Histol Histopathol (2001) 16: 1013-1019
DOI: 10.14670/HH-16.1013
http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Transcriptional mRNA of BMP-2, 3, 4 and 5 in trigeminal nerve, benign and malignant peripheral nerve sheath tumors

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Summary. The aim of our study was to document whether relationships existed among bone morphogenetic proteins (BMPs), peripheral nerve and neoplastic lesions of nerve sheath tumors. The mRNA transcriptions of BMP-2, 3, 4 and 5 in 10 cases of schwannoma, three cases of malignant schwannoma and two cases of trigeminal neuralgia were detected using an *in situ* hybridization technique. Our results demonstrated that the myelin sheaths of Schwann cell from the peripheral neuroectomy of trigeminal neuralgia positively expressed mRNA of BMP-2, 3, 4, and 5. The most interesting finding was that the nerve fibers of trigeminal nerve showed only BMP-2 positive staining. All of the neoplastic lesions of nerve sheath showed a consistent but variant expression of BMP-2, 3, 4, and 5. The expression signals of BMP-2, 3, 5 mRNA in malignant schwannoma were relatively lower than in benign lesions except for the expression of BMP-4 mRNA. Our results indicated that selected members of BMPs were expressed in the peripheral nerves that might contribute to the health maintenance, proliferation, regeneration and neoplastic transformation of the peripheral nerve system. Furthermore, the effects of BMP-2, 3, 4 and 5 on peripheral nervous system during neoplastic transformation might be widespread, diverse and antagonistic.

Key words: BMPs, nerve sheath tumor, malignant, trigeminal nerve, Schwann cell, *in-situ* hybridization

Introduction

Bone morphogenetic proteins (BMPs) are a rapidly expanding family closely related to the transforming growth factor-ß (TGF-ß) superfamily. BMP irreversibly induces the differentiation of perivascular mesenchymal-type cells into cartilage and bone-forming cells, induces ectopic bone formation *in vivo* and enhances the healing of bone defect and bone formation (Sampath et al., 1992; Wozney, 1992; Kingsley, 1994). Over the past two decades, the knowledge about BMPs including their purification, cloning, physicochemical characteristics and application have greatly expanded. At least six of the BMPs (BMP 2-7) can clearly act as initiators of normal bone and cartilage formation and the others may also function at the later stages of skeletal growth and differentiation (Sampath et al., 1992; Wozney, 1992; Kingsley, 1994). Although BMPs have been identified by their presence in bone, cartilage and dentin-inductive extracts derived from bone, cartilage and tooth, the activities of the BMPs are not restricted to hard tissue formation. BMP family consists of a large number of genetically related molecules, conserved in evolution, playing key roles in the development of the kidneys (BMP-3, 4 and 7), heart (BMP-2, 4, 6 and 7), lungs (BMP-3, 4 and 5), small intestines (BMP-3 and 7), sperm (BMP-8A) and limb bud (BMP-2, 4, 5 and 7) (Vukicevic et al., 1994; Sasai et al., 1995; Kawakami et al., 1996; Barlow and Francis-West, 1997; Glinka et al., 1997; Hemmati-Brivanlou and Melton, 1997; Godin et al., 1998; Zhao et al., 1998). Recently, increasing evidence suggests that there is a regulatory role of BMPs in neurogenesis (Mehler et al., 1997). BMP ligands and receptor subunits are present throughout neural development within discrete regions of the embryonic brain and within neural crest-derived migratory zones (Fann and Patterson, 1994; Hawley et al., 1995; Varley and Maxwell, 1996; Xu et al., 1996; Lo et al., 1997; Bengtsson et al., 1998). Previous studies have indicated that BMPs regulated neuronal survival and phenotypic maturation from more lineage-restricted peripheral and central nervous systems progenitor populations (Fann and Patterson, 1994; Iwasaki et al., 1996; Mehler et al., 1997).

