Measurement of total and local bone morphogenetic protein concentration in bone tumours

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Summary. Bone morphogenetic protein (BMP) has been shown to be one of the significant factors in the prognosis of bone tumours. In normal development BMP induces new bone formation and later takes part in fracture healing, but its function in malignant tumours is not known. In this study the concentration of bone morphogenetic protein was measured in primary bone tumours by two methods. Local staining intensity was detected immunohistologically by the avidin-biotin-peroxidase method determining the highest dilution of anti-serum against bovine bone morphogenetic protein. The total amount of BMP in a tumour sample was measured by an enzyme-linked immunosorbent assay technique after digesting the tissue with collagenase to remove proteins from the connective tissue. Immunohistochemical staining showed that bone morphogenetic protein was present in the cytoplasm and in reactive bone formed by malignant cells. The local concentration was highest in the tissue of giant cell tumours compared to chondrosarcoma, osteosarcoma and benign bone tumours. The total amount in malignant bone tumours was 2.4 times higher compared to benign bone tumours.

Résumé. Il a été démontré que la protéine morphogénétique osseuse (BMP) est un facteur important dans le pronostic des affections osseuses. Dans un développement osseux normal, cette protéine induit la formation de nouveau tissu osseux et contribue, par exemple, dans les fractures, à la

consolidation. Dans les affections malignes, la fonction précise de la BMP reste inconnue. Dans la présente étude, la concentration de protéine morphogénétique osseuse a été mesurée dans des tumeurs osseuses primaires à l'aide de deux méthodes. L'intensité de coloration locale de la BMP a été détectée immunohistochimiquement par la méthode avidine-biotine-péroxydase, déterminant le titre le plus élevé de dilution d'antisérum par rapport à la BMP bovine (bBMP); le résultat de la coloration a été positif. Le total de protéine morphogénétique osseuse présent dans un échantillon de tumeur a été mesuré à l'aide d'un dosage enzymatique immuno-absorbant (ELISA) après digestion du tissu avec de la collagénase pour retirer les protéines du tissu conjonctif. La coloration immunohistochimique a montré que la BMP se localisait dans les cytoplasmes et dans le tissu osseux réactif formé par les cellules malignes. La plus forte concentration locale de BMP a été trouvée dans le tissu des cellules géantes de la tumeur dans chondrosarcome, l'ostéosarcome et les tumeurs osseuses bénignes. Le total de BMP présent dans les tumeurs osseuses malignes était 2.4 fois supérieur à celui décelé dans les tumeurs osseuses bénignes.

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Introduction

Bone morphogenetic proteins (BMPs) are morphogenes capable of inducing new cartilage and bone in ectopic sites [23]. Attempts have been

Table 1. Details of the tumours and BMP measurements of the samples studied. Local BMP concentration in tissue sections presented as the reciprocal of the highest dilution of antiserum giving positive immunostaining. Total BMP concentration repre-

sents the amount in the whole tissue sample studied. When two figures are given, the first represents intracellular BMP and the second extracellular in reactive bone formed by malignant cells

Case	Origin of tumour samples			BMP concentration		
	Diagnosis	Sex	Age	Local (relative)	Total ($\mu\text{g/g}$)	Mean $\pm$ SEM
1	Osteosarcoma	M	19	200	8	10 ± 3
2	Osteosarcoma	M	19	200	11	
3	Osteosarcoma	M	27	200	10	
4	Chondrosarcoma	F	57	200	18	
5	Chondrosarcoma	F	83	200, 5000	6	
6	Chondrosarcoma	M	80	5	7	10 ± 4
7	Chondrosarcoma	M	48	200	7	
8	Chondrosarcoma	M	77	200	10	
9	Giant cell tumour	F	16	100, 2000	8	
10	Giant cell tumour	M	77	500	7	
11	Giant cell tumour	F	40	100, 1000	17	4 ± 1
12	Enchondroma	F	45	200	3	
13	Osteoblastoma	M	27	200	5	

made to find and produce this active bone inducing protein since it might provide an alternative source for bone grafts [3, 8, 13]. Structurally the BMPs belong to the transforming growth factor of the β (TGF- β) superfamily. Nine different BMPs have been identified by their amino-acid sequence homology and 7 showed osteoconductivity when investigated by bioassay [4, 33]. The significance of the various BMPs in bone inductivity is not clear [20, 28], although it has been assumed that BMP-2 and BMP-4 are mainly responsible [29].

