BMP signaling pathway may play important roles in the proliferation rather than in the invasive activity and the ossification in canine OSA.

INTRODUCTION

Osteosarcoma (OSA) is one of malignant mesenchymal tumors characterized by proliferation of neoplastic osteoblasts with production of bone and osteoid tissues [75]. Although OSA has high potential for metastasis and poor clinical prognosis [13], the mechanisms of the progression of OSA including its growth and metastasis remain unclear.

Bone morphogenetic proteins (BMP)s belong to transforming growth factor (TGF)-β superfamily, and were originally identified as an important factor for endochondral ossification [72, 73, 82, 83]. Activated BMPR phosphorylates Smad 1/5/8 after the formation of heterodimer of BMPR II with BMPR IA or IB, and thereafter the Smad complex is then translocated to the nucleus and binds specific sites in DNA. Noggin inhibits the pathway by preventing BMPs from binding to BMPR [23]. Noggin is suggested as a factor associated with regulation of BMP-induced differentiation in mesenchymal cells [1]. BMPs not only induce the bone formation but also stimulate the proliferation of mesenchymal cells [3]. In addition, BMP-2 is a factor that stimulates proliferation of neoplastic cells [53, 71]. In the 20 isotypes of BMPs, BMP-2 and BMP-4 have 92 % homology [14]. While there are a few evidences that BMP signaling pathway induced by BMP-4 is related to malignant

behavior of OSA [7, 89], the entire mechanisms of the progression of OSA remain unknown.

Extraskeletal osteosarcoma (ESO) has the same aggressive biological behavior as bone-derived OSA and commonly shows pulmonary metastasis [52]. Recently, we established a cell line, MCO-Y4 [42], from canine mammary gland OSA, which is considered to an ESO. MCO-Y4 has an ossifying ability *in vivo*, and the cells are immunopositive for BMP-2/4 and BMPRII, suggesting that the cell line may be a useful tool to clarify the relationship between BMPs and OSA.

The present study examined the association between the expressions of BMPs or BMPRII and the formation of tumor matrix in canine OSA. The biological functions of BMP-4 on MCO-Y4 cells are also investigated *in vitro* and *in vivo*.

Materials and Methods

Cell line and treatment: MCO-Y4 [42], was maintained in Dulbecco's Modified Eagle's medium (DME) and Ham's Mixture F-12 (Sigma) 10 % fetal calf serum (FCS), 100 IU /ml penicillin and 100 μg/ml streptomycin. Cells were kept in a humidified incubator with 5% CO_2 at 37℃. For the analysis of the effect of BMP-4 on

proliferation of MCO-Y4, 100 or 500 μg/ml of human recombinant BMP-4 was added to the medium. Then, the cells were detached and counted using hemotocytometer after 2 days from the addition. Other cells were also rinsed and scraped into RIPA buffer for Western blot analysis. In addition, to examine the role of noggin in BMP signaling pathway, MCO-Y4 cells were incubated with 10 μg/ml of human recombinant noggin. The treated cells were scraped into RIPA buffer for Western blot analysis.

Spontaneous OSA cases: Twenty-nine biopsy and 3 necropsy canine cases diagnosed as OSA and extraskeltal osteosarcoma (ESO) were collected. All samples fixed in 10% formalin were embedded in paraffin. Some tissues were decalcified by formic acid solution. Parrafin sections of 4 μm thick were made and stained with hematoxylin and eosin (HE). The diagnoses of the tumors were classified according to the World Health Organization (WHO) International Histological Classification of Bone and Joint Tumors [75] and of Mammary Gland Tumors of Domestic Animals [55]. The amount of tumor matrix including osteoid, bone or cartilage was scanned per field in ×100 using imaging processing software (WinRoof ver.3.1, Mitani corporation, Tokyo, Japan).

Antibodies and recombinant proteins: For immunohistocheminstry, goat polyclonal antibodies for human BMP-2/4 and BMPR II (Santa Cruz, Delware avenue, CA, USA), and biotinylated rabbit serum against goat immunogloblin (DAKO-Japan, Tokyo, Japan) were purchased. For western blot analysis, rabbit anti-phospho Smad 1/5/8 antibody (Cell Signaling Technology, Beverly, MA, USA) and biotinynated swine anti-rabbit swine (Fab) IgG (Dako-Japan) were purchased. Mouse monoclonal antibody against BMP-4 was purchased from Chemicon (Temecula, CA, USA) for immunocytochemistry. Recombinant human noggin and BMP-4 were purchased from R&D systems (Minneapolis, MN, USA) to examine the effects of the proteins on proliferation, invasiveness and tumorigenesis. The recombinant proteins were reconstituted in the 5% serum culture medium. Normal goat serum used for *in vivo* experiments was obtained from clinically healthy goat.

