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Expression of bone morphogenetic proteins in salivary pleomorphic adenomas

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Abstract Salivary pleomorphic adenomas are often associated with chondroid tissue formation. We investigated the relationship between chondroid tissue formation and the expression of bone morphogenetic proteins (BMPs), which are strong inducers of ectopic bone and cartilage formation. Fifteen pleomorphic adenomas and seven normal salivary glands were examined genetically and immunohistochemically. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that BMP-1, BMP-2, BMP-3, BMP-4, and BMP-7 mRNAs were overexpressed in 10 (66.7%), 9 (60.0%), 1 (6.7%), 8 (53.3%), and 12 (80.0%), respectively, of the 15 pleomorphic adenomas. Overexpression of BMP-2 mRNA was observed in pleomorphic adenomas. Marked chondroid formation or expression of type II collagen was frequently observed in pleomorphic adenomas that overexpressed BMP-2 mRNA. Immunohistochemically, BMP-2 was detected in modified myoepithelial cells aroud chondroid tissue and basement membranes. These results suggest that BMPs, and expecially BMP-2, have a role in chondroid formation in pleomorphic adenomas.

Key words Pleomorphic adenoma · Bone morphogenetic proteins · Cartilaginous formation · Myoepithelial cell

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Introduction

Pleomorphic adenoma is the most common type of tumour in the salivary gland. The morphological diversity exhibited by this tumour includes a conjunction of patterns with epithelial and mesenchyme-like components [25, 28]. The chondroid area seen in the mesenchyme-like component has prompted some pathologists to suggest that pleomorphic adenoma is a true mixed tumour. This supposition has, however, been challenged by recent ultrastructural and immunohistochemical studies suggesting that neoplastic myoepithelial cells within the tumour transform into chondroid tissues [4, 5, 17]. Thus, the mechanism of chondroid tissue formation in pleomorphic adenomas remains controversial.

In 1965, Urist reported that demineralized bone tissues induced ectopic cartilage and bone formation when implanted at intramuscular and subcutaneous sites [26, 27]. The putative factor involved in this process was termed the bone morphogenetic protein (BMP), but the molecular nature of BMP has only recently been elucidated. In the past few years, eight different human cDNAs for BMPs, BMP-1 [33], BMP-2 (BMP-2A) [33], BMP-3 (osteogenin) [12, 33], BMP-4 (BMP-2B) [33], BMP-5, BMP-6 (Vgr-1) [13], BMP-7 (OP-1) [20, 23] and BMP-8 [1] have been cloned. BMP-2 through BMP-8 belong to the transforming growth factor-β (TGF-β) superfamily [13, 20, 33, 32]. Several lines of evidence indicate that BMPs play an important role in both chondrogenesis and osteogenesis [9, 25, 29, 33]. It is of particular interest that several epithelial cells have been found to express BMP mRNAs during embryogenesis and organogenesis (S. Nishimatu, A. Suzuki, A. Shoda, K. Murakami, N. Ueno, personal communication) [14, 22, 24]. Recently, Hatakeyama et al. revealed the immunolocalization of BMPs in salivary gland pleomorphic adenomas [6].

These recent findings suggest that the epithelial tumour cells in pleomorphic adenomas are involved in chondroid tissue formation via the synthesis of BMPs. To investigate this hypothesis, we examined BMP

mRNA expression and the synthesis of its protein product in pleomorphic adenomas. Our results confirm that salivary pleomorphic adenomas express several kinds of BMP mRNA, and that BMP-2 mRNA expression is closely related to type II collagen expression in this tumour.

Materials and methods

Fifteen surgical specimens from salivary pleomorphic adenomas resected during the 1989–1991 period were obtained from the pathology files of the Faculty of Dentistry, Tokyo Medical and Dental University, Tokyo, Japan. Seven normal salivary gland specimens were obtained at either operation or autopsy. One-half of each specimen was immediately snap-frozen and stored in liquid nitrogen before use for reverse transciption-polymerase chain reaction (RT-PCR) assay. For immunohistochemical study, the other half of the surgical specimen was either directly frozen in O.C.T. compound, or pre-fixed in 4% paraformaldehyde in PBS (phosphate-buffered saline) at 4° C for 12 h and then frozen in O.C.T. compound. Following storage at –80° C, 6-μm sections were cut with a cryostat.