BMPs have been shown to contribute to abnormal bone formation and its occurrence in malignant tissues can be used to determine prognosis [13, 33, 34], in addition to other factors such as sex [5, 16, 18], its site [2, 5, 6], its size [2, 5, 16], the duration of symptoms and histological subtype [5, 16, 17]. Yoshikawa et al detected BMP in only some samples of osteosarcoma; their patients with BMP-producing osteosarcoma had lung metastases more often than those with osteosarcoma which did not produce BMP [33, 34]. These results show the importance of BMP in the clinical evaluation of bone diseases.

Recently immunohistochemical analysis has been widely used to detect BMP in bone and soft tissue malignant tumours [13, 33, 34]. In the present study our aim was to develop a new technique to quantify BMP in small amounts of bone tumour tissue and to compare its properties with those of the more commonly used immunohistochemical analysis.

Materials and methods

Thirteen samples of tissue from primary bone tumours were obtained from surgical operations in our hospitals. There were 3 osteosarcoma, 5 chondrosarcoma, 3 giant cell tumours, and 2 benign bone tumours. Two of the samples of osteosarcoma were from the same patient, one before and one after chemotherapy (cases 1 and 2). The mean age was 49 years (range 16 to 83 years). The diagnosis, age and sex are shown in Table 1.

Preparation of antiserum against BMP.

75 μg of highly purified bovine BMP (bBMP) [10] was mixed with 0.1 ml of Freund's complete adjuvant (Sigma Chemical Co, St Louis, USA) and injected intracutaneously into the abdomen of a mouse. Similar injections were given weekly with Freund's incomplete adjuvant for 5 weeks. After collection of blood and separation of the serum, the antibody was measured using an enzyme-linked immunosorbent assay (ELISA) with 0.25 μg of highly purified bBMP as antigen.

Western blot.

This assay was performed to confirm antibody specificity against bBMP. 0.5 mg of highly purified bBMP was dissolved in 6 M urea, diluted in 30 mM sodium buffer at pH 9.6, mixed with equal volumes of nonreduced sample loading buffer and boiled for 5 minutes. The bBMP was loaded for 12% (acrylamide) SDS-PAGE analysis. Proteins from SDS-PAGE gels were transferred electrophoretically to a Hybond-C extra nitrocellulose sheet (Amersham International plc, Buckinghamshire, England). For immunodetection the filter was blocked with Tris buffered saline (TBS, 0.5 M NaCl in 20 mM HCl, pH 7.5) containing 1% Triton X-100 and 10% nonfat milk powder. The filter was then reacted with bBMP-antiserum diluted in 1% Tween-20 TBS (TTBS) overnight at +4 °C. Bound antibodies were detected using the avidin-biotin-peroxidase (ABC) method (ABC-Elite, Kit, Vector Laboratories Inc, Burlingame, USA).

Fig. 1. Western blot assay of bBMP under nonreducing conditions. Three different bands with molecular weights of 30 kD, 67–69 kD and 90 kD can be seen.

Local BMP concentration.

A piece of bone tumour tissue, 1 cm³ in size, was fixed in formalin and then embedded in paraffin. Sections, 7 µ thick, from each sample were stained by the ABC method (ABC-Elite Kit, Vector Laboratories Inc). Mouse antiserum to highly purified bBMP was applied overnight at +4 °C. The antiserum was diluted in 1% bovine serum albumen (BSA) in phosphate buffered saline (PBS) pH 7.6 with 150 mM NaCl at 1:5, 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000, 1:5000, and 1:8000. Normal rabbits serum in dilution buffer served as control. The specificity of staining was controlled by blocking the anti-serum with bBMP (1 mg/ml) and positivity observed microscopically by 2 individuals. The local BMP concentration in the tissue section was presented as the reciprocal of the highest dilution which gave positive staining.

Total BMP concentration.

BMP was purified from extra- and intracellular tumour tissue by the collagenase digestion method [9]. The samples were cut into pieces about 2 mm³ which were digested with type 1 collagenase (Sigma Chemical Co, St Louis, USA) in 20 mM Tris-HCl buffer, pH 8.0 with 0.1 M NaCl, 50 mM CaCl₂, 50 mM MgCl₂, 1 mM N-ethylmaleimide, 0.1 mM phenylmethanesulfonyl fluoride and 0.1 mM benzamidinium-HCl at +37 °C overnight with continuous shaking. The mixture was then centrifuged and the supernatant dialysed against distilled water at +4 °C overnight. The precipitate was dissolved in 30 mM sodium bicarbonate buffer pH 9.6.