Immunohistochemistry for BMP-2/4 and BMPRII in 31 OSA cases: For imunohitochemical analysis, deparaffinized sections were incubated with 0.5% hydrogen peroxidase in methanol at room temperature for 20 min to block endogenous peroxidase activity, and incubated with 3% bovine serum albumin (BSA) at 37℃ for 20 min. Sections were then incubated with primary antibodies of polyclonal human

BMP-2/4 (1:20, Santa Cruz) or BMPR II (1:20, Santa Cruz) at 37 ℃ for 40 min, and then sections were incubated with biotinylated rabbit serum against goat immunogloblin (1:20, DAKO-Japan) at 37 ℃ for 40 min, followed by reaction with avidin-biotin-peroxidase complex (ABC) reagents (PK4000, Vectastain, Burlingame, CA, USA) at 37 ℃ for 30 min. The attached antibodies were visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, St.Louis, MO, USA) and counterstained with Mayer's hematoxylin. The results of immunohistochemistry were quantified by counting 1000 cells using semi-quantitative analysis as follows: (-); 0%, (±); 0-5%, (+); 5-10%, (2+); 10-50%, (3+); >50%.

mRNA expression by RT-PCR: Total RNA was extracted from MCO-4 cells with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacture's instrument. The sequence of the primers and the expected sizes of the amplification products are as follows.

BMP-4 (forward)	5'-GATCTTTACCGGCTCCAGTCT-3'	
BMP-4 (reverse)	5'-CTGGGGCTTCATAACCTCAT-3'	325bp

BMPRII (forward)	5'-GATATGCAGGTTTCTGGTGTC-3'	
BMPRII (reverse)	5'-AGTTCAGCCATCCTCTCTTC-3'	170bp
β-actin (forward)	5'-GAGAAGCTGTGCTACGTCGC-3'	
β-actin (reverse)	5'-CCAGACAGCACTGTGTTGGC-3'	275bp

First strand cDNA was synthesized by reverse transcription with Superscript (Invitrogen) and oligo (dt). PCR mixture was prepared using a AccuPower® PCR Premix (BIONEER, Deajeon, Korea). Reverse transcription–polymerase chain reaction (RT-PCR) was performed (initial denaturing step of 1 min at 95℃, increasing cycle numbers of 1 min at 95℃, 1 min at 55℃ and 2 min at 72℃). PCR products were evaluated by UV transillumination of the electrophoretic pattern in 1.5% agarose gels stained with ethidium bromide.

Immunocytechemistry for BMP-4 in MCO-Y4: The neoplastic cells plated on eight wells chamber slide (LAB-TEK, Christchurch, New Zealand) were fixed in 10% formalin for 10 min at room temperature. The cells were incubated with monoclonal

antibody against BMP-4 (1:20, Chemicon) for 60 min. The cells were then incubated with Envision polymer reagents (Dako-Japan) for 40 min at 37 ℃. The attached antibodies were visualized with DAB, (Sigma) and counterstained with Mayer's hematoxylin.

Invasion assay: The effect of BMP-4 on the invasion activity of MCO-Y4 cells was examined using the BD BioCoat^{TM} Matrigel^{TM} Invasion Chamber (Becton Dickenson, Bedford, MA, USA).

The 5% serum culture medium containing 5×10^4 cells/ml was placed in the upper chamber and 750 μl of the medium containing recombinant BMP-4 (500 μg/ml) or an equal volume of the medium was added to the lower well. After 48hr incubation, the cells on the upper chamber removed using a cotton swab. The cells that had invaded the membrane were stained with Diff-Quick^{TM} stain and the number of cells was counted under the microscope at 400 magnification.

Western blot analysis: Western blot analysis was performed according to the previous report [53]. Briefly, cells were homogenized in RIPA buffer (Santa Cruz) and incubated for 1hr. The extracts were centrifuged at 10,000×g for 10 min, and protein

concentration was measured by Bradford methods. The supernatants were separated by SDS-12.5% polyacrylamide gel electrophoresis and were transferred to a polyvinylidiene difluoride (PVDF) membrane (Atto, Tokyo, Japan). The membrane was incubated with 5% non-fat dried milk in Tris-buffered saline with 0.05% Tween 20 (TTBS) at 4℃ overnight to block non-specific binding. After incubation with the rabbit polyclonal antibody for anti-human phospho Smad 1/5/8 (1:250, Cell Signaling Technology) at 37℃ for 1 hr, the blots were reacted with biotynated swine anti-rabbit swine (Fab) IgG (1:20, Dako-Japan) at 4℃ for 1hr. The membrane was reacted with ABC reagents at 37℃ for 40 min. The attached antibodies were visualized using DAB (Sigma).

Effect of anti-BMP-2/4 antibody, anti-BMPRII antibody, recombinant noggin or recombinant BMP-4 on tumor growth in sever combined immunodeficiency (SCID) mice: MCO-Y4 cells were co-injected subcutaneously into female SCID mice with anti-BMP-2/4 antibody, anti-BMPRII antibody, recombinant noggin or recombinant BMP-4. The antibodies and recombinant proteins were co-injected using Affi-Blue agarose beads as previously reported [53]. In brief, 25 μg of the beads were incubated with 20 μl of 100 μg/ml of anti-BMP-2/4 or BMPRII antibody, 20 μl of 10 μg/ml of

recombinant noggin or 20 μl of 500 μg/ml of recombinant BMP-4 for 2h. The coated beads were co-injected with 5×10^7 MCO-Y4 cells into the SCID mice. The 5% serum culture medium (control 1) or normal goat serum (control 2) was co-injected with MCO-Y4 cells. The mice that the cells treated either with the culture medium, recombinant noggin or recombinant BMP-4 (n=3 in each experiment) were killed 61 days post inoculation. In the meantime, mice recieved the cells with goat normal serum, anti-BMP-2/4 antibody, or BMPRII antibody (n=3 in each experiment) were killed 80 days post inoculation. Then, the size of removed xenotransplanted tumors was measured in three dimentions (length × width × depth). The animal experiments were approved by our institutional guidelines for animal care and use committee (admission number: 2004-062-3).