Both routinely processed paraffin sections and frozen sections stained with haematoxylin and eosin were examined for the presence of chondroid tissues. The degree of chondroid tissue formation was expressed as follows: – no chondroid component, +– chondroid component comprised less than 5% of total stroma, + 5–20%, ++ 21–50%, and +++ more than 50%.

Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method [3] and reverse-transcribed into single-stranded cDNA using random hexamer as a primer and Moloney murine leukaemia virus reverse transcriptase (BRL, Gaithersburg, Md.). Synthesized cDNA 1 μl was used for each PCR. Oligonucleotide primers used for PCR were synthesized on an Applied Biosystems Model 391 DNA synthesizer. Primer sequences and annealing temperatures used are shown in Table 1. Twenty-five cycles of denaturation (94° C, 30 s), annealing (each optimal temperature, 30 s), and extension (72° C, 45 s) were carried out in a thermal processor (Taitec, Japan). One quarter of each PCR product was analysed electrophoretically on 2% agarose gels. As a negative control for each experiment, water alone was used as the RNA template and was carried through all PCR stages. Each experiment was performed twice, under the same conditions to confirm reproducibility.

Unfixed frozen sections were post-fixed in 4% paraformaldehyde in PBS at 4° C for 10 min. All sections were immunostained by the avidin–biotinylated peroxidase complex method [7]. A mouse monoclonal antibody h3b2/17.8.1, provided by Dr. E.M. Alderman (Genetics Institute), was used to detected BMP-2. This monoclonal antibody was made by standard procedures using full-length recombinant human BMP-2 as the antigen. This monoclonal antibody reacts with BMP-2 and BMP-4 by Western blot analysis [38]. A murine monoclonal antibody against human type II collagen (clone II-4C11; Fuji Yakuhin Kogyo, Japan) was used to identify type II collagen. A S-100 protein polyclonal antiserum (Z311; Dako Japan, Kyoto, Japan) and an anti-alpha smooth muscle actin monoclonal antibody (M0851; clone 1A4, Dako Japan, Kyoto, Japan) were used to identify modified myoepithelial cells. Anti-BMP-2 antibody was diluted to 1:250, anti-type II collagen antibody to 1:500, anti-S-100 protein antiserum to 1:400, and anti-alpha smooth muscle actin antibody to 1:50. Immunoreaction was visualized with biotinylated or anti-mouse IgG antibody (Vector Laboratories, Burlingame, Calif.).

Results

Of the 15 pleomorphic adenomas, 7 exhibited various amounts of chondroid tissues in routinely processed surgical materials. In frozen sections, however, only 2 specimens contained areas of chondroid, presumably because of the irregular distribution of chondroid tissues within the tumours. As 2 of the 15 pleomorphic adenomas showed moderate cellular atypia, partial loss of fibrous capsules, and a relatively homogeneous growth pattern, these were classified as atypical variants of pleomorphic adenoma.

Normal salivary glands expressed mRNAs for each BMP after 40 amplification cycles, though the levels of expression differed among BMPs (data not shown). No type II collagen mRNA was detected in normal salivary glands under the same conditions of amplification. Because overamplification can produce a plateau effect, we limited the number of amplification cycles for semiquantifying each mRNA assay to 25. The signal intensity of the RT-PCR product was evaluated as follows: – no expression, +– very weak expression, + weak expression, ++ moderate expression, +++ strong expression. For each mRNA examined any signal expression stronger than that observed in normal salivary glands was defined as overexpression.