The protein concentration of the supernatant was measured by Lowry's method [14] and adjusted to 1 mg/ml. It was then fractionated using a Superdex 200 high performance liquid chromatography (HPLC) gel filtration column (Pharmacia Diagnostics, Umeå, Sweden). The collected fractions were then dialysed against 30 mM sodium carbonate buffer, pH 9.6, at +4 °C overnight.

ELISA plate (Nunc MicroWell Plate, Nunc A/S, Roskilde, Denmark) wells were coated with the collected gel filtered fractions and dissolved precipitates, and for the standard curve with 0.005 µg, 0.01 µg, 0.025 µg, 0.05 µg of BMP dissolved in carbonate buffer for 3 hours at +37 °C. The plates were blocked by incubating with with 4% nonfat milk powder in 30 mM sodium carbonate buffer, pH 9.6 for 50 minutes at +37 °C. They were then incubated overnight at +4 °C with bBMP-antiserum. As the second antibody, alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulins (Dako, Glostrup, Denmark) were incubated for 3 hours at +37 °C. Phosphatase substrate (Sigma Diagnostics, St Louis, USA) was administered and the colour reaction measured spectrophotometrically at 405 nm. The results were presented as µg of BMP/g of total protein. The dilution buffer served as a control for the antiserum.

Data are expressed as mean ± SEM.

Fig. 2.a Positive immunohistochemical staining of a tumour section. Strongly positive dark areas (arrows) can be seen within the cells of a chondrosarcoma (ABC strain, ×100). **b** A negative control of chondrosarcoma. Counterstaining with Harris's haematoxylin. (ABC stain, ×100).

Results

Immunohistochemical characterisation of antiserum

In Western blot analyses the antiserum reacted with three different bands with molecular weights of 30 kD, 67–69 kD and 90 kD. The assay of highly purified bBMP with bBMP-antiserum is shown in Figure 1. In ELISA, the antiserum detected 0.025 µg of highly purified bBMP at a dilution of 1:8000.

Immunohistochemical staining of BMP

This was used to demonstrate the existence and the local concentration of BMP in bone tumour tissue samples, and was positive in all of them (Fig. 2a). The frequency of the cytoplasm staining positively varied considerably. In those stained intensively, the BMP-positive cell groups appeared adjacent and surrounded by negatively stained cells. Those sections which showed reactive bone formation by

Fig. 3. The tumour was fractionated after collagenase digestion with an HPLC gel filtration column and fractions of 1.8 ml were collected. The BMP concentration was measured using ELISA and results presented as optical density. Fractions with an optical density higher than 0.1 were pooled for total BMP measurement.

malignant cells also showed positivity in that area. All of the controls were negative (Fig. 2b). When the relative local amount of BMP was detected in cytoplasm in 13 cases, the titre was 1:200 in 9, 1:100 in 2, and 1:500 and 1:5 in one case each. In 3 cases where reactive bone formation by malignant cells was seen, the titre in that area was 1:1000, 1:2000 and 1:5000. Table 1 summarises the lowest dilutions in which immunostaining for BMP was positive in all samples.

Detection of total BMP

The dissolved collagenase-digested precipitate contained no BMP as measured by ELISA. When collagenase-digested supernatant was fractionated with the HPLC gel filtration column, high molecular weight fractions (>250 kD) showed positivity in ELISA when exposed to bBMP-antiserum. A typical HPLC fraction curve from collagenase-digested tumour tissue and the BMP content of the fraction are shown in Figure 3. The concentration varied to some extent between the different samples. The total concentration of BMP in the samples of osteosarcoma and chondrosarcoma varied from 6–18 µg/g of protein, average 10 µg (SEM ± 3). There was no significant difference between samples taken before and after chemotherapy. In giant cell tumours the amount varied from 8–17 µg, average 11 µg (SEM ± 4). In 2 benign tumours, the content was on average 4 µg (SEM ± 1). The BMP concentration was 2.4 times higher

Fig. 4. The distribution of BMP concentration between different tumour samples. The amount in different collagenase-digested tumours was measured using ELISA. In benign tumours the amount of BMP was lower than in malignant.

in malignant bone tumours compared with benign, when giant cells tumours were excluded. The results of ELISA for total concentration of BMP in tissue samples are given in Figure 4 and Table 1. The specificity of ELISA was ascertained by including controls for the binding reactions which were negative.

Discussion

In 1978, tissue from osteosarcoma and chondrosarcoma was reported to induce new bone formation when lyophilised samples were transplanted to mouse muscle [7]. BMP was found to have prognostic significance in osteosarcoma [33, 34], which has been the most commonly investigated source of BMP in tumour tissue [1, 7, 22, 23, 24, 29, 31] and only some malignant bone tumours have been reported to contain BMP [13, 32, 33, 34, 35, 36]. The absence of bone and osteoid tissue is presumably the explanation for BMP not being detected in fibrosarcoma [35]. The measurement of BMP could be valuable in the diagnosis of borderline cases [13]. The reason why all malignant bone tumours do not contain BMP is uncertain, but research continues [1].

Conventional biochemical techniques, such as extraction with 4 M GuHCl combined with

bioassay for osteoconductivity, have been used to detect BMP [1, 33]. In normal bone the amount is only 20 µg/10 kg [29], and the content in bone tumours has been assumed to be low, but methods of quantification are not available. Measurement techniques have so far needed several kilograms of material, which is not practical in bone tumours, except by implanting the tumour several times in mice in order to increase its mass [22, 23, 24]. Nowadays immunohistochemical staining with avidin-biotin-peroxidase is the most widely used and successful method for the qualitative detection of BMP [10, 13, 35, 36].

Antibodies against bBMP [13, 22], murine osteosarcoma BMP [15] and human BMP (hBMP) have been used in previous research. In this study we used a purified mixed bBMP as antigen in producing antiserum. The reason for using bovine rather than human bone as a source of crude BMP is mainly ethical; recent clinical studies with bBMP have produced satisfactory results in human experiments [18, 19], and the antiserum of bBMP and hBMP crossreact 100% [26]. As the bone-inductive activity and importance of various BMPs remain unresolved [29, 31], an antiserum against a crude BMP mixture is closer to the normal state when total amounts of BMP are measured. The Western blot analysis produced three different bands with molecular weights of 30 kD, 67–69 kD and 90 kD obtained as the higher molecular weights represent BMP aggregates.

Since the investigation of BMP in bone and soft tissue malignant tumours has rapidly become important, we have tried to develop an accurate and reproducible quantitative method. The results of immunohistochemical staining showed that only part of the tumour cells contained BMP, and these were located in groups which were not necessarily near to each other. It, therefore, might be possible to overlook positive samples if a given section does not contain positive cell groups. This might be overcome by using numerous sections, although the possibility would still remain. The ELISA technique, which was developed for measuring BMP in small amounts in tissues, can overcome this disadvantage. This is essential because in the diagnosis of bone cancer, these procedures are undertaken before operation and chemotherapy using small samples. Since it is possible that BMP is inactivated during chemotherapy, we identified and quantified it in one sample before and after treatment, and found no difference in BMP content.

Different bone tumours, when stained immunohistochemically with antiserum, showed only

minor variations in local BMP concentration, but giant cell tumours had slightly higher concentrations when compared to other tumours. There was only one exception: in a poorly differentiated chondrosarcoma the highest dilution for positive immunostaining was only 1.5, but the total concentration was the same as other chondrosarcoma samples. Reactive bone formation by malignant cells stained positively in all three giant cell tumours and the local BMP in the reactive bone appeared to be very high compared to that in cell cytoplasm. Despite this high local concentration, the total BMP in these tumours did not differ from that in others. When analysing and comparing results, giant cell tumours were excluded since they cannot be strictly classified as either benign or malignant. Although often classified as benign, these tumours have been reported to metastasise to the lungs in 9% of cases [12]. The total amount of BMP in our giant cell tumours was very high, but this did not correlate to the local concentration.

Our preliminary results in measuring the total BMP content showed a 2.4 times difference between malignant and benign tumours with the malignant tumours containing more BMP than the benign. These results were not statistically significant, but we believe that BMP has an essential role in the malignancy of bone tumours.

The techniques we have used are reliable and reproducible and it is obvious that some tumour cells produce or contain more BMP than others. Furthermore, the BMP in these cells may have different biological and physiochemical properties. It is not clear whether intracellular or extracellular BMP is more important in relation to the clinical features and whether structural differences are involved. These questions need to be pursued by further research.

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