Statistical analysis: Analysis of variance (ANOVA) was carried out for statistical analysis. A t-test was carried out for the comparison between the groups. The minimum level of significance was concidered $P < 0.05$.

RESULTS

Histopathological features and expression of BMP-2/4 and BMPRII in spontaneous

canine OSA: The dominant neoplastic cells of 31 cases were classified as osteoblastic cells (14 cases), osteoblastic plus chondroblastic cells (4 cases), osteoblastic cells plus fibroblastic cells (9 cases), and osteoblastic cells plus chondroblastic cells plus fibroblastic cells (4 cases) (Table 1). The vascular invasion of the neoplastic cells composed of the osteoblastic cells with osteoid was observed in 4 cases of the biopsy cases. The metastatic lesions were composed of the osteoblastic, chondroblastic and fibroblastic cells with osseous trabeculae of woven bone and/or chondroid matrix. The mean tumor matrix occupation was 46.6±2.7 % (ranging from 16.1 to 66.9 %) (Table 1). As shown in Table 1, the osteoblastic cells lining the trabeculae of woven bone or osteoid showed intense immunoreactivity for BMP2/4 and BMPR II (Figs. 1a and b). The chondroblastic cells were also intensely positive for both markers (Figs. 1c and d), and the fibroblastic cells showed mild to moderate immunoreactivity for BMP2/4 and BMPR II (Figs. 2e, f). The neoplastic cells in the vessels were also intensely positive for BMP2/4 and BMPR II. The metastatic lesions in the necropsy cases showed as same immunoreactivity for BMP2/4 and BMPR II as those seen in the primary lesions. The tumor matrices were negative for BMP2/4 and BMPR II.

The expression of BMP-4 and BMPRII signaling pathway in MCO-Y4 cells:

Immunocytochemically, the intense expression of BMP-4 was observed in MCO-Y4 cells (Fig. 2a). RT-PCR revealed the expression of BMP-4 mRNA (325 bp) and BMPRII mRNA (170 bp) in MCO-Y4 cells (Fig. 2b). Western blot analysis demonstrated the immunopositive bands for phospho-Smad 1/5/8 at 60kD in MCO-Y4 cells, corresponding to the molecular weight of human phospho-Smad 1/5/8 (Fig. 2c). The phospho-Smad 1/5/8 protein level was decreased in the experiment group that the cells had been treated with recombinant noggin. No significant change in phospho-Smad 1/5/8 expression was detected in the group that the cells had been treated with recombinant BMP-4 versus control group (Fig. 2c).

Effect of anti-BMP-2/4, BMPRII, recombinant noggin or BMP-4 on tumor growth in SCID mice: The effect of BMP signaling pathway on the xenotrasplantaion of MCO-Y4 cells into SCID mice was examined. The results of xenotransplanted tumors are indicated in Fig. 3. Although the xenotransplanted tumors in the groups that MCO-Y4 cells had been treated either with anti-BMP2/4, anti-BMPRII or recombinant noggin slowly grew and were found 2 wks after the inoculation (approx. $0.1 \times 0.1 \times 0.1$ cm), the mice injected MCO-Y4 cells with recombinant BMP-4 formed the xenotransplanted tumors 7 days after the injection. In the mice treated with recombinant BMP-4 the xenotransplanted tumors were larger than those in the mice

injected with the 5 % serum culture medium (Table 2). The tumors observed in the groups that MCO-Y4 cells had been treated with anti-BMP2/4 antibody, anti-BMPRII antibody or recombinant noggin were less than half of the size of the xenotransplanted tumors formed in the mice inoculated with the normal goat serum (Table 2). No metastatic lesions were observed in all of the groups.

Effect of BMP-4 on proliferation and invasion activity of MCO-Y4 cells: The growth effect induced by recombinant BMP-4 (100 or 500 μg/ml) for 2 days was not observed (Fig. 4a). In addition, although the tendency of stimulating invasiveness on MCO-Y4 cells co-cultured with recombinant BMP-4 (500 μg/ml) was detected, there were no significant differences of the invading cells between the experimental and control group (Fig. 4b).

DISCUSSION

It is well recognized that the functions of TGF-β superfamily proteins are originally identified as inhibitory factors and related to cell proliferation and differentiation [40]. In human tumors, TGF-β is suggested as an important factor to inhibit the tumor growth by inducing apoptosis of tumor cells in the early stage of tumorigenesis. However,

Jennings and Pietenpol [40] proposed that BMP signaling pathway might be associated with tumor progression in the later stages and that TGF-β signaling pathway might lose its growth-inhibitory potential by inducing ECM production and activating proteases. Several studies using various OSA cell lines have indicated that TGF-β may be related to the proliferation of these cell lines [46, 68, 70], suggesting that Smad-dependent pathway may be important for the progression of OSA.