Figure 1 shows the mRNA expression typical of each BMP and type II collagen by RT-PCR in normal salivary glands and pleomorphic adenomas. As shown in Fig. 1 and Table 2, 10 of the 15 pleomorphic adenomas (66.7%) overexpressed BMP-1 mRNA, and 9 of them

Table 1 Primer sequences (*s* sense strand, *a* antisense strand)

Primer	Sequence	Annealing		
		Location	Temperature	Amplicon
BMP1-1	ACG TTT CCA TCG TTC GTG AG	727–746 (s)		
BMP1-2	ACC TCC ACA TAG TCG TAC CA	1174–1155 (a)	56° C	448 bp
BMP2-1	ATG GAT TCG TGG TGG AAG TG	994–1013 (s)		
BMP2-2	GTG GAG TTC AGA TGA TCA GC	1342–1323 (a)	58° C	349 bp
BMP3-1	ATG GGA TAG CCA CAT CAG AG	1136–1155 (s)		
BMP3-2	GGC ATG CTC CAG AGC AAT AA	1530–1511 (a)	56° C	395 bp
BMP4-1	AGC ATG TCA GGA TTA GCC GA	1143–1162 (s)		
BMP4-2	TGG AGA TGG CAC TCA GTT CA	1541–1522 (a)	58° C	399 bp
BMP7-1	ATG GTG GCT TTC TTC AAG GC	829–848 (s)		
BMP7-2	TTC AGG ATG ACG TTG GAG CT	1253–1234 (a)	56° C	425 bp
COL2-1	TAC TGG AGT GAC TGG TCC TA	1131–1150 (s)		
COL2-2	GAA TCC TCT CTC ACC ACG TT	1482–1463 (a)	56° C	352 bp
GAPDH1	CCA TGG AGA AGC TGG GGG	199–217 (s)		
GAPDH2	CAA AGT TGT CAT GGA TGA CC	394–374 (a)	54° C	196 bp

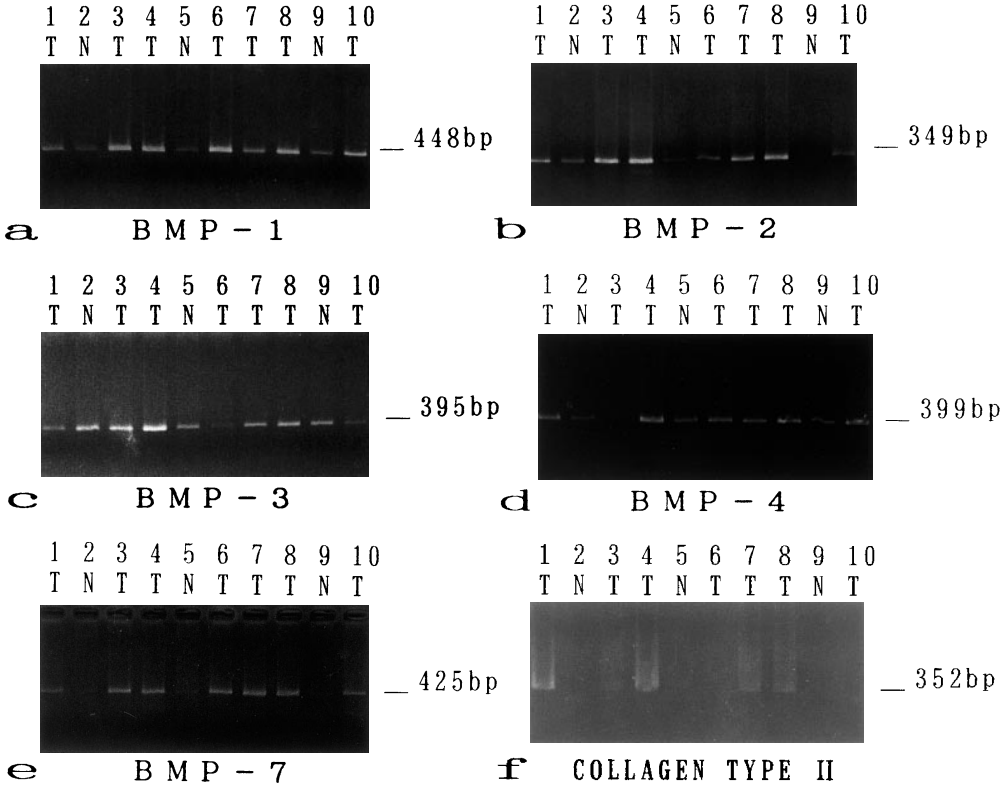

Fig. 1e–f Expression of bone morphogenetic protein (BMP) and type II collagen mRNAs. *N* normal glandular tissue, *T* tumour tissue. *Lanes 1, 4, 6, 7, 8* and *10* are pleomorphic adenomas, and *lane 3* is an atypical pleomorphic adenoma. *Lane numbers* correspond to the numbers in Table 2. Semi-quantiative RT-PCR analysis of **a** BMP-1 mRNA, **b** BMP-2 mRNA, **c** BMP-3 mRNA, **d** BMP-4 mRNA, **e** BMP-7 mRNA, and **f** type II collagen mRNA

