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# An immunohistochemical study of bone morphogenetic protein in pleomorphic adenoma of the salivary gland

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**Abstract.** Bone morphogenetic protein (BMP) is a potent induction factor for new bone formation including heterotopic chondro-ossification in soft tissues. The immunohistochemical reaction for BMP was studied in 23 cases of pleomorphic adenoma of salivary gland by using a monoclonal antibody produced by hybridoma technique. Positive BMP immunoreactivity was seen in 87% of tumours. Immunohistochemical expression of BMP was observed in modified myoepithelial cells (88% cases), luminal tumour cells of tubulo-ductal structures (78% cases) and chondroid cells in hyaline tissue (22% cases). The authors concluded that the simultaneous presence of glycosaminoglycans as matrix substance and S-100 protein for calcium signalling are associated with BMP-mediated cellular activity of modified myoepithelial cells in the formation of chondroid structures in pleomorphic adenomas of the salivary glands.

**Key words:** Pleomorphic adenoma – Bone morphogenetic protein – Immunohistochemistry

## Introduction

Bone morphogenetic protein (BMP) is an induction factor in the process of heterotopic bone formation in non-osteogenic soft tissues. Ectopic ossification and cartilage formation induced by BMP in non-osteogenic tissues and BMP induced differentiation of osteoprogenitor cells in vitro studies have been reported (Nathanson 1985; Kawamura and Urist 1988; Mahy and Urist 1988; Yamaguchi et al. 1991). The expression of BMP using monoclonal antibody in normal and diseased skeletal tissues and osteosarcoma has also been reported (Yang and Jin 1990; Jin and Yang 1990).

Pleomorphic adenomas of the salivary glands are composed of tumour cells showing duct-like structures accompanied by modified or neoplastic myoepithelial cells which are often transformed into chondroid cells. The chondroid cells and modified myoepithelial cells express S-100 protein which interacts with calcium signalling (Mori et al. 1987, 1989, 1990; Ninomiya et al. 1989) and produce glycosaminoglycans (GAGs) as matrix substance (Shibutani et al. 1990). The present study describes the presence of BMP in pleomorphic adenomas of the salivary glands and a possible role of BMP in induction of chondroid structures in the tumour is discussed.

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## Materials and methods

BMP isolated from bovine cortical bone described by Urist et al. (1984) was used in the production of anti-BMP monoclonal antibody (BMP-mAb) as previously reported (Fan et al. 1989), Briefly, Balb/c mice were immunized by 250 μg of the complex mixed with Freund's adjuvant. Three days after a booster dose, the spleen cells from immunized mice were fused with myeloma cell line SP2/0, and hybridomas were obtained by precipitation from spent peritoneal culture fluid with ammonium sulphate and fractionation on a DE-52 column. One clone of the hybridoma cell line yielding a mAb $IgG_{2a}$ was isolated. The analysis of the mAb by Western blot assay showed a specific band corresponding to the pattern species between 30 kDa and 34 kDa (Yang and Jin 1990). The specificity of this mAb has also been determined by using BMP activity inhibition test by Yang and Jin (1990).

Surgically resected tumour specimens from 23 cases of pleomorphic adenoma of salivary glands were examined for the presence of BMP. Serial sections 4 μm thick, cut from the formalin-fixed, paraffin-embedded blocks were deparaffinized and immersed in methanol solution containing 0.3% hydrogen peroxide for 30 min to block endogeneous peroxidase activity. The sections were washed in 0.01 M phosphate-buffered saline (0.01 M PBS pH 7.4) for 10 min. This was followed by non-specific background blocking with diluted normal rabbit serum (1:200) for 30 min, and washed in PBS. The sections were treated with mAb-BMP (1:50) overnight at 4° C, washed in PBS, treated with biotinylated, affinity-isolated, mouse-immunoglobulin at room temperature, washed and finally treated with avidin-biotin complex for 30 min at 37° C. For visualization of the antigen-antibody complex, the sections were immersed in 0.03% 3-3 diaminobenzidine tetrahydrochloride solution containing 0.005% hydrogen peroxide for 5 min. Finally, the sections were counterstained with haematoxylin.

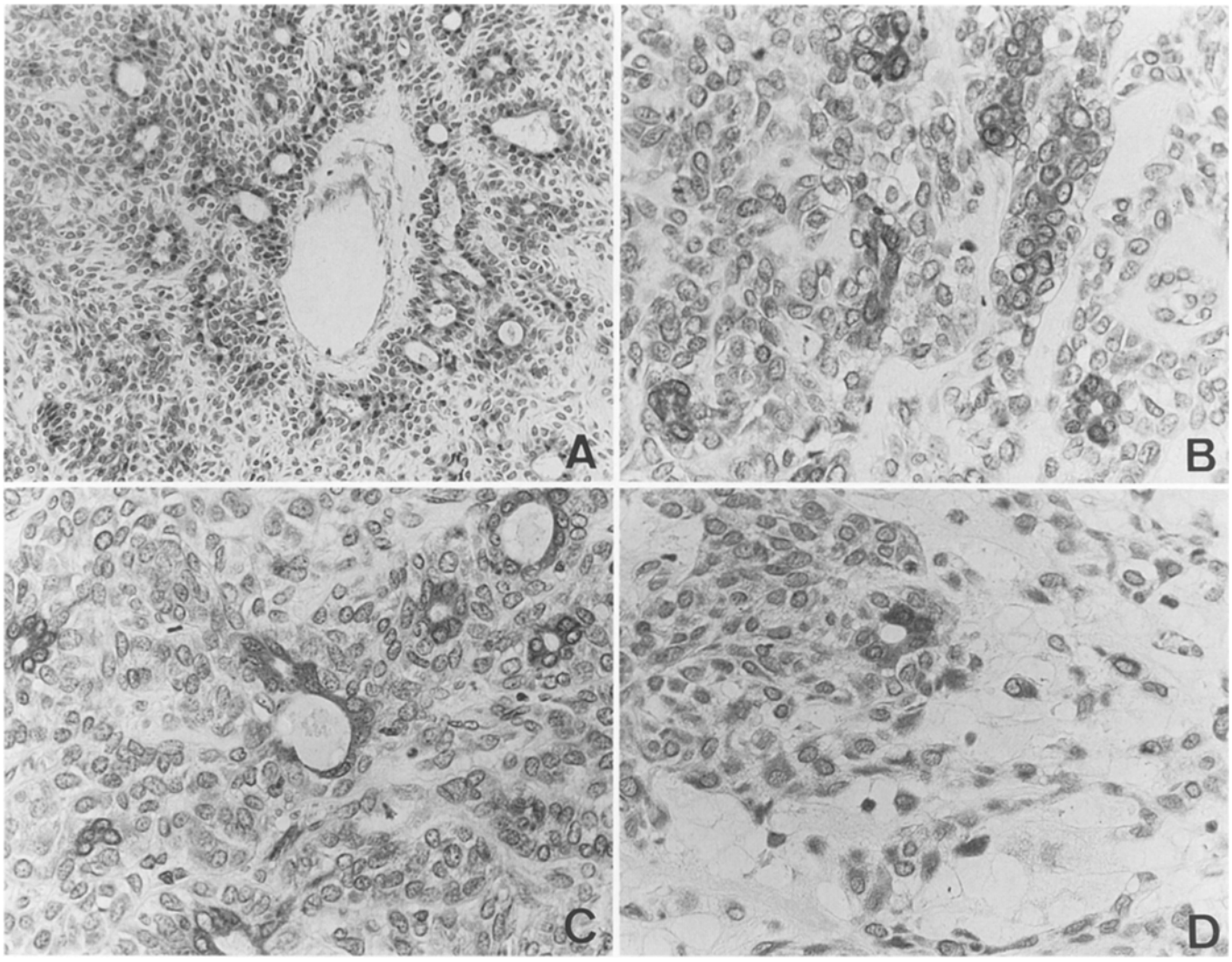


**Fig. 1 A–D.** Bone morphogenetic protein (BMP) staining in pleomorphic adenoma. Tubulo-ductal structures of pleomorphic adenoma are the most common histological feature and BMP immunostaining shows positive staining in luminal cells. **A** ×100; **B–D** ×200

Control specimens were as follows: the normal submandibular salivary gland (negative control), and BMP induced experimental chondro-osseous tissue in mouse thigh muscle, Kamegai et al. 1990b (positive control, Fig. 3B).

## Results

Normal salivary glands showed no BMP staining. Twenty cases of pleomorphic adenoma (88% cases) showed positive immunostaining. The immunohistochemical staining of BMP was localized to the luminal cells of tubulo-ductal structures (78% cases). A limited number of luminal tumour cells in tubulo-ductal structures and in cyst-like cavities of pleomorphic adenoma were stained positively for BMP immunoreactivity (Fig. 1A, D).

Positive immunostaining of BMP in modified myoepithelial cells was seen in 88% of the cases. Numerous modified myoepithelial cells in the tubulo-ductal structures, usually the spindle-shaped cells arranged in fasicles, stained positively (Fig. 2A, B). In myoepitheliomatous focui, spindle shaped cells, intermingled in diffusely proliferating sheets, were strongly positive for BMP (Fig. 2A, B). Polygonal and triangular cells in small islands and large modified myoepithelial masses (Fig. 2C, D) and plasmacytoid cells (Fig. 2F) also showed a marked BMP immunostaining.

Chondroid cells in hyaline areas in 22% cases were also positive for BMP (Fig. 3A).

## Discussion

The cloning of DNA for BMP has identified seven types of BMP, and BMP-2 to BMP-7 are members of transforming growth factor-$\beta$ superfamily (Wozney et al. 1988, 1989; Celeste et al. 1990).

In non-chondro-osseous tissue, chondrogenesis or osteogenesis is associated with the production of BMP, as insertion of synthetic BMP in situ induces osteogenesis preceeded by chondrogenesis. In this regard, BMP production in the cells of pleomorphic adenoma of the salivary gland was evaluated in the present study as these tumours show chondroid tissue formation from modified myoepithelial cells (Kusafuka et al. 1992). It has

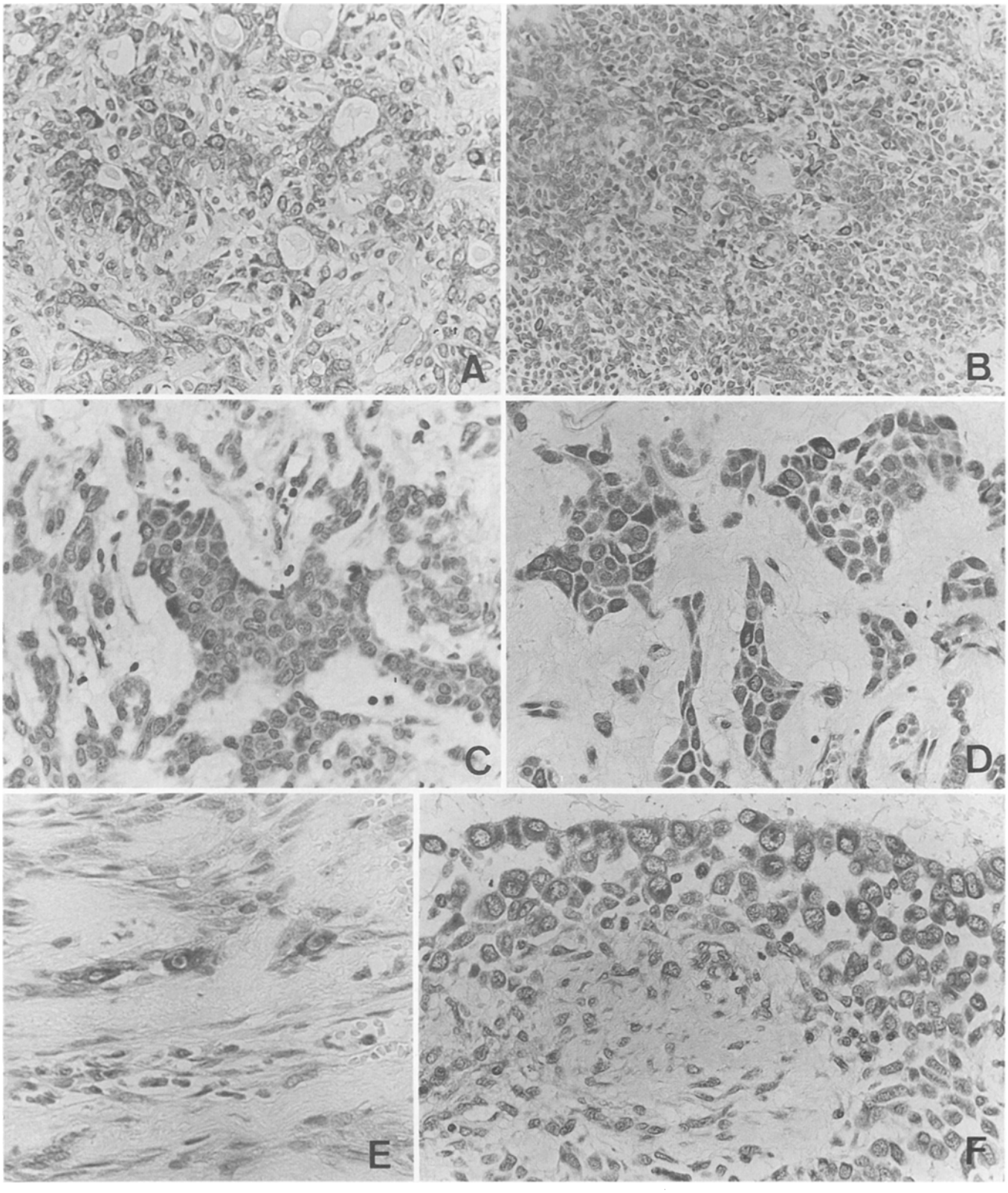


**Fig. 2A–F.** BMP immunoreactivity in modified myoepithelial cells. Spindle-shaped, modified myoepithelial cells (**A, B**) and triangular and polyhedral modified myoepithelial cells (**C, D**) in pleomorphic adenoma stain markedly for BMP. The cells in hyaline structure (**E**) and plasmacytoid myoepithelial cells (**F**) show BMP staining. **A, B** ×100; **C–F** ×200

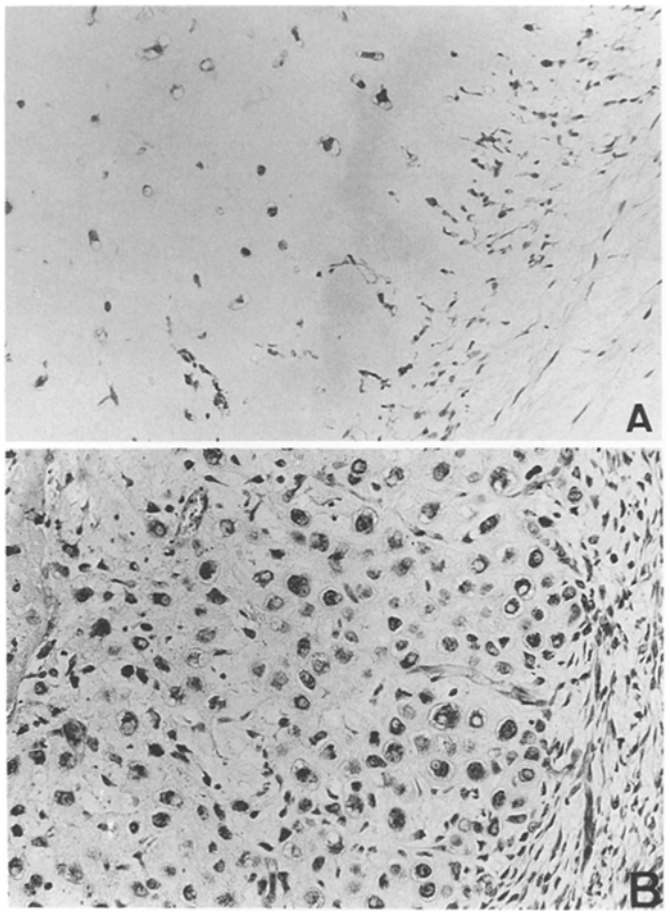


**Fig. 3A, B.** Metaplastic chondroid cells (**A**) of pleomorphic adenoma show positive staining for BMP. BMP expression is markedly found in cartilage cells in BMP induced chondro-osseous tissue in thigh mouse tissue (3 week specimen, positive control, **B**). **A, B** ×100

been reported that modified or neoplastic myoepithelial cells of pleomorphic adenomas and myoepithelioma express S-100 protein and its $\alpha$, $\beta$ subunits, keratin, vimentin, glial fibrillary acidic protein and neuron specific enolase (Mori et al. 1987, 1989, 1990; Ninomiya et al. 1989; Huang et al. 1992). Chondroid cells in the tumour are present in the modified myoepithelial cell related foci in hyaline areas. Chondrocytes are consistently positive for S-100 protein in BMP induced osteogenesis of rat muscle and during electric callus formation (Kamegai et al. 1990a). S-100 protein is a calcium binding protein, and the presence of S-100 protein in chondrocytes may be associated with calcium signalling mechanism in precalcified tissues. In the present study, BMP immunoreactivity was found in a limited number of modified myoepithelial cells, plasmacytoid and fibrous cells in hyaline structures suggesting certain modified myoepithelial cells produce BMP which, in turn, may result in chondroid changes.

The normal distribution of BMP in normal and neoplastic human tissues is not well-described in the literature. However, it has been identified in bone-forming cells and human osteosarcoma cells (Jin and Yang 1990; Yang and Jin 1990). Except for human, bovine and tooth anlage, no BMP immunoreactivity is seen in normal muscle, nerves, oral mucosa salivary glands, heart, lungs, liver, kidney, spleen, and other human soft tissue tumours. GAGs are widely distributed in connective tissue as well as in cartilage and bone and have been reported in normal epithelia and their neoplasms (Izzo et al. 1985). In pleomorphic adenoma GAGs or proteoglycans are localized in modified myoepithelial foci and plasmacytoid or chondroid cell areas (Sibutani et al. 1990) and may be associated with matrix formation and subsequent chondroid changes. It is probable that chondro-osseous changes involve multifactorial interactions of BMP production, GAGs synthesis and initial calcium signalling by S-100 proteins. However, ossification is rarely found in pleomorphic adenoma, since ossification requires abundant vascularization. The absence of angiogenesis in the hyaline structures of pleomorphic adenomas may be one of the explanations for the relatively low incidence of ossification, despite abundant chondroid changes.

The luminal tumour cells of pleomorphic adenomas were usually, but not invariably positive to BMP immunostaining. The biological role of positive reactivity for BMP in luminal tumour cells is uncertain. Immunohistochemically, luminal tumour cells are positive to cytokeratin, but negative to S-100 protein and vimentin and the cells have been suggested to originate from intercalated duct cells. The significance of BMP staining in luminal cells may be associated with a supplementary factor, paracrine in action, affecting the outer layer cells or modified myoepithelial cells.

The pattern of immunohistochemical localization of BMP resembled the distribution of S-100 protein as previously reported (Mori et al. 1987, 1989, 1990) where the immunoreactivity is localized in both the nuclei and cytoplasm. The significance of this localization cannot be explained in the present study, although Western blotting has shown the specificity of the antibody as recognizing 30–34 kDa component of the BMP molecule.

The production of BMP in a cultured submandibular adenocarcinoma cell line (HSG-S8) has been reported by Hatakeyama et al. (1991). Over-expression of BMP-2 mRNA determined by the reverse transcriptase-polymerase chain reaction method has been found in pleomorphic adenoma, particularly in chondroid tissue (Hatakeyama et al. 1992). Metaplasia of Schwann cells derived from neuroectodermal neural crest or schwannoma is accompanied by chondroid or osteoid tissue formation (D'Agostino et al. 1963). The origin of the modified myoepithelial cells of pleomorphic adenoma has been suggested to be related to neuroectodermal cells (Huang et al. 1992) but our findings suggest that metaplastic potential of modified myoepithelial cells is responsible for chondroid change. BMP may play a significant role in this yet incompletely understood process.

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