Yoshikawa *et al.* [89] indicated that BMP2/4 expression was detected in about 60% of human OSA examined. In the report, only fibroblastic cells were positive for BMP-2/4, in contrast to osteoblastic or chondroblastic cells, with no apparent difference in the amount of tumor matrix between bone morphogenetic activity (BMA)-positive and BMA-negative OSA [88]. Thus, they concluded that BMPs might have only a limited role in ossification in OSA. In the present study, the moderate to intense expression of the BMP2/4 and BMPRII were observed in all types of the neoplastic cells, and mild to moderate expression of BMP-2/4 and BMPRII was observed in the fibroblastic cells. In addition, the degree of BMPs- and BMPRII-expression in canine OSA tended not to be associated with the amount of extracelular matrix. Therefore, these results may support the previous hypothesis that BMP signaling pathway in canine OSA has the functions rather than ossification [42].

Gobi *et al.* [21] revealed that BMP and BMPR mRNA expression had been detected in human OSA cell lines. This observation indicated that there would be a mechanism that might be associated with the simultaneous activation of BMPs and BMPRs in OSA. We previously observed that recombinant noggin had inhibited the proliferation of MCO-Y4 cells [42], suggesting that BMP signaling pathway might be associated with the proliferation of OSA cells. In the present study, the phospho-Smad 1/5/8 expression was decreased in the MCO-Y4 cells treated with recombinant noggin compared to the cells without treatment with the recombinant protein. Hence, BMP signaling pathway may be related to the progression of OSA [7].

Although MCO-Y4 showed the tendency of stimulating growth, there were no significant differences between in the MCO-Y4 cells treated with or without recombinant BMP-4. Recently, Smad-independent pathway has described as a novel signaling pathway [26, 62, 80]. The binding of BMPs to BMPR leads to activate an alternative mitogen-activated protein kinase (MAPK) [26, 62]. In the present study, the stimulation of the expression of the phospho-Smad 1/5/8 protein in the cells incubated with recombinant BMP-4 were not demonstrated, it is possible that MCO-Y4 cells may have Smad-independent pathway and BMP-4 may induce the pathway under *in vitro* condition. Further studies are required to clarify the relationship between

MCO-Y4 and Smad-independent pathway and the involvement of Smad-dependent or –independent pathway for proliferation of MCO-Y4 cells.

Several studies revealed that BMP proteins might be suppressive factors for cellular proliferation [10]. However, Rothmmer *et al.* [71] indicated that BMP was overexpressed in melanoma and associated with the promotion of cell invasion and migration. Langenfield *et al.* [53] reported that BMPs might be involved in the growth of non-small cell lung carcinomas. In the present study, recombinant noggin or antibodies to block the BMP signaling pathway suppressed the growth of the xenotransplanted tumors, and recombinant BMP-4 induced the xenotransplanted tumor growth. Yoshikawa *et al.* [89] demonstrated that the co-expression of BMP2/4 and BMPR II proved to be associated with a poor prognosis for patients with OSA. Moreover, the expression of BMPRII in human OSA is correlated to metastasis of the tumor [24]. Hence, the present data suggested that, BMP signaling pathway might play roles in the proliferation of canine OSA, supporting that BMP signaling pathway may be associated with progression of OSA [7].

Inhibition of differentiation (Id) proteins, which are regulators of basic helix loop-helix (bHLH) transcriptional factors that stimulate cell cycle [64, 65, 81] are induced by Smad 1/5 signaling pathway [59]. BMPs upregulate Id proteins in

mesenchymal cells [35, 63]. Also, Id proteins have been suggested as factors which play roles in the malignancy of human tumors [27, 54, 59]. It is a possibility that the interaction between these proteins may be needed to form the tumor. Further studies are required to examine the interactions between Id and Smad pathway in MCO-Y4 cells in order to clarify the progression of OSA.

Invasiveness is an essential and important ability to form metastatic lesions. BMP signaling pathway may be correlated to the invasiveness in cancers [53, 71]. In the present study, recombinant BMP-4 had no stimulating effect on MCO-Y4 to invade into the collagen membrane. Other factors such as urokinase plasminogen activatior receptor (uPAR) or matrix metalloproteinases tissues inhibitor (TIMP)-1 have been suggested as important factors of the neoplastic osteoblastic cells in metastasis or recurrence [16, 18, 19]. The significant differences in size of the xenotransplanted tumors between BMP-4 treatment group and control were observed. There were no formations of metastatic lesions in the *in vivo* experiment. Thus, the current studies suggest that BMP-4 may only stimulate the growth of the xenotransplanted tumor rather than invasiveness and metastasis.

In conclusion, our studies demonstrated that BMP2/4 and BMPRII expressed in all types of neoplastic cells in OSA, may promote the xenotransplanted tumor growth

in SCID mice without the formations of metastatic lesions. These results suggest that BMP signaling pathway may play important roles in the progression of OSA, rather than in the invasive activity and the formation of tumor matrix in canine osteosarcoma. Also this experimental system may be useful for analysis of the mechanisms of OSA growth in human.

Table 1. Immunoreactivity of the neoplastic cells for BMP-2/4, -6 and BMPR Ⅱ.

Type of the tumor	*Cell morphology	Immunoreactivity									
		BMP-2/4					BMPR Ⅱ				
		-	±	+	2+	3+	-	±	+	2+	3+
Osteoblastic (n = 14) (Tumor matrix: 44.99% ±4.48)	Os				4	10					14
Osteoblastic and chondroblastic (n = 4) (Tumor matrix: 38.5% ±7.05)	Os				4						4
	Ch				4						4
Osteoblastic and Fibroblastic (n = 9) (Tumor matrix: 47.61% ±3.81)	Os				2	7					9
	F			4	5				3	6	
Osteoblastic and chondroblastic and fibroblastic (n =4) (Tumor matrix: 51.67% ±10.10)	Os				2	2					4
	Ch				2	2					4
	F				4				1	3	

*Os = osteoblastic; Ch = chondroblastic; F = fibroblastic

(-): 0%, (±): 0-5%, (+): 5-10%, (2+): 10-50%, (3+): >50%.
Tumor matrix=%±SE.

Table 2. The size of the xenotransplanted tumors

Culture medium	Recombinant noggin	Recombinant BMP-4
0.45±0.26	0.048±0.026 *	1.60±0.31*
Goat serum	**Anti-BMP2/4**	**Anti-BMPRII**
0.99±0.24	0.4±0.48 * *	0.075±0.040 * *

The size of the xenotransplanted tumors were measured in 3 dimentions (length × width × depth). Data was expressed as mean±SE (n=3). BMP-4 stimulated the xenotransplanted tumor growth versus control, and recombinant noggin suppressed the xenotransplanted tumor growth versus control (* $P<0.05$). The growth of the xenotransplanted tumors were significantly inhibited in experimental versus control (* * $P<0.05$). A t-test was carried out to compare the means of each groups.

Fig. 1

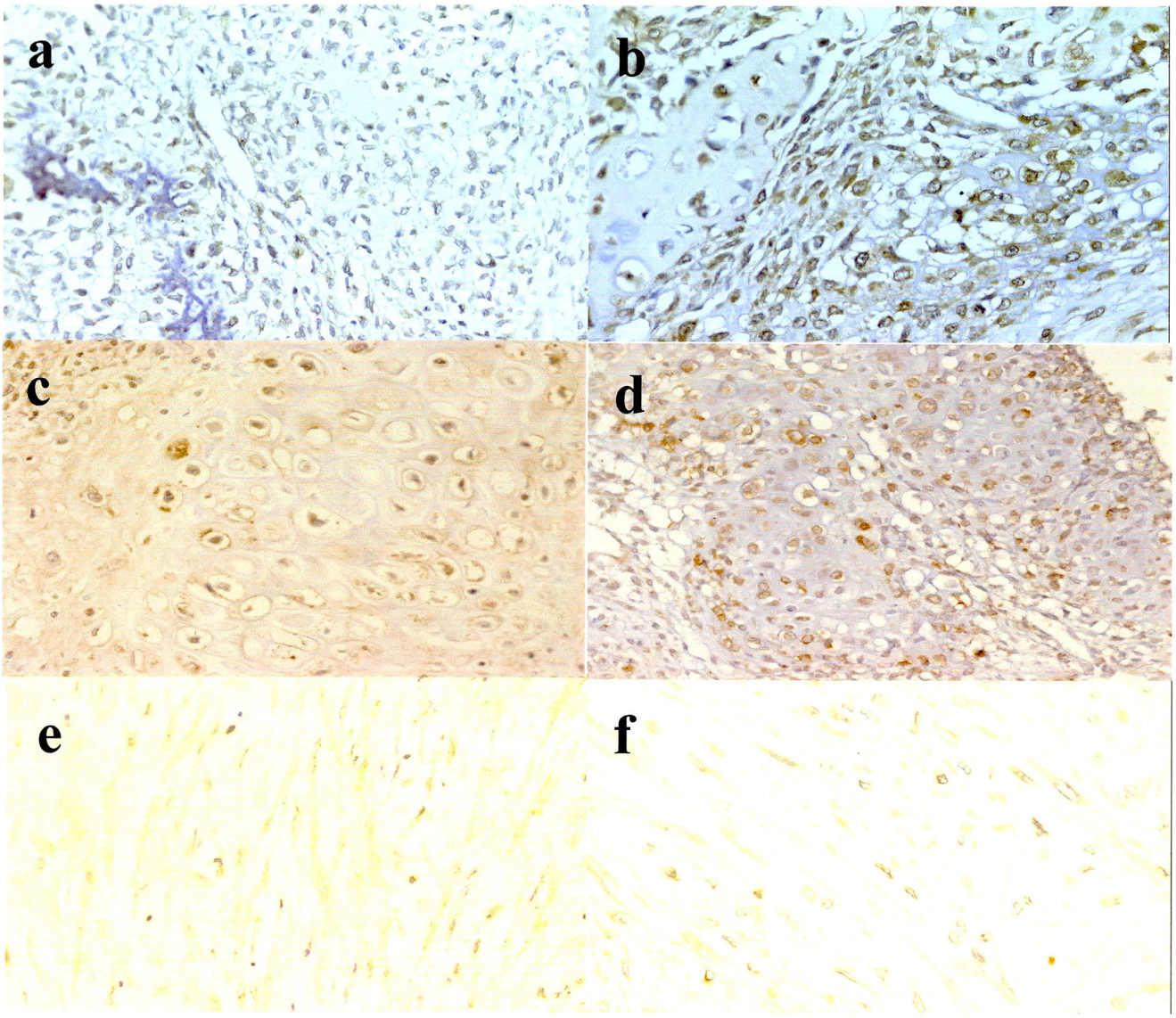
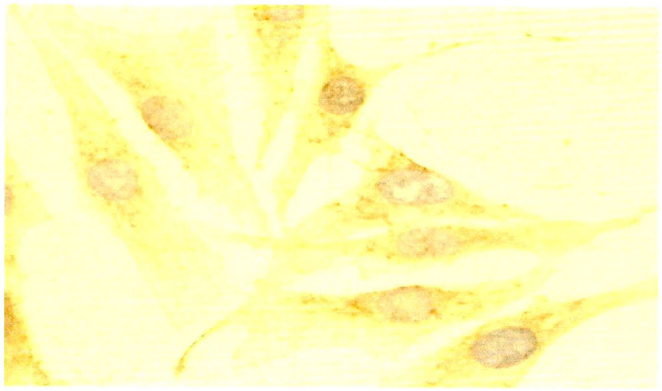