Table 2 Expression of bone morphogenetic proteins (*BMPs*) and type II collagen (*paro* parotid gland, *smg* submandibular gland, *plt* palate, *fom* floor of mouth, *N* normal gland, *PA* pleomorphic adenoma, *PA·r* recurrent tumour of pleomorphic adenoma, *aPA* atypical pleomorphic adenoma)

No.	Age (years)	Sex	Site	P.D.	Chondroid index[a]	RT-PCR[b]						Immunostain[b]	
						BMP-1	BMP-2	BMP-3	BMP-4	BMP-7	Collagen type II	BMP-2	Collagen type II
PA	62	M	paro	N	–	±	–	+	±	–	–	ND	ND
2	38	F	paro	N	–	+	+	++	+	±	–	–	–
SM	62	M	smg	N	–	±	±	+	±	–	–	ND	ND
5	16	F	smg	N	–	+	+	+	+	±	–	±	–
9	33	F	smg	N	–	+	±	++	+	±	–	±	–
17	68	M	smg	N	–	+	+	+	++	±	–	ND	ND
PL	62	M	plt	N	–	ND	+	++	+	+	–	ND	ND
1	38	F	paro	PA	++	++	++	+	+++	+	++	+	–
10	53	M	paro	PA	–	++	+	+	++	+	–	+	–
14	47	F	paro	PA·r	–	+	++	±	+	±	–	++	–
20	27	F	paro	PA	++	++	+	–	+	±	–	ND	ND
4	16	F	smg	PA	+++	+++	+++	+++	+++	++	++	++	±
7	53	M	smg	PA	+++	++	++	++	++	++	+	++	++
9	33	F	smg	PA	±	+++	+++	++	++	++	+	+	–
19	51	F	smg	PA	–	+	+++	+	++	+	–	++	–
6	62	F	plt	PA	++	+	+	++	++	–	–	++	–
13	57	F	plt	PA	±	+	+++	–	±	+	–	++	–
18	57	M	plt	PA	–	++	+	–	++	+	–	+	–
21	39	F	plt	PA	–	++	+	–	–	++	–	ND	ND
22	61	M	plt	PA	±	–	±	–	–	–	–	ND	ND
3	75	F	plt	aPA	–	+++	+++	++	–	++	±	++	–
11	78	F	plt	aPA	–	+	+	–	+	++	+	++	+

[a] Chondroid component/stromal component ratio: – 0%, ± <5%, + 5~20%, ++ 21~50%, +++ >50%
[b] – No expression, ± very weak expression, + weak expression, ++ moderate expression, +++ strong expression, ND not done

(60.0%) overexpressed BMP-2 mRNA. Strong overexpression of this mRNA was frequently seen in pleomorphic adenomas with abundant chondroid. The level of BMP-3 mRNA expression in normal salivary glands was much higher than that of other BMP mRNAs. There was 1 pleomorphic adenoma, which had been taken from a submandibular gland, showed strong overexpression of BMP-3 mRNA. Interestingly, 6 of 7 pleomorphic adeno-