Tumors of neural tissue arise in connection with the

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sheaths of peripheral nerves, neuroglia and nerve cells. Tumors of the nerve sheath are neurilemmoma and neurofibroma. Although there is growing evidence to indicate the regulatory role and neurotrophic capacity of BMPs in neurogenesis, there are no published report about the relationships between BMPs and nerve sheath tumors and between BMPs and peripheral nerves. Thus, the purpose of the present study was to investigate the expression of BMPs in the peripheral nerve and nerve sheath tumors and to evaluate the possible roles of BMPs in the progression and malignant transformation of nerve sheath tumors.

Materials and methods

Samples

The samples of nerve sheath tumor were obtained from biopsy of surgical patients at the Department of Oral Histology and Pathology, Qin Du Stomatological Hospital, Fourth Military Medical University, Xi'an, People's Republic of China. Lesions were all located in the oral maxillofacial region, consisting of ten cases of schwannoma (neurilemmoma) and three cases of malignant schwannoma. Ages of patients ranged from 25 to 64 years old. Two control specimens of trigeminal nerve fragments were collected by peripheral neuroectomy from cases of trigeminal neuralgia. All samples were fixed in 10% neutral formalin and embedded in paraffin blocks. Five μm thickness sections were cut and stained with hematoxylin and eosin for histopathological assessment and processed for *in situ* hybridization.

Preparation of BMPs cDNA probes

Plasmid pSP65-BMP-2 containing human BMP-2 cDNA fragment (1.58kb), plasmid PGEX-2T-BMP-3 containing human BMP-3 cDNA fragment (780bp; obtained from Dr. JM Wozney, Genetics Institute, Inc., Cambridge, MA, USA), Bluescript KS-BMP4 containing human BMP-4 cDNA fragment (1.8kb; obtained from Dr. JQ Feng, Division of Endocrinology, University of Texas Health Science Center, San Antonio, Texas, USA) and pBmp5UT containing human BMP-5 cDNA fragment (246bp; obtained from Dr. DM Kingsley, Department of Developmental Biology, Stanford University, Stanford, California, USA) were labeled with digoxigenin according to method of random primed labeling using a DIG-Labeling Kit (Roche Diagnostics, Germany). The specificity of the BMP cDNA probes was established in our previous study (Lu et al., 2000).

In situ hybridization (ISH)

In situ hybridization was carried out with RNase-free materials and solutions. *In situ* hybridization kit (Roche Diagnostics, Germany) was used in the following procedures according to the manufacturer's instructions with some modifications. Briefly, sections were dewaxed with xylene, rehydrated in sequential ethanols and rinsed in 0.1% diethylpyrocarbonate (DEPC) and phosphate-buffered saline (PBS). Sections were treated with 0.2 mol/L HCl solution for 15 min and 0.3% Triton X-100 in PBS for 10 min followed by digestion with 10 μg/ml proteinase K (Sigma Chemical Co., USA) for 10 min at 37 °C, washed in 0.2% glycine in PBS for 10 min and post-fixed in 4% PFA for 30 min. Subsequently, sections were dehydrated and dried. Hybridization buffers containing 40 ng/ml DIG-labeled BMP-2, 3, 4, 5 cDNA probes were applied appropriately to each corresponding section. Labeled probes were heated for 10 min in a boiling water bath before used. After hybridization for 16 h at 42 °C, sections were washed sequentially in 2xSSC (standard saline citrate), 1xSSC (150 mmol/l sodium citrate; 15 mmol/l NaCl, pH 7.0) at 37 °C, 0.5xSSC and buffer I (100 mmol/l Tris-HCl; pH 7.5; 150 mmol/l NaCl). Sections were incubated with 2% normal goat serum for 30 min and subsequently incubated with anti-digoxigenin Fab/alkaline phosphatase (AP) conjugate for 2 h, then immersed in buffer I with shaking, followed by an equilibration step in buffer III (100 mmol/l TrisHCl; pH 9.5; 100 mmol/l NaCl; 50 mmol/l $MgCl_2$), and later in BCIP (5-bromo-4-chloro-3-indolyphosphate)/NBT (4-nitro blue tetrazolium chloride)/levamisole colour solution in the dark for up to 4 h. The colour reaction was stopped with 10 mmol/L Tris-HCl and 1 mmol/L EDTA (pH 8.0). Sections were processed in graded ethanols, xylene, and then mounted. For better histological observation, some of the sections were counterstained in methyl green, washed in 3 changes of distilled water and 3 changes of 100% butanol and then finally processed in xylene and then mounted.