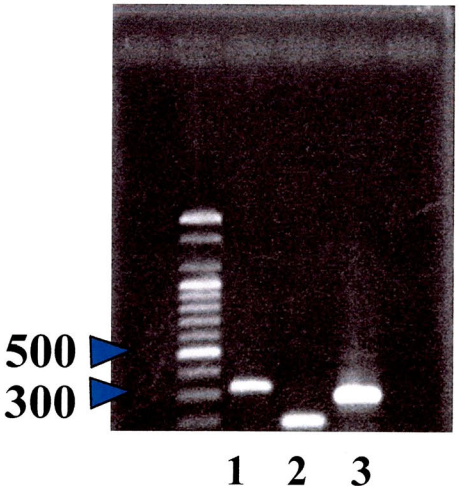
Fig. 1. Immunohistochemical expression of BMP-2/4 and BMPRII in 31OSA cases. a, b The osteoblastic cells lining osteoid showed intense immunoreactivity for BMP2/4 and BMPR II, respectively. c, d The chondroblastic cells were intensely positive for BMP2/4 and BMPR II, respectively. e, f The fibroblastic cells showed mild to moderate immunoreactivity for BMP2/4 and BMPR II, respectively. Note that no tumor matrices were positive for all markers. Original magnification, ×200.

Fig. 2

a



b



c

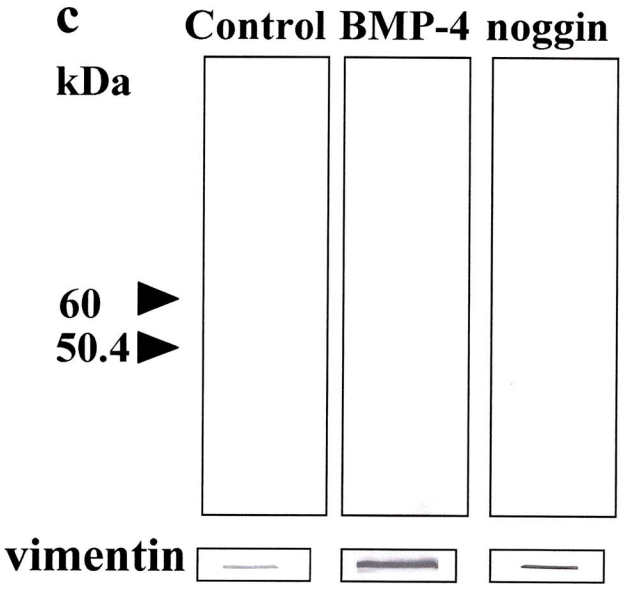


Fig. 3

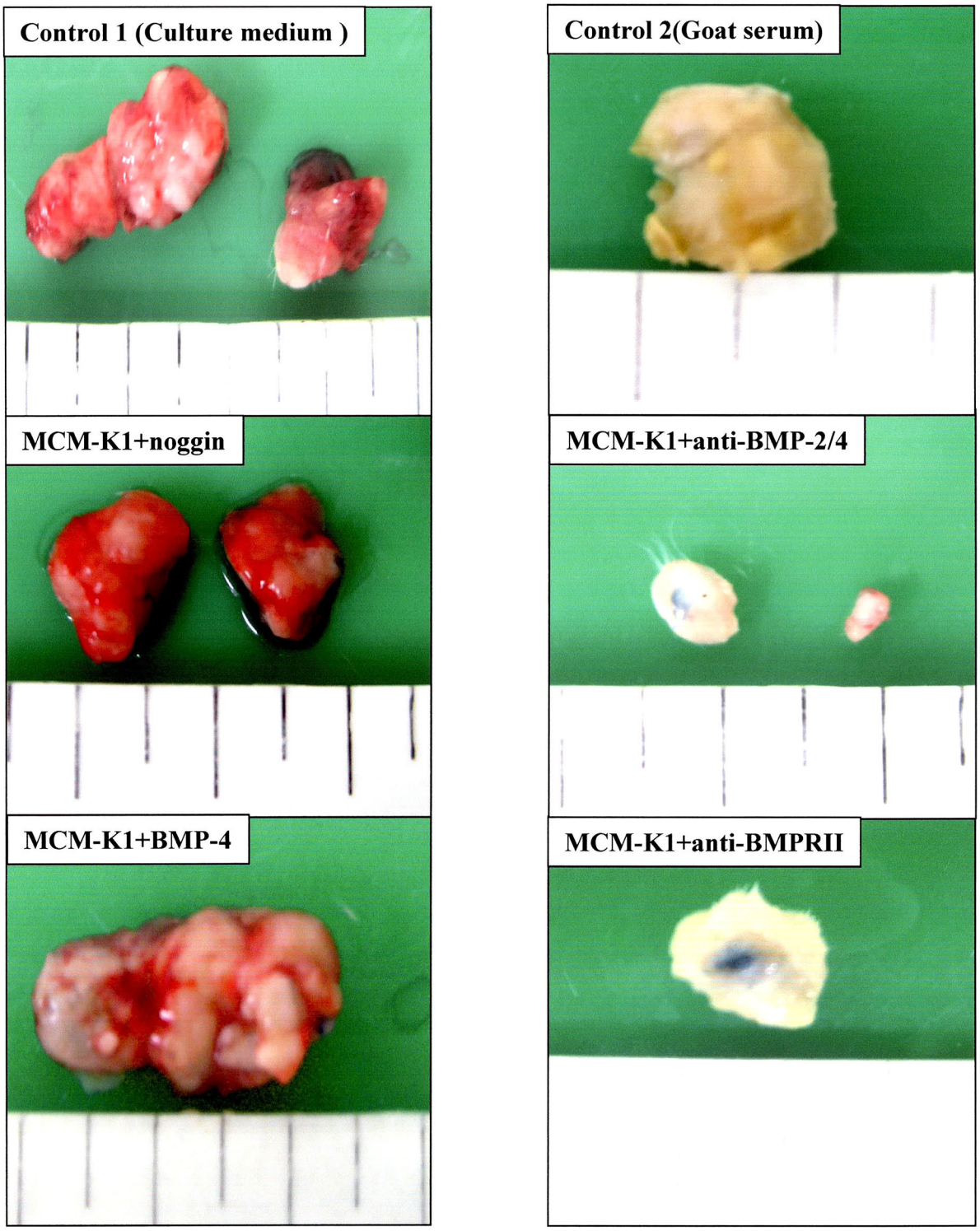

Control 1 (Culture medium)
Control 2(Goat serum)
MCM-K1+noggin
MCM-K1+anti-BMP-2/4
MCM-K1+BMP-4
MCM-K1+anti-BMPRII

Fig. 2. The expression of BMP signaling pathway in MCO-Y4 cells. **a** Immunohistochemical expression of BMP-4 and BMPRII in MCO-Y4 cells. The intense expression of BMP-4 and BMPRII was observed in MCO-Y4 cells. **b** RT-PCR analysis for BMP-4 and BMPRII in MCO-Y4 cells. BMP-4 mRNA expression was detected at 325 bp and BMPRII mRNA expression was detected at 170 bp in MCO-Y4 cells. **c** Western blot analysis for phospho-Smad 1/5/8 protein in MCO-Y4 cells. Immunopositive bands for phospho-Smad 1/5/8 at 60kDa were detected in MCO-Y4 cells, (*lane 1*). The band of phospho-Smad 1/5/8 was not detected in the recombinant noggin treated group (10μg/ml), (*lane 2*). No significant change was detected in the recombinant BMP-4 treated group (500μg/ml), (*lane 3*).

Fig. 3. Effect of anti-BMP-2/4, BMPRII, recombinant noggin or BMP-4 on tumor growth in SCID mice. Anti-BMP2/4 antibody, anti-BMPRII antibody or recombinant noggin suppressed tumor growth in MCO-Y4 cells. Recombinant BMP-4 stimulated tumor growth in MCO-Y4 cells.

Fig.4

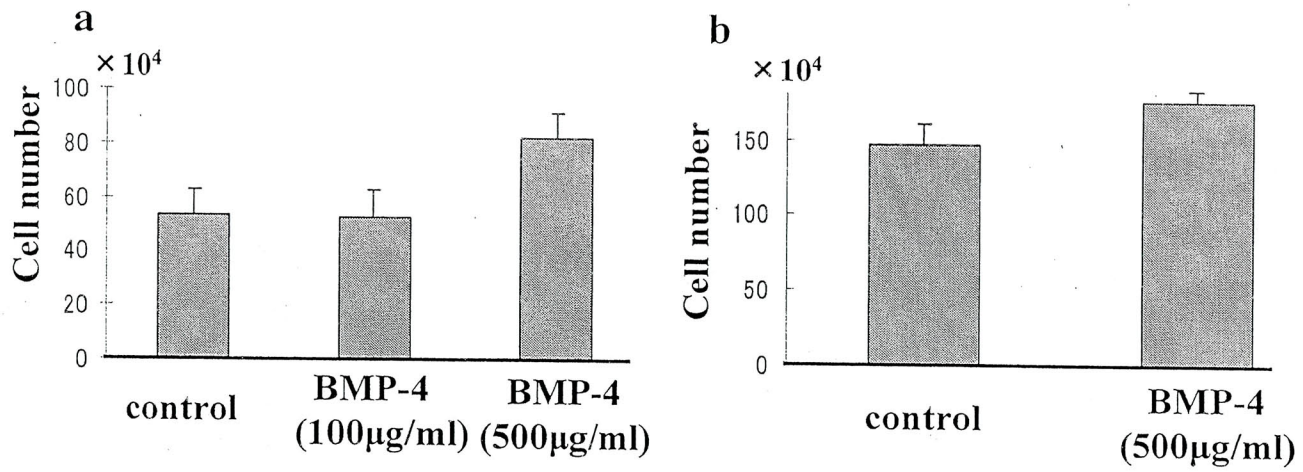


Fig. 4. Effect of BMP-4 on proliferation and invasiveness of MCO-Y4 cells. a The significant growth stimulation was not observed in the BMP-4 treated group. b No stimulation of the invasiveness was observed in the BMP-4 treated group compared to control.

CONCLUSION

The series of the studies revealed that fundamental roles of several osteogenic or chondrogenic factors including ChM-I ad BMPs in the ectopic mesenchymal tissues and its malignant tumor in canine mammary gland tumors.

In spontaneous canine mammary gland tumors, immunoreactivity to BMP-6 was observed in intraductal and interstitial myxomatous myoepithelial cells in complex adenomas and benign mixed tumors. Although the most intense immunoreactivity to ChM-I was observed in the proliferative myoepithelial cells adjacent to ectopic cartilage and mature chondrocytes, interstitial myxomatous myoepithelial cells showed weak immunoreactivity to ChM-I, suggesting that BMP-6 might be involved in all stages of metaplasia of myoepithelial cells to cartilages or bone and that ChM-I might especially participate in the latter process of cartilage formation, and also in endochondral ossification. Therefore, the results of the study revealed that co-localization of ChM-I and BMP-6 in myoepithelial cells might be associated with mesenchymal metaplasia of canine mammary gland tumors. In addition, ChM-I and BMP-6 may also be associated with proliferation of myoepithelial cells in terms of the immunoreactivity for both markers in myoepithelial cells in simple adenoma.

MCO-Y4 that has been newly established from a canine mammary gland OSA is characterized by proliferation of cells and ossification ability in the xenotransplanted

tumors. In addition, the cells are immuno-positive for BMP-2/4 and BMPRII. The potential of BMP signaling pathway for the association with proliferation was also confirmed by growth assay. The results of the study indicate that BMP signaling pathway may play roles in proliferative activity in MCO-Y4 cells. Consequently, MCO-Y4 may be a useful tool for the further study for the malignant behavior of canine OSA.

To better understand the role of BMP signaling pathway in canine OSA, the study was conducted. BMP-4 and BMPRII are expressed in all types of the neoplastic cells in canine spontaneous osteosarcoma immunohistochemically. BMP-4 induced xenotransplanted tumor growth in SCID mice without the formations of metastatic lesions. However, BMP signaling pathway has no functions *in vitro* on invasive activity. BMP signaling pathway may play roles in the proliferation of neoplastic cells, and the association of BMP signaling pathway with the invasiveness or metastasis may be minor in canine OSA. The results support the hypothesis that BMP-4 may be involved in progression of human OSA. Thus, BMP signaling pathway may be an important factor for more targeted therapies of OSA.

All these results of the series of studies would help clarify the functions of BMP proteins in canine mammary gland tumors. It is clear that BMPs may have the

relationship with the ossification of mammary gland tumors in terms of the original function of BMP. The data may be useful to clarify the mechanisms of ossification not only in canine mammary and human salivary gland tumors. Considering the pleiotropic functions of BMP including carcinogenesis, the expression of BMP in myoepithelial cells of simple adenoma may be the important observation, suggesting that BMPs may be associated with other cellular functions that are not related to ossification. Because the growth stimulation induced by BMP signaling pathway was confirmed in the a cell line derived from mammary gland OSA, BMPs in myoepithelial cells may also contribute to the other functions such as malignant transformation to OSA by autocrine or heterocrine manner. Hence, the author proposes that BMPs in canine mammary gland tumors may have the other important roles associated with proliferation as well as ossification, especially of myoepithelial cells, which would lead to a malignant form, OSA.

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