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Immunocytochemical localization of BGP in human bones in various developmental stages and pathological conditions

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Summary. Bone γ-carboxyglutamic acid containing protein (BGP) was isolated from human bone and anti-BGP antibody was produced in rabbits. Localization of BGP was investigated immunocytochemically by light and electron microscopy in human bones in various developmental stages and pathological conditions. In the bones of a 12 week fetus, osteoblasts stained strongly in areas of bone formation. However in bones of late fetal stages and in newborns and adults, BGP was localized predominantly in the osteoid and bone matrix in the ossifying front. Osteoblasts and osteocytes also stained positive, but less dominantly than in the early fetus. Electron microscopy showed that BGP was localized in the ER and Golgi cisternae of osteoblasts and osteocytes, and the collagen fibers of the osteoid and bone matrix. The intensity and distribution of staining were not significantly different in osteoporosis and osteoarthritis. These observations indicate that BGP is synthesized by osteoblasts most actively in early fetal life and is then deposited on collagen fibers of the osteoid and bone matrix.

Key words: Bone Gla protein – Osteocalcin – Immunocytochemistry – Calcification – Osteogenesis

Introduction

BGP (osteocalcin) is a major component of non-collagenous bone proteins. It was isolated from the bones of various kinds of animals such as chickens (Hauschka et al. 1975), cows (Price et al. 1976), swordfish (Price et al. (1978), rats (Otawara et al. 1980), monkeys (Hauschka et al. 1975) and humans (Poser et al. 1980). BGP contains two or three γ-carboxyglutamic acid moieties which have the capability to bind calcium ions (Poser et al. 1979). Although BGP is considered to participate in the process of bone mineralization, its exact role in bone formation is unclear. As to the cells responsible for the production of BGP, it has been demonstrated that the osteoblastic cell lines, derived from osteogenic sarcomas, produce BGP (Price et al. 1980). Recently, several studies have been performed on the distribution of BGP within bone tissue and the localization of BGP in osteoblasts, osteocytes and bone matrix (Bianco et al. 1985; Bronckers et al. 1985; Camarda et al. 1987). In this study, we investigated the immunocytochemical localization of BGP in human bones in various developmental stages and pathological conditions using anti-human BGP antibody. We found that osteoblasts stained most intensely in the early fetus, and that the osteoid and bone matrix stained dominantly from late fetus to adult.

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Materials and methods

BGP was extracted by the method of Gundberg et al. (Gundberg et al. 1984) with some modifications. Human cortical bones were obtained from the amputated material of patients (15–30 years old) with osteogenic sarcoma. After the removal of adhering tissue, the bones were pulverized in liquid nitrogen with a magnetically driven mill (Spex Industries). The bone powder (100 g wet weight) was first washed with an 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; phenylmethylsulphonyl fluoride, 0.5 mM). It was then suspended in a solution containing 0.5 M EDTA and the protease inhibitors, and stirred for 24–40 h. The supernatant was dialyzed against 50 mM NH_4HCO_3 containing the protease inhibitors and gel-filtrated at 4° C with a column (2.9 × 90 cm) of Sephadex G-100 equilibrated with 50 mM NH_4HCO_3. Ion exchange chromatography was carried out on the protein peak fraction, having a molecular weight of about 10000, on DE-53 at 4° C with a linear gradient of NH_4HCO_3 (70 mM–700 mM). The peak fraction was re-

chromatographed on DE-53 with a linear gradient NH_4HCO_3 (200 mM–500 mM). In each step of purification, the total amount of protein was determined by the method of Lowry et al. (Lowry et al. 1951), and the purity of the BGP was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). An aliquot of the final preparation of BGP was analyzed for amino acid composition by hydrolyzing it in 6N HCl at 110° C for 24 h.

For the preparation of antisera, BGP (1 mg/ml) was emulsified with 750 mg of polyvinyl pyrolidone (MW: 40000) and 2 ml of Freund's complete adjuvant (Difco.) and administered subcutaneously to rabbits. Multiple subcutaneous injections of the same amount of BGP mixed with polyvinyl pyrolidone and Freund's incomplete adjuvant followed every two weeks (Worobec et al. 1972; Worobec et al. 1972). Sera were tested for the presence of anti-BGP antibody by enzyme-linked immunosorbent assay (ELISA) on microtiter plates (Dynatech). The antibody was purified by affinity chromatography using the purified BGP conjugated with cyanogen bromide activated Sepharose 4B.

The specificity of the antisera was determined by the Western blotting method. The purified BGP and the EDTA-guanidine HCl extract of bones, which contains all noncollagenous proteins (Price et al. 1983) were electrophoresed on Phastgel (Pharmacia) and transferred to a nitrocellulose sheet (Toyo Chemical. Co., pore size 0.1 μm). It was sequentially treated with 5% skim milk, the rabbit anti BGP antibody, the biotynilated swine anti-rabbit IgG 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 et al. 1967).

The bones used for immunolocalization of BGP were the femur of fetuses aborted at 12 weeks and 17 weeks of gestation, the femur of a 40 week stillborn, the growth-plate of the distal end of the femur removed from a 2 month old victim of atrial septal defect, an osteoporotic vertebra from a 70 year old man who died of silicosis and the distal end of the femur from 4 cases of osteoarthritis (40–70 year old). The tissues were quickly fixed in the periodate-lysine-paraformaldehyde fixative (Maclean et al. 1974) at 4° C for 6 h. Part of the tissue was then rinsed in 20% polyethylene glycol (MW: 20000 ± 5000), dissolved in PBS, and frozen in liquid nitrogen. The other part of the tissue was demineralized with the solution containing 0.3 M EDTA and 15% glycerol at −5° C for 5 days, changing the solution daily (Mori et al. 1986). Then the tissues were serially rinsed in 20% sucrose and 20% polyethylene glycol solutions, and subsequently frozen on liquid nitrogen.

For immunohistochemistry 4 μm thick sections of the tissues were cut on a cryostat and mounted on albumin coated slides. The sections were then rinsed in 0.6% H_2O_2 and methanol for 30 min to block endogenous peroxidase activity. The sections were serially incubated with 5% skim milk, the affinity purified rabbit anti-BGP antibody, and peroxidase-conjugated Fab fragments of goat antibodies to rabbit-IgG, followed by rinsing in the complete Graham-Karnovsky medium (0.03% 3,3′-diaminobenzidine 0.01% H_2O_2 in 0.05 M Tris-HCl, pH 7.6) (Graham et al. 1967). The sections were mounted on glycerin-agar.

The bones of the 12 week fetus were used for immunoelectron microscopic studies. The tissues, fixed in the same fixative, were dehydrated at −20° C through graded ethanol and embedded in LR White at −4° C. The ultrathin sections were incubated at 4° C with 2.5% bovine serum albumin for 20 min then with the anti BGP antibody for 3 h. The bound IgG was visualized by incubation of the grids with goat anti-rabbit IgG coupled with 15 nm gold particles.

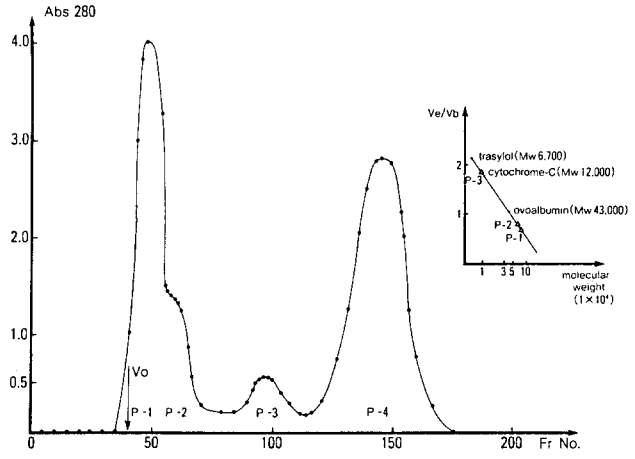

Fig. 1. Sephadex G-100 column chromatography of 0.5 M EDTA-extracted human cortical bone and the molecular weight of its eluate calibrated with standard proteins. About 100 mg of protein was applied to a 2.9 × 90 cm column at 4° C flowing at 17 ml/h. Molecular weight of p-3 was about 10000

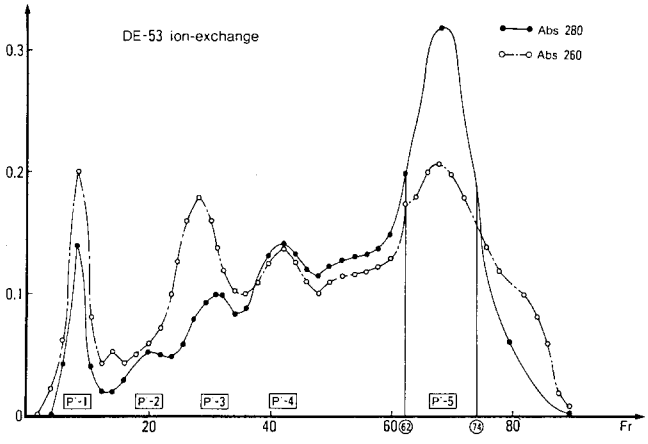

Fig. 2. DE-53 column chromatography of Sephadex G-100 eluate. About 30 mg of P-3 fraction (Fig. 1) was applied to the column (1.5 × 36 cm) and eluted at 4° C with linear gradient of NH_4HCO_3 (70 mM–700 mM). Flow rate was 30 ml/h. Molecular weight of P′-5 was 12000

Results

The EDTA extract of the bones was eluted into four major fractions on Sephadex G-100 gel filtration (Fig. 1). The third fraction, containing the proteins of which molecular weight ranged from 14000 to 8000, was applied to ion exchange chromatography and separated into five fractions (Fig. 2). Rechromatography of the fifth fraction on DE-53 resulted in a single symmetrical peak fraction (Fig. 3). SDS-PAGE demonstrated that the purified BGP migrates to a single band with an apparent molecular weight of 12000 (Fig. 4e). Table 1 shows the amino acid composition of typi-

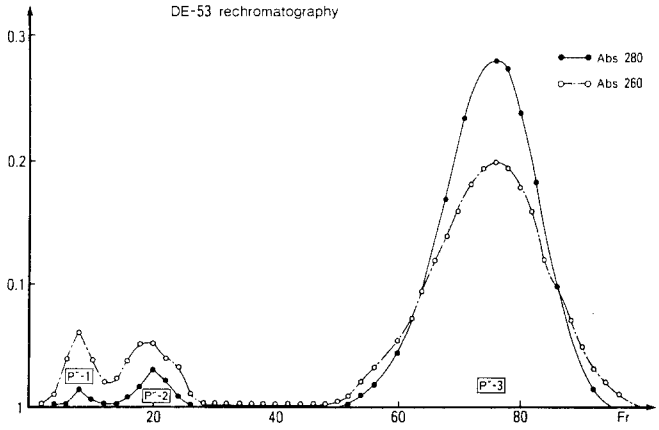

Fig. 3. DE-53 column chromatography of P″-5. About 10 mg of P′-5 fraction (Fig. 2) was applied to the same sized column and eluted at 4° C with linear gradient of NH_4HCO_3 (200 mM–500 mM). Rechromatography of DE-53 resulted in a single symmetrical peak fraction

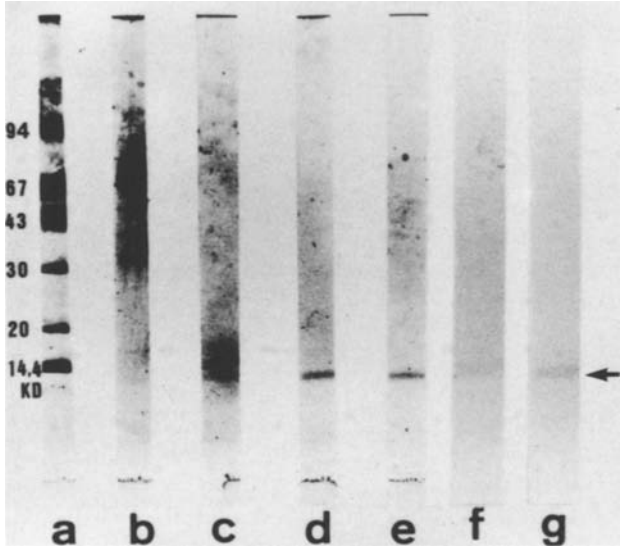

Fig. 4. 8–20% linear gradient of SDS-PAGE. Lane a is calibration. Lane b is EDTA-extracts of human bones. Lane c is P-3 fraction of Sephadex G-100. Lane d is P′-5 fraction of DE-53 ion exchange column chromatography. Lane e is the fraction of DE-53 rechromatography. BGP was demonstrated as a single band (*arrow*) (Mw: 12000). Coomassie Brilliant Blue staining. Lane f is EDTA, guanidine-HCl extract reacted to anti-human BGP antibody (Western blotting). Lane g is purified BGP reacted to the anti-human BGP antibody (Western blotting)

cal purification of BGP in the final step. It was almost identical to that reported by Poser et al.

Western blotting analysis demonstrated that the purified antibody reacted with human BGP. An identical single immunoreactive band (Mw: 12000) was observed in the crude bone extract (Figs. 4f and g).

Table 1. Amino acid composition of the final step of preparation; Amino acid composition of purified BGP

	Residues/Mol.
Asp	7
Thr	1
Ser	1
Glu	8
Pro	5
Gly	3
Ala	3
half-Cys	2
Val	3
Met	0
ILe	1
Leu	5
Tyr	4
Phe	2
Lys	0
His	1
Arg	3
Trp	–

Table 2. Localization of BGP in each stage of developing human bones

	12 week fetus	17 week fetus	25 week fetus	new-born	osteo-porosis	osteo-arthritis
Fibrous tissue	–	–	–	–	–	–
Cartilage	–	–	–	–	–	–
Osteoid	–	+	+	+	+	+
Bone matrix	–	+	+	+	+	+
Fibroblast	–	–	–	–	–	–
Chondrocyte	–	–	–	–	–	–
Marrow cell	–	–	–	–	–	–
Osteoclast	–	–	–	–	–	–
Osteoblast	+	+	+	+	+	+
Osteocyte	+	+	+	+	+	+

(+): positive staining of BGP
(−): negative staining of BGP

In the bones of an early fetus (12 weeks of gestation), the osteoblasts were strongly positive in the areas of endochondral and intramembranous bone formation (Fig. 5a and Table 2). The cytoplasm of osteoblasts showed granular staining of BGP. The staining of osteoid and bone matrix were either negative or very weakly positive for BGP. The osteoclasts, chondrocytes, cartilage matrix, and fibrous tissue of bone marrow were all negative.

In the bones of middle (17 weeks of gestation) and late (40 weeks) fetuses and newborn, the osteoid (unmineralized matrix) of endochondral and intramembranous bone stained most intensely (Fig. 5b and Table 2). The mineralized matrix stained weakly. When the tissues were demineral-

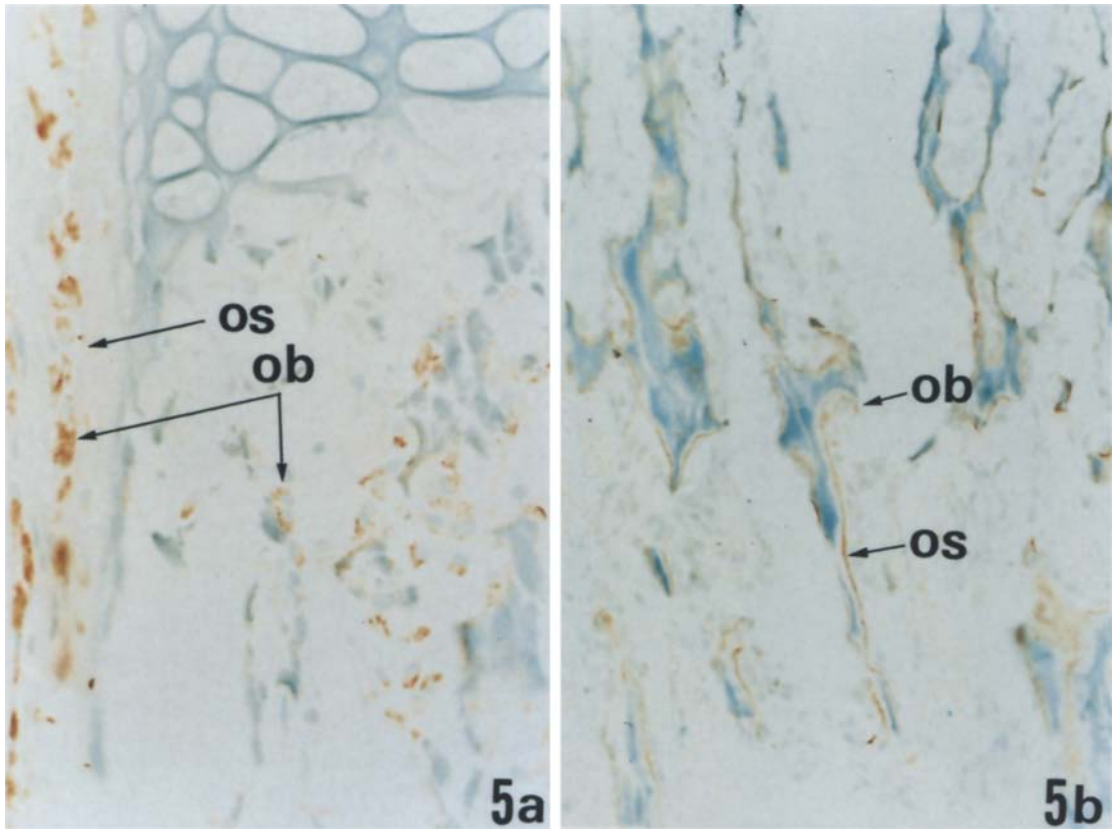

Fig. 5. Growth plate of the femur of developing human bones (undemineralized). **(a)** The bone tissue of a 12 week fetus immunostained with the anti-human BGP antibody. Osteoid (OS). Osteoblasts (Ob). The osteoblasts stained strongly but the osteoid and bone matrix are negative. **(b)** The bone tissue of a 17 week fetus immunostained with the anti-human BGP antibody. Osteoid (OS). Osteoblasts (Ob). The osteoid stained most intensely

ized, staining intensity of the mineralized matrix became enhanced, whereas the staining of other tissue components remained unchanged. Osteoblasts and osteocytes stained positive, but less dominantly than those in the early fetus. The staining pattern of BGP in the bone of osteoporosis and osteoarthritis was not significantly different from that of late fetuses and newborns (Table 2). However, when the Fab fraction of normal rabbit IgG was used instead of the anti-BGP antibody, only a minimal nonspecific reaction was observed.

In electron microscopy the gold particles were seen in the ER and Golgi cisternae of osteoblasts and osteocytes by the immunogold method (Figs. 6a, b). In the mineralized matrix and osteoid, the gold particles were evenly seen along the collagen fibers, but there was no relationship between a distribution pattern of the gold particles and the whole zone of the collagen fibers or early mineralization nodules. The matrix vesicles did not show any enhanced localization of the gold particles (Fig. 7).

Discussion

Human BGP was easily purified by the method of Gundberg et al. (Gundberg et al. 1984) with some modifications. One of the modifications made in this study was the use of larger amounts of protease inhibitors (0.5 mM PMSF and 10 mM benzamidine) when compared with the original method of Gundberg et al. (Gundberg et al. 1984). The second modification was that we repeated the ion exchange chromatography, which was benificial for elimination of minor contaminants, in the last step of purification. The efficiency of purification was comparable to that of the report of Gundberg et al. (Gundberg et al. 1984). Using human BGP as the antigen, we successfully produced the monospecific anti-human BGP antibody which was useful for localization of BGP in human bones. Western blotting analysis demonstrated that this antibody reacted to a single band (MW: 12000) equivalent to the purified human BGP in EDTA-guanidine HCl extract of bone which con-

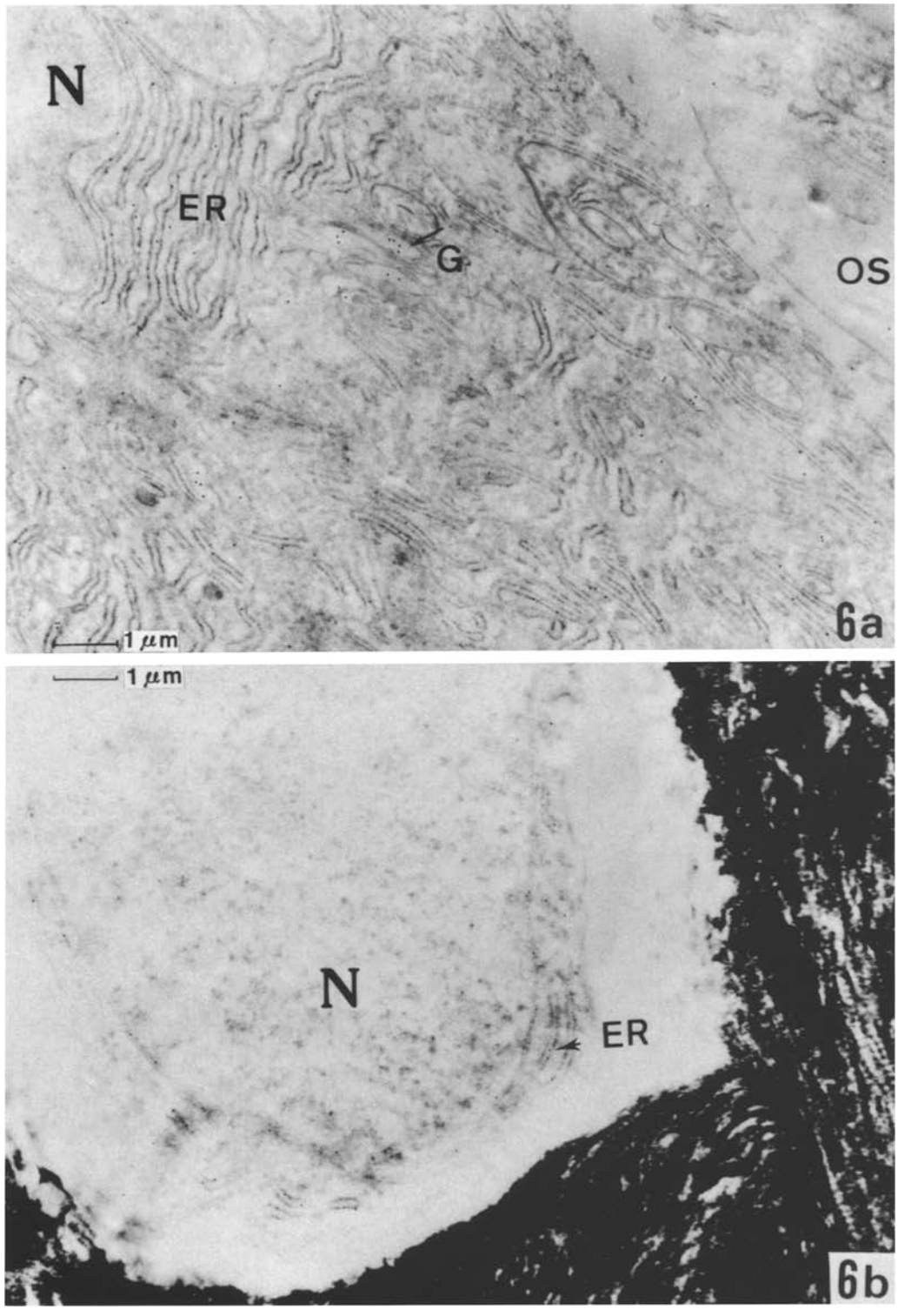

Fig. 6. The electron micrographs stained for BGP. (**a**) Osteoblast. Gold particles are seen in the ER and Golgi cisternae. Nucleus (N). Golgi cisternae (G). Osteoid (OS). (×10000) (**b**) Osteocyte. Gold particles are seen in the ER. (×10000)

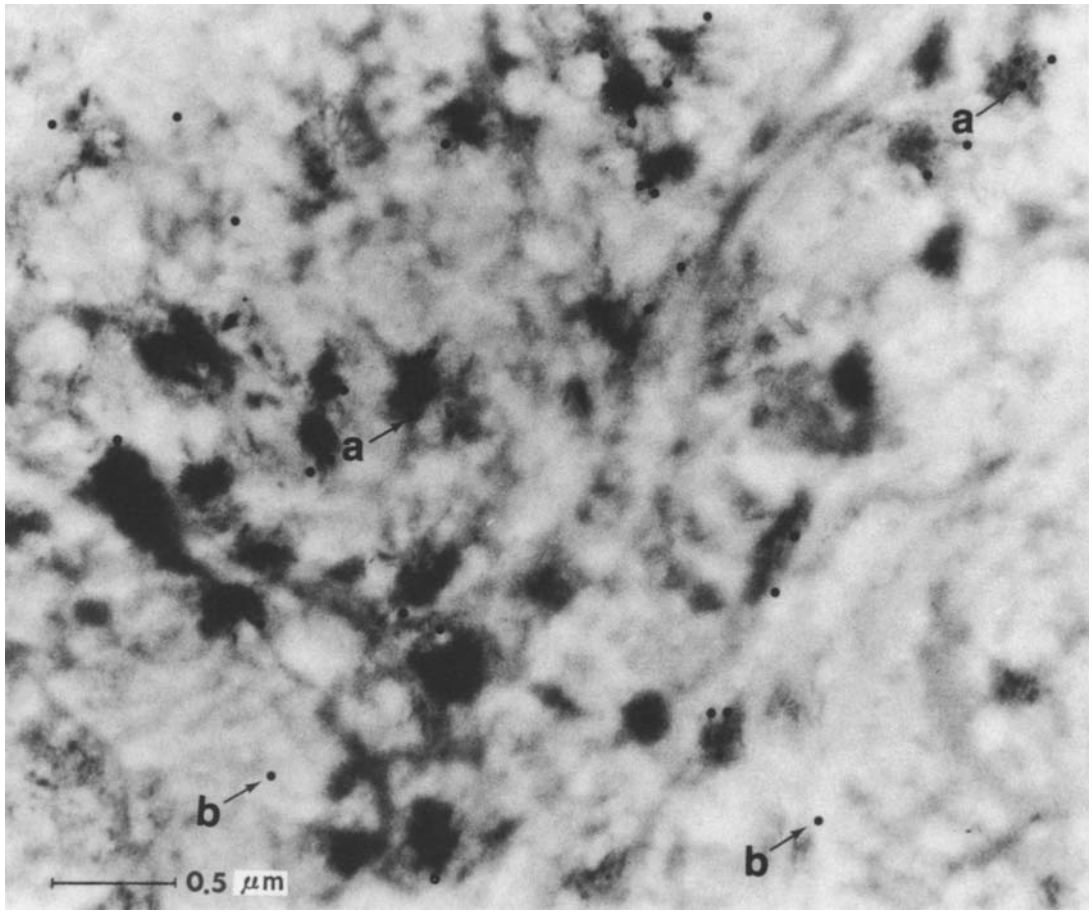

Fig. 7. Undemineralized bone with immