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Production of monoclonal antibodies specific for human bone γ-carboxyglutamic acid containing protein

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Summary. Murine monoclonal antibodies specific for human bone γ-carboxyglutamic acid containing protein (BGP) were produced against BGP purified from young adult human long bones. The amino acid composition of purified protein corresponds with that of human BGP, and Western blot analysis revealed that the antibodies reacted most intensely with the cytoplasm of osteoblasts and less intensely with the cytoplasm of osteocytes, but did not react with any other cells, such as chondrocytes, or osteoclasts. Because of their ability to react with routinely processed tissue sections and their marked reactivity with human osteoblastic cells, the antibodies are expected to be a useful tool for studying the process of ossification in human bones and for the immunohistochemical diagnosis of human osteogenic tumours.

Key words: Bone Gla protein – Osteocalcin – Monoclonal antibody – Calcification – Osteogenic sarcoma

Introduction

Bone γ-carboxyglutamic acid containing protein (BGP, or osteocalcin) is a Ca^{2+} binding protein which comprises 10–20% of the non-collagenous proteins in bone (Haushka and Reid 1978). The major properties of BGP are the specific γ-carboxyglutamic acid dependent binding with Ca^{2+} and the high affinity binding to hydroxyapatite. Therefore, BGP is likely to play a central role in the mineralization of bone (Poser et al. 1980), but the exact function of the protein in bone formation remains unknown (Hauschka and Reid 1978; Haushka et al. 1983). Several attempts have been made to prepare antibodies specific for BGP, but the antibodies obtained so far are restricted to polyclonal antibodies against bovine BGP (Bianco et al. 1985) and rat BGP (Bronckers et al. 1985; Camarda et al. 1987). We have previously raised a polyclonal antibody against human BGP, but cross-reactivity with other proteins has not been ruled out entirely (Ohta et al. 1989). In the present study, we produced monoclonal antibodies against human BGP for the first time. Immunohistochemical localization of human BGP using the monoclonal antibodies was shown to be strongly reactive with osteoblasts in normal human bone, but did not react with either chondrocytes, osteoclasts, fibroblasts, marrow cells, cartilage matrix, or fibrous tissues. Osteoblastic cell lines derived from osteogenic sarcoma have been reported to produce BGP (Price and Baukol 1980) and, therefore, the monoclonal antibodies developed here would be useful not only for the analysis of BGP function in the process of mineralization of human bone but also for the immunohistochemical and serum diagnosis of human osteogenic tumours.

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Materials and methods

The procedure for purification of human BGP followed the method of Gundberg et al. (1984) with some modifications, as previously described (Ohta et al. 1989). Briefly, human cortical bones were pulverized in liquid nitrogen with a magnetically driven mill (Spex Industries, Metuchen, N.J.) and the bone powder (100 g wet weight) was washed with ice-cold Tris-buffer solution (pH 7.6) containing protease inhibitors (benzamidine, 10 mM; 6-aminocaproic acid, 10 mM; *p*-hydroxymercuribenzoic acid, 0.1 mM; phenylmethylsulphonylfluoride, 0.5 mM). The bone powder was then suspended in a solution of 0.5 M ethylenediaminetetra-acetic acid (EDTA) containing the protease inhibitors and stirred for 24–48 h. The supernatant was dialysed against 50 mM NH_4HCO_3 containing the protease inhibitors and gel-filtrated at 4° C with a Sephadex G-100 column (2.9 × 90 cm) equilibrated with 50 mM NH_4HCO_3. Ion exchange chromatography with a linear NH_4HCO_3 gradient (200–500 mM) was carried out on the protein peak fraction, which had a molecular weight of about 10 kDa on DE-53 at 4° C. The total amount of protein was determined and the purity of the BGP was examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). It was then hydrolysed in 6N HCl at 110° C for 24 h to prepare the sample for amino acid analysis. All chemicals used were purchased from Sigma (St. Louis, Mo.).

BALB/cj mice were injected intraperitoneally with 50 μg purified human BGP emulsified in 0.5 ml of 50% Freund's complete adjuvant. Four days after priming, a booster injection in Freund's

incomplete adjuvant was given once a week for 5 weeks. Three days after the last injection, the mouse was sacrificed and the spleen was removed under sterile conditions. Spleen cells and NS-1 mouse myeloma cells were fused at a ratio of 5:1 using the procedure described by Köhler and Milstein (1976). The screening of supernatants from wells exhibiting hybrid growth was performed by enzyme-linked immunosorbent assay (ELISA) using the technique of Engvall (1982) and cloned by the limiting dilution method. The wells of immunoplates (MS-3796F, Sumitomo Bakelite Co., Tokyo, Japan) were coated with 20 μg/ml BGP and incubated for 24 h at 4° C. After rinsing with 1% normal horse serum to block nonspecific reaction, the wells were serially incubated with supernatant of hybridoma, biotinylated goat antibody to mouse Ig and avidin-biotin complexes (Vector Laboratories, Burlingame, Calif.). The reaction was visualized by incubation with the buffer [0.2 mg/ml ABTS (2,2′-azinobis, 3-ethylbenzthiazoline sulphonic acid), 0.05% (v/v) H_2O_2 and 0.1 M citrate, 0.1 M K_2HPO_4, pH 4.5] and the products of the enzymatic reactions were followed by measuring the optimal density at 492 nm with an autoreader MPR-A4 (Toyosotatsu, Tokyo, Japan).

The immunoglobulin subclass of the produced antibodies were determined by the enzyme assay method, using a peroxidase-conjugated mouse immunoglobulin set (The Binding Site Ltd., Birmingham, UK).

The hybridoma cells were then injected into steropristane primed mice ($1.0–1.5 \times 10^6$ cells/mouse). Two to 4 ml ascitic fluid was obtained from each mouse. The specificity of the antibodies recovered was determined by the following two methods. The first was by the Western blotting method as previously described (Ohta et al. 1989). Briefly, the EDTA-guanidine HCl extract of the bones, which contains all non-collagenous proteins (Price et al. 1983) were electrophoresed on Phastgel (Pharmacia, Uppsala, Sweden) and transferred to a nitrocellulose sheet (Toyo Chemical Co. Tokyo, Japan; pore size 0.1 μm). It was sequentially treated with 1% normal horse serum, the monoclonal antibodies, biotinylated goat antibody to mouse Ig (TAGO, Burlingame, Calif.), and avidin-biotin complex. The localization of BGP was visualized by rinsing the nitrocellulose membrane in Graham-Karnovsky medium; 0.03% 3,3′-diaminobenzidine, 0.01% H_2O_2 in 0.05 M Tris-HCl, pH 7.6 (Graham and Karnovsky 1967). The second procedure was the purification of the antigen with affinity column chromatography using 5 mg anti-BGP IgG covalently linked to 10 ml cyanogen bromide activated Sepharose (March et al. 1974). The anti-BGP Sepharose was equilibrated with 0.05 M Tris-HCl containing 0.02% NaN_3, pH 7.3 and then added to a solution containing 100 mg human bone extract equilibrated in the same buffer. After overnight incubation at 4° C, the suspension was placed into the column. When the absorbance at 280 nm of effluents was at baseline, the column buffer was switched to 0.1 M glycine (pH 2.8), and fractions were collected into tubes containing 1 M Tris-HCl (pH 8.5). Glycine fractions containing protein as determined by absorbance at 280 nm were pooled, dialysed against 50 mM NH_4HCO_3 and subsequently lyophilized. Finally, the protein was hydrolysed in 6 N HCl at 110° C for 24 h, and analysed for amino acid composition using an automated amino acid analyser.

The tissues used for immunolocalization of BGP were the distal end of the femur, heart, lung, oesophagus, intestine, liver, spleen, pancreas, kidney, adrenal gland, thyroid, urinary bladder, and uterus. These tissues were removed at autopsy from two 2-month-old cases of atrial septal defect, 4- and 8-month-old cases of pneumonia, and 16- and 20-year-old cases of osteosarcoma.

The procedure for tissue fixation was as previously described (Ohta et al. 1989). For immunohistochemistry, paraffin sections from each tissue were rinsed with 0.6% H_2O_2 in methanol for 30 min to block endogenous peroxidase activity. The sections were serially incubated with 1% normal goat serum, monoclonal anti-BGP antibody, biotinylated goat antibody to mouse Ig, and avidin-biotin complex followed by incubation with the complete Graham-Karnovsky's medium; 0.03% 3,3′-diaminobenzidine, 0.01% H_2O_2 in 0.05 M Tris-HCl, pH 7.6 (Graham and Karnovsky 1967). The sections were mounted with glycerin-agar.

Results

Five monoclonal antibodies (1C3, 5E10, 7F4, 10A4, and 10E8) were established. The subclass of 1C3 and 5E10 was IgG 2a, while those of 7F4 and 10A4, and 10E8 were IgM and IgG 2b respectively.

The monoclonal antibodies reacted specifically with a single protein of 12 kDa on SDS-PAGE when immunoblotted against crude bone extract (Fig. 1). Amino acid analysis demonstrated that the protein purified by affinity column chromatography was rich in glutamic acid and aspartic acid but poor in serine and threonine (Table 1).

Immunohistochemical analysis of the human bone tissues demonstrated that the osteoblasts were strongly

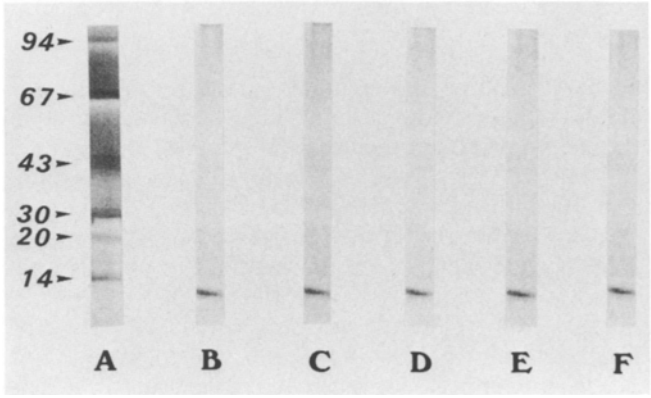

Fig. 1. Western blotting with monoclonal antibodies with 8–20% linear gradient gel of SDS-PAGE. *Lane A* is the calibration. *Lanes B–F* are EDTA, guanidine-HCl extracts of human bone reacted to 1C3, 5E10, 7F4, 10A4, 10E8, respectively

Table 1. Amino acid composition of affinity-purified BGP with 10E8 from human bone extracts

	Residues/ 1000res.[a]	Residues/ mol. wt.[b]	Residues/ mol.
Gla	41.4[c]	2.02	2
Asp	116.7	5.68	6
Thr	8.2	0.40	0
Ser	7.8	0.38	0
Glu	118.7	5.78	6
Pro	123.1	5.99	6
Gly	63.3	3.08	3
Ala	55.8	2.72	3
Half-Cys	35.6	1.73	2
Val	63.4	3.09	3
Met	6.5	0.32	0
Ile	22.1	1.08	1
Leu	103.0	5.01	5
Tyr	94.9	4.62	5
Phe	39.2	1.91	2
Lys	8.0	0.39	0
His	20.8	1.01	1
Arg	73.5	3.58	4
Trp	4.5	0.22	0

Mol. wt., Molecular weight
[a] These values are the averages of two 24-h hydrolyses
[b] Residues per molecule expected from molecular weight
[c] Determined by alkaline hydrolysis

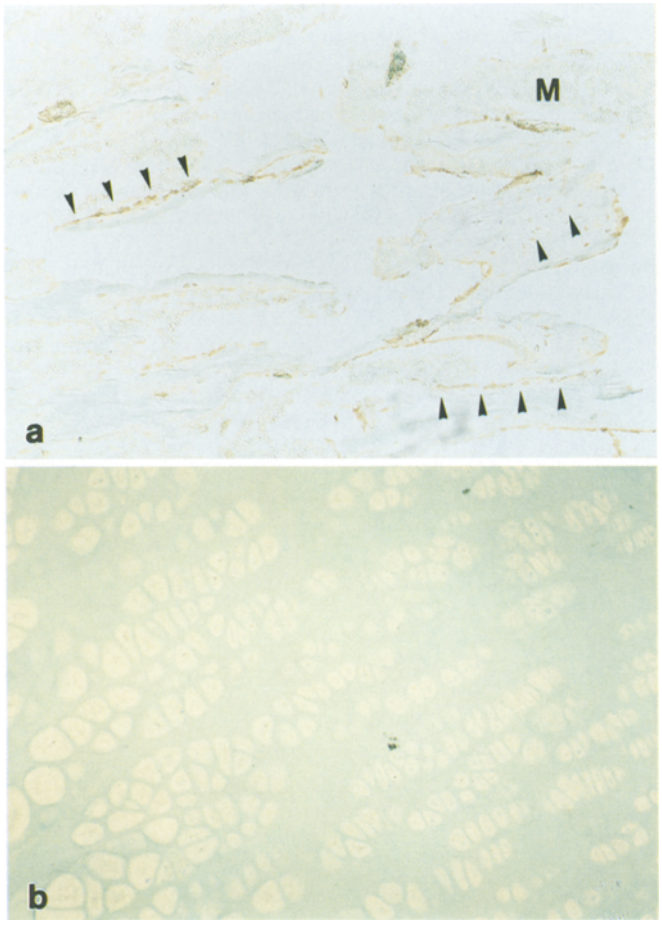

Fig. 2a, b. Reactivity of monoclonal antibody (10E8) with normal human bone. **a** Osteoblasts and osteocytes (*arrows*) were positive. **b** Chondrocytes and **a** marrow cells (*M*) are negative

Table 2. Immunoreactivity of monoclonal antibodies to human normal tissues (all of them negative)

Organ	No. of cases	No. of positive cases				
		IC3	5E10	7F4	10A4	10E8
Heart	6	0	0	0	0	0
Lung	6	0	0	0	0	0
Oesophagus	4	0	0	0	0	0
Stomach	6	0	0	0	0	0
Colon	4	0	0	0	0	0
Liver	4	0	0	0	0	0
Spleen	4	0	0	0	0	0
Pancreas	4	0	0	0	0	0
Kidney	4	0	0	0	0	0
Adrenal gland	4	0	0	0	0	0
Thyroid	6	0	0	0	0	0
Urinary bladder	6	0	0	0	0	0
Uterus	6	0	0	0	0	0

positive for BGP, whereas the osteocytes were weakly positive. However, the antibodies were unreactive to chondrocytes, osteoclasts, fibroblasts, marrow cells, cartilage matrix, fibrous tissue, and osteoid (Fig. 2). There was no difference in the immunohistochemical reaction of the antibodies (data not shown). In all other human tissues tested, antibodies against human BGP did not show cross-reactivity (Table 2).

Discussion

We report here a procedure to produce murine monoclonal antibodies against human BGP. BGP is a major non-collagenous protein of bone composed of 49 amino acids. It contains three γ-carboxyglutamic acid residues, through which BGP is believed to bind specifically with Ca^{2+} and hydroxyapatite (Poser and Price 1979). Thus, it is considered to have an important role in calcification of bone.

Since our goal is to elucidate the biological role of BGP on bone formation under physiological and pathological conditions in humans and, if possible, to utilize the anti-BGP antibody in the diagnosis of human bone tumours, we generated monoclonal antibodies against human BGP.

A major problem in the development of these antibodies is the difficulty in obtaining native antigen without degradation. To minimize this, we modified the method of Gundberg et al. (1984) by adding larger amounts of protease inhibitors throughout the process of isolation and purification of human BGP. The amino acid composition of the purified protein corresponds well with that of human BGP as reported by Poser et al. (1980). By using the protein as an immunogen, we have generated five monoclonal antibodies against human BGP.

The reaction of the five monoclonal antibodies with a single band of 12 kDa on SDS-PAGE analysis of EDTA guanidine-HCl extracts of normal human bone supports the conclusion that the five monoclonal antibodies are specific. This conclusion is further strengthened by the results of the anti-BGP affinity column, which indicated that the only protein retained by the column is human BGP as evidenced by amino acid analysis of the retentate. Therefore, all five monoclonal antibodies are specific for human BGP.

Immunohistochemical examination of normal human bone revealed that the antibodies reacted intensely with osteoblasts and less intensely with osteocytes, but were not reactive with any other cells or tissues tested such as chondrocytes, osteoclasts, or fibrous tissues.

Selective reactivity of the monoclonal antibodies with human BGP suggested that the antibodies are not only useful for the studies of BGP in the mineralization process of human bone, but may also prove useful in the serum and immunohistochemical diagnosis of human bone diseases, especially of osteogenic tumours.

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