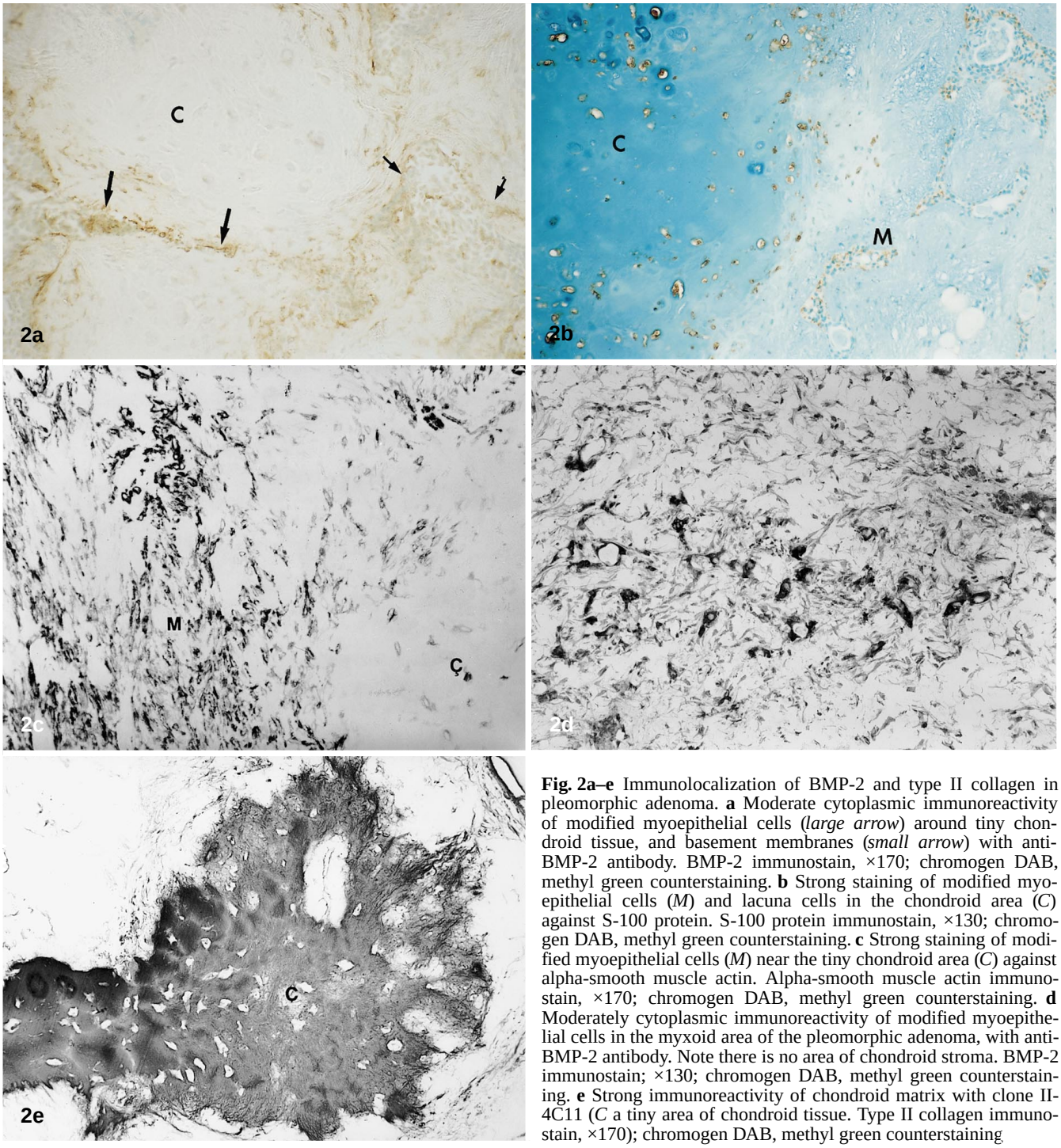

Fig. 2a–e Immunolocalization of BMP-2 and type II collagen in pleomorphic adenoma. **a** Moderate cytoplasmic immunoreactivity of modified myoepithelial cells (*large arrow*) around tiny chondroid tissue, and basement membranes (*small arrow*) with anti-BMP-2 antibody. BMP-2 immunostain, ×170; chromogen DAB, methyl green counterstaining. **b** Strong staining of modified myoepithelial cells (*M*) and lacuna cells in the chondroid area (*C*) against S-100 protein. S-100 protein immunostain, ×130; chromogen DAB, methyl green counterstaining. **c** Strong staining of modified myoepithelial cells (*M*) near the tiny chondroid area (*C*) against alpha-smooth muscle actin. Alpha-smooth muscle actin immunostain, ×170; chromogen DAB, methyl green counterstaining. **d** Moderately cytoplasmic immunoreactivity of modified myoepithelial cells in the myxoid area of the pleomorphic adenoma, with anti-BMP-2 antibody. Note there is no area of chondroid stroma. BMP-2 immunostain; ×130; chromogen DAB, methyl green counterstaining. **e** Strong immunoreactivity of chondroid matrix with clone II-4C11 (*C* a tiny area of chondroid tissue. Type II collagen immunostain, ×170); chromogen DAB, methyl green counterstaining

mas of the palatal glands expressed no BMP-3 mRNA. The 1 of the 7 that did express BMP-3 mRNA was an atypical variant of pleomorphic adenoma, and the expression level was almost same as that observed in normal palatal glands. Overexpression of BMP-4 and BMP-7 mRNAs was seen in 8 (53.3%) and 12 (80.0%) of the 15 pleomorphic adenomas, respectively.

Expression of type II collagen mRNA was observed in 6 of the 15 pleomorphic adenomas (40.0%). Areas of chondroid were seen on routinely processed paraffin sections or frozen sections in 5 of these 6 specimens. No normal salivary gland showed positive signals for type II collagen mRNA.

There were no distinct differences in expression of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene among the specimens. This was taken as proof that a similar volume of RNA had been applied to each reaction.

Immunodetectable BMP-2 was seen in 8 of 13 typical pleomorphic adenomas (53.8%; Fig. 2). The degree of immunoreactivity against BMP-2 correlated with RT-PCR-induced BMP-2 mRNA expression (Table 2). The reaction products of BMP-2 were localized mainly in the cytoplasm of modified myoepithelial cells in myxoid components. Modified myoepithelial cells around the chondroid component exhibited moderate immunoreactivity, while lacuna cells in the chondroid area were only weakly reactive. Moderate immunoreactivity against BMP-2 was observed in basement membranes of solid tumour nests.

Only 2 cases showed chondroid components on frozen sections. In these 2 cases, a positive reaction against type II collagen was observed in the chondroid matrix and around the lacunae in a homogeneous pattern. One atypical pleomorphic adenoma with no chondroid area on routine sections showed moderate immunoreactivity against type II collagen. This case expressed type II collagen mRNA with RT-PCR.

In pleomorphic adenomas, modified myoepithelial cells expressed S-100 protein and alpha-smooth muscle action. Also, S-100 protein was immunolocalized in the lacuna cells of the chondroid areas and modified myoepithelial cells. In addition, alpha-smooth muscle actin was observed in the modified myoepithelial cells around the chondroid areas, whereas no signals of alpha-smooth muscle actin were seen in the lacuna cells of the chondroid areas. These findings were compatible with previous reports.

Discussion

Salivary pleomorphic adenomas exhibit various histological features of mesenchyme-like tissues including chondroid areas. Several ultrastructural and immunohistochemical studies have provided evidence that chondroid areas are composed of epithelial cells, perhaps modified myoepithelial cells [4, 5], while failing to discriminate lacuna cells in the chondroid areas from authentic chondrocytes [17]. Cartilage is composed of various extracellular matrices produced by chondrocytes. Type II collagen is an important matrix component that distinguishes cartilage from other tissues. We found that 6 of the 15 pleomorphic adenomas examined in this study expressed type II collagen mRNA. The production of type II collagen in chondroid areas was confirmed immunohistochemically. Landini also used immunohistochemical techniques to demonstrate the presence of type II collagen in chondroid areas of pleomorphic adenomas [10]. These findings strongly support the supposition that chondroid areas seen in pleomorphic adenomas are cartilaginous in nature.

Huggins reported that transplantation of uro-epithelium and bladder epithelium into subcutaneous sites induced ectopic cartilage and bone formation [8]. Others have also demonstrated that transplantation of several epithelial tumour cell lines leads to ectopic cartilage and bone formation [30, 33]. These observations suggest that epithelial cells synthesize BMPs or BMP-like molecules which induce undifferential mesenchymal cells to differentiate into chondrocytes and osteoblasts. Indeed, Ozkaynak et al. demonstrated marked mRNA expression of OP-1 (BMP-7) in the kidney and urinary bladder [20]. They speculated as to the involvement of OP-1 in the process of ectopic cartilage and bone formation in response to implantation of uro-epithelium or bladder epithelium. Ogase et al. showed that gastric adenocarcinoma cell line MKN45 expressed BMP-1, 2, 4, and 7 and that this cell line induced bone tissue by diffusion chamber assay [18]. These findings imply that BMPs are produced not only by osteogenic cells but also by some epithelial cells.

In the present study, we investigated the expression of mRNAs for BMP-1, BMP-2, BMP-3, BMP-4, and BMP-7. These mRNAs were overexpressed, as compared with levels in normal salivary glands, in several pleomorphic adenomas. Among these BMPs, BMP-2 mRNA was frequently overexpressed in adenomas with conspicuous chondroid formation and/or type II collagen expression. We also used immunohistochemistry to demonstrate the presence of BMP-2 in modified myoepithelial cells surrounding a chondroid area. These findings indicate that, among BMPs expressed in pleomorphic adenomas, BMP-2 is the most closely related to chondroid formation. Hatakeyama et al. and Yang et al. reported that the luminal cells of tubuloglandular structures were conspicuously immunostained with anti-BMP antibodies, but these anti-BMP antibodies have not been well characterized until now [6, 35]. On the other hand, Lianjia et al. reported modified myoepithelial cells expressed BMP, and that modified myoepithelial cells were related to chondrogenesis in pleomorphic adenoma [11]. S-100 protein produced by modified myoepithelial cells is calcium-binding protein, which may be related to chondrogenesis in pleomorphic adenomas. Yang and Jin reported antibody against BMPs, but they did not record that this antibody recognized which kind of BMPs. In the present study, antibody against BMP-2 recognized BMP-2/4 on Western blot analysis [35]. In addition to BMP-2, mRNAs of BMP-1 and BMP-4 were also overexpressed in almost all pleomorphic adenomas with chondroid formation. Recently Wang et al. reported that recombinant proteins for BMP-1 and BMP-4, as well as BMP-2, induced cartilage formation when implanted at subcutaneous sites [29]. It is likely that these BMPs, BMP-1, BMP-2 and BMP-4, interact to promote the formation of chondroid tissues in salivary pleomorphic adenomas. Lyons et al. suggested that coordinated expression of several TGF-β-like growth factors, including BMPs, is essential to the development of specific organs [14].

The pleomorphic adenomas without obvious chondroid elements expressed BMP-2 mRNA, and its product was localized primarily in the areas of sheet-like growth composed of spindle-shaped tumour cells and occasional basement membranes. Lyons et al. have demonstrated the expression of mRNAs for BPM-2, BMP-4, and Vgr-1

(BMP-6) in several tissues during nonosseous organogenesis [14, 15]. Their findings suggest multiple functions of BMPs depending on specific tissue and cell types. Thus, although BMPs do not induce cartilage formation in pleomorphic adenomas without chondroid elements, they may have signifiant roles in the differentiation of neoplastic cells or the production of extracellular matrices by neoplastic epithelial cells.

All the normal salivary glands expressed BMP-3 mRNA at weak to moderate levels. This suggests that BMP-3 has a function in normal salivary glands. Almost all tumours of the parotid and submandibular glands expressed BMP-3 mRNA at similar or lower levels, whereas typical pleomorphic adenomas of the palate did not express this mRNA. This may be related to the morphological characteristics of pleomorphic adenomas of the palate, which infrequently show the typical chondroid or chondromyxoid histology [28].

There are two possible mechanisms of chondroid tissue formation in pleomorphic adenomas. First, BMPs produced by epithelial tumour cells may induce mesenchymal stromal cells to differentiate into chondrocytic cells. Another possible explanation is that epithelial tumour cells, perhaps modified myoepithelial cells, acquire a chondrocyte-like phenotype and synthesize type II collagen in response to their own BMPs. Indeed it has been demonstrated that a number of embryonic epithelial cells synthesize type II collagen transiently [2, 31]. To understand the mechanism of chondroid formation in pleomorphic adenomas, it is important to determine which cell types in the tumour respond to BMPs by acquiring chondrocytic phenotypes. Lyons et al. demonstrated the specific expression of Vgr-1 in hypertrophic chondrocytes during chondrogenesis [14]. This suggests the involvement of Vgr-1 in the chondroid formation observed in pleomorphic adenomas. The expression of BMP-6, the human counterpart of mouse Vgr-1, in pleomorphic adenomas is presently under investigation in our laboratory.

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