Sections of osteosarcoma that demonstrated consistent expression of BMP-2 mRNA were included in each batch of staining to serve as positive control. Negative controls were processed similar to the above protocol with the omission of the probes in the hybridization buffer prior to hybridization procedure or after treating sections with RNase (100 μg/ml).

Results

No positive staining was seen in the control sections without applying probes. Negative staining or obviously decreased signal was observed in the control sections treated with mRNase prior to hybridization.

There was a consistent BMP-2, 3, 4 and 5 positive staining in the trigeminal nerve. Both the horizontal and longitudinal section of the fragments of trigeminal nerve showed the expression of BMPs signals. Generally, the hybridization signal was highest in BMP-2 than that in BMP-3, 4 and 5. The most interesting finding was that the nerve fibers of trigeminal nerve showed only BMP-2 mRNA positive staining (Fig. 1A,B). The myelin sheaths of Schwann cell were obviously stained with BMP-2, 3, 4 and 5, whereas the staining appearance of BMP-3, 4, 5

presented a reticular form (Figs. 2A,B, 3A,B). The various levels of positive signals were also detected in the epineurium, perineurium, and endoneurium of peripheral nerve.

The results of *in situ* hybridization revealed that almost all of the lesions showed variant positive staining for BMP-2, 3, 4 and 5. Microscopically the schwannomas showed two distinct cellular patterns designated as Antoni type A and Antoni type B. Antoni type A tissue consists of cells like fibrocytes together with intercellular collagenous tissue. Almost all of the tumor cells (fibrocyte-like cells) were BMP-2, 3, 4 and 5 positively stained, whereas the intensity of BMP-4 and 5 staining was higher than that of BMP-2 and 3 (Fig. 4). The positive signals were obviously seen in the palisading area, or the region of accumulated tumor cells (Figs. 4, 5A). There was no detectable staining in the matrix of tumor and collagenous fiber. The staining intensity was relatively and slightly weak in cells of Antoni B tissue when compared with Antoni A tissue. Nevertheless, the tumor cells in Antoni B tissue also showed BMP-2, 3, 4 and 5 positively staining (Fig. 5B). The fibroblasts of schwannoma were negatively stained.

Almost all of the malignant lesions showed positive hybridization signals for BMP-2, 3, 4 and 5 except the varied staining intensity of *in situ* hybridization. When comparing the expression of mRNA signals of BMP-2, 3 and 5 except for BMP-4 with benign and malignant schwannomas, the latter showed reduced signals (Figs. 6, 7 and 8B). The high level of BMP-4 mRNA signal was observed in the neoplastic cells of both malignant and benign lesions (Fig. 8A).

Discussion

We have previously shown that the expressions of BMPs were also present in the neoplasms of bone, tooth, cartilage, glandular tissue and other tissues (Jin and Yang, 1990a-c; Yang et al., 1993a,b). In particular, we found that BMP could influence the progression and prognosis of osteosarcoma, chondrosarcoma and benign and malignant salivary gland tumors. Experimental analysis of BMPs action during neural development is still in its infancy. However, rapid and dramatic progress have been accomplished in elucidating the role of this expanding TGF-ß subclass in wide diverse cellular events during early, intermediate and later stages of neurulation, morphogenesis, lineage elaboration and phenotypic maturation (Liem et al., 1995; Varley and Maxwell, 1996; Hemmati-Brivanlou and Melton, 1997; Mehler et al., 1997; Bengtsson et al., 1998). However, it still remains unknown about the expressions and the roles of BMPs in the postnatal nerve system, particularly in the peripheral nervous system (PNS). Thus, the aim of our present study was to investigate the patterns of expression and distribution of BMPs in the postnatal and neoplasms of the PNS, and to determine the possible roles of BMPs in the generation and progression of the neoplasms of PNS. Our results showed that there was a consistent BMP-2, 3, 4 and 5 positive hybridization signals in the myelin sheaths of Schwann cell of the trigeminal nerve. Generally, the positive signal was highest in BMP-2 staining than in BMP-3, 4, and 5. The most interesting finding was that the nerve fibers of trigeminal nerve expressed BMP-2 mRNA only.

The specific cellular binding proteins of BMP-2 on neuronal cells seems particularly interesting as several of the TGF-ß superfamily members have recently been shown to possess neurotrophic activity (Iwasaki et al., 1996; Mehler et al., 1997). BMP-2 induces the neuronal differentiation of rat pheochromocytoma PC12 cells with concomitant expression of three neurofilament proteins (Iwasaki et al., 1996) and in concert with tumor necrosis factor (TNF), it plays an essential role in regulating the regeneration of peripheral nerves through an indirect mechanism by which it stimulates nerve growth factor (NGF) production in fibroblasts (Hattorl et al., 1996). Mabie et al. (1997) found that the BMPs act as potent inductive factors in postnatal glial lineage commitment that initiate a stable program of astroglial differentiation. Our findings further demonstrated that BMP-2, 3, 4 and 5, particularly BMP-2 and 4 might play an important role in the healthy maintenance and pathological changes of peripheral nerve by means of autocrine or paracrine pathway. We propose that the selected members of BMP family might be able to influence the status of peripheral nerves through Schwann cells (SCs). SCs of neural crest-derived cells, have been demonstrated to be active participants in the recovery from peripheral nerve damage and promoting CNS axonal regeneration (Levi et al., 1997). A number of studies have demonstrated that the addition of SCs within channels or grafts dramatically improves the regeneration of PNS neurites. SCs produce both neurotrophic factors such as NGF, brain derived neurotrophic factor and ciliary neurotrophic factor, and the extracellular matrix molecules such as laminin and collagen. Our present results further proved that SCs could synthesize and secrete BMP-2, 3, 4 and 5, that might have selective effects on the nerve fibers. Our study also showed the sole expression of BMP-2 in the nerve fiber of trigeminal nerve. Although it is still not clear on how the detected transcription mRNA of BMP-2 was synthesized and transported, it seemed that there was an interaction between nerve fiber and SCs. BMP-2 appearing to be another neurotrophic factor such as NGF or basic fibroblast growth factor, which might play a critical role in the growth and proliferation of both nerve fiber and SCs.

Benign schwannoma is a rather common tumor derived from SCs, which form the nerve sheath. Malignant nerve sheath tumor usually arises as a result of either *de novo* or malignant change in pre-existing neurofibromas, particularly in cases of neurofibromatosis. Structurally, malignant nerve sheath tumors consist of fusiform cells and fibers, closely resembling fibrosarcoma. Occasionally, a palisading arrangement that represent the typical morphological appearance of

Schwannoma is present and it aids in the diagnosis of the malignant lesion (Casadei et al., 1995; Kindblom et al., 1995; Chrysomali et al., 1997; Hasegawa et al., 1997). Relatively little is known about the molecular genetic alterations that underlie their formation (Halling et al., 1996). Up to date, there is no published report on the relationship between BMPs and benign or malignant nerve sheath tumors. Our results revealed that almost all of the lesions showed variant transcriptional mRNA signals of BMP-2, 3, 4 and 5 in both tumor cells in Antoni type A or B tissues. The positive signals were observed in the palisading area or in the area of the accumulated tumor cells. Although all of the malignant lesions showed positive staining, the malignant schwannoma showed reduced transcriptional mRNA signals of BMP-2, 3 and 5 when compared with the benign lesions, except for the expression of BMP-4. It was not yet clear why the expression signals of BMP-2, 3, 5 were decreased in the malignant than in the benign lesions. Previous reports have indicated that BMPs might exert selective effects and might have opposite influences on the proliferation, differentiation and morphogenesis of neural cells (Fann et al., 1994; Hawley et al., 1995; Xu et al., 1996, 1997; Hemmati-Brivanlou and Melton, 1997; Lo et al., 1997; Mehler et al., 1997). BMP-2 and BMP-6 could induce mRNAs for distinct sets of neuropeptides and neurotransmitter synthetic enzymes in rat neural crest-derived sympathetic neurons (Fann et al., 1994). BMP-2 and BMP-4 were able to induce expression of MASH1 and to promote autonomic neuronal differentiation in neural crest stem cells (Lo et al., 1997). Several reports indicates that BMP-4 might be a neural inhibitor (Hawley et al., 1995; Sasai et al., 1995; Hemmati-Brivanlou and Melton, 1997; Xu et al., 1997). Whereas Xu et al. (1996, 1997) have shown that BMP-4 was a ventralizing factor in Xenopus body patterning. Several studies have suggested that other types of BMP could fulfill the same neural inhibitory activity of those dominant negative BMP ligands that have a pleiotropic inhibitory effect on all BMPs (Hawley et al., 1995; Hemmati-Brivanlou and Melton, 1997). Some studies suggested that the action of BMP factors in the developing nervous system is dependent on the species, particular cell type and developmental stage being examined (Fann et al., 1994; Varley et al., 1996). Our results indicated that changes in the expression of BMP-2, 3, 4 and 5 occurred during the neoplastic and malignant transformation of Schwann cells and the expressions of BMP-2, 3 and 5 were relatively and slightly reduced. The effects of BMP-2, 3, 4 and 5 on PNS might be widespread, diverse and antagonistic.

Although the expression of BMPs in both benign and malignant nerve sheath tumors was evidently shown, the roles of BMPs during the neoplastic transformation of SCs and progression of nerve sheath tumors were not clearly understood. We cannot exclude the possibility that there might be mutant types of BMPs gene during neoplastic or malignant transformation of peripheral nerve. Nevertheless, our results indicated that the positive hybridization signals of BMPs might be an indicator in the differentiation of malignant schwannoma and fibrosarcoma. It is accepted that foci of cartilaginous and osseous modulation could be found in some malignant schwannomas. Although we did not observe the formation of tumorous cartilage or bone in our collected lesions, our present results indicated that BMPs secreted by tumorous cells of benign and malignant schwannomas might contribute in the bone or cartilage-induction of some malignant schwannomas.

In conclusion, we demonstrated that the expression of BMP-2, 3, 4 and 5 might be a common event in the peripheral nerve system. Almost all of the tumor cells of the benign and malignant nerve sheath tumors showed variant expressions of BMP-2, 3, 4 and 5. The effects of BMP-2, 3, 4 and 5 on PNS might be widespread, diverse and antagonistic. Our results indicated that BMP-2, 3, 4 and 5, particularly BMP-2 and BMP-4 might play critical roles in the normal and pathological changes of PNS. BMP-2, 3, 4 and 5 were involved in the neoplastic transformation of the Schwann cells of PNS and they might contribute to the neoplastic and malignant transformation of peripheral nerve cells. Further studies are needed to clarify the effect of each individual BMPs and SMADs in the growth, regeneration or degeneration

Fig. 1. Trigeminal nerve showing positive hybridization signals of BMP-2 in the myelin sheath and nerve fibers. x 100

Fig. 2. Longitudinal **(A)** and horizontal **(B)** sections of trigeminal nerve showing the positive hybridization signals of BMP-3 mRNA in the myelin sheath of Schwann cells. x 100

Fig. 3. The longitudinal section of a trigeminal nerve. BMP-4 **(A)** and BMP-5 **(B)** are obviously expressed in the myelin sheath of nerve. x 100

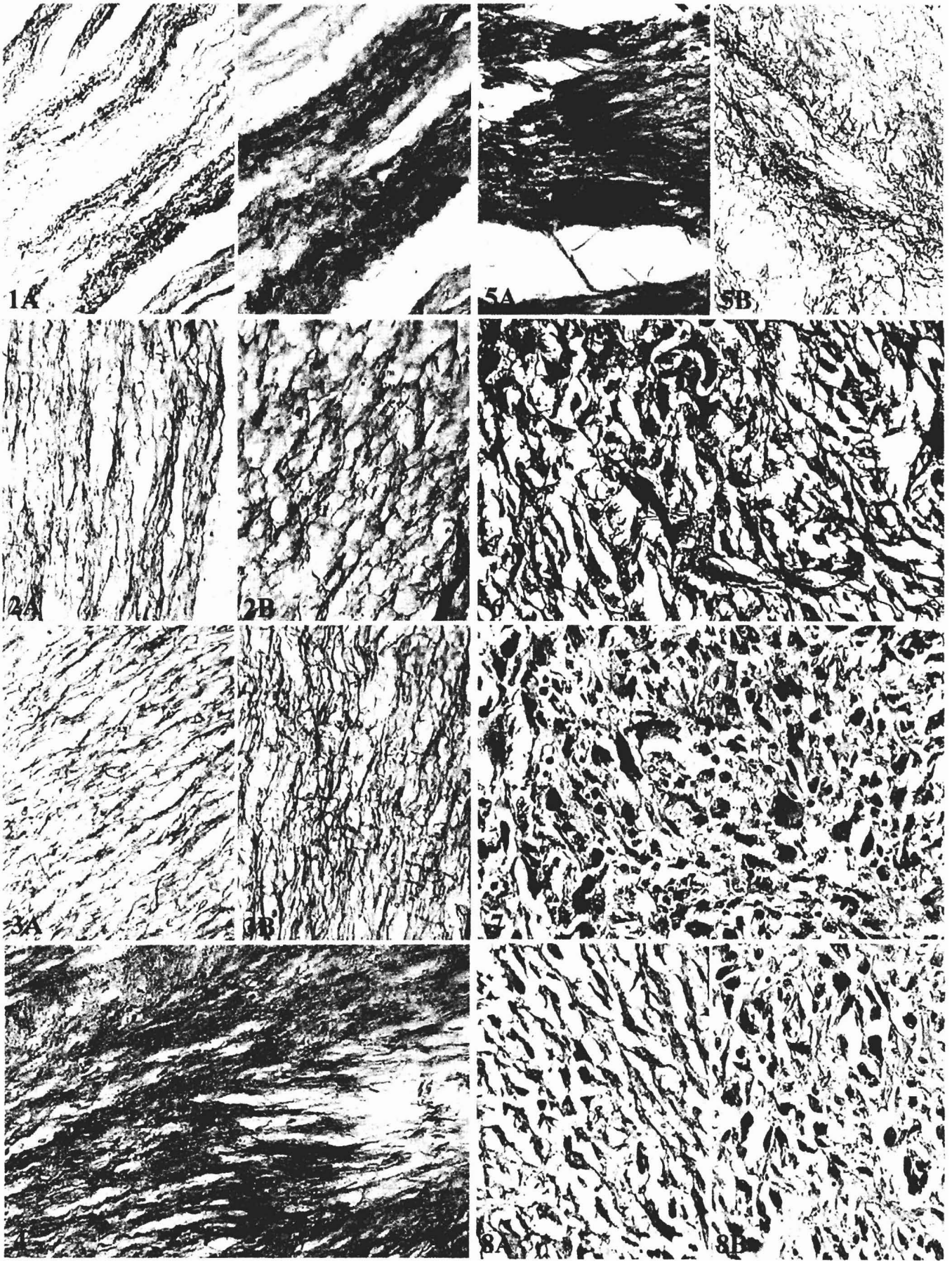
Fig. 4. The tumor cells of schwannoma showing overexpression of BMP-4 mRNA. x 100

Fig. 5. A. Palisading area of the schwannoma is strongly positive for BMP-2 mRNA. **B.** Schwannoma cells in Antoni B area showing positive for BMP-5 mRNA. x 100

Fig. 6. Tumor cells of malignant schwannoma showing BMP-2 positively stained. x 200

Fig. 7. Tumor cells of malignant schwannoma showing BMP-4 positively stained. x 200

Fig. 8. A. The BMP-3 positive signal is shown in the cytoplasm of tumor cells of malignant schwannoma (no counter-stain). **B.** While BMP-5 positive signal is shown in some of the tumor cells of malignant schwannoma. x 200


1A
1B
5A
5B
2A
2B
3A
3B
7
4
8A
8B

of PNS and the relationship between each individual BMPs and clinico-pathological features of benign and malignant schwannomas.

Acknowledgements. We would like to thank Mr. Johnny Leung for his photographic work in this manuscript. This work was supported by Internal Competitive Research Grant (A.52.37.PC15), F.H.S.S., Hong Kong Polytechnic University.

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Accepted May 23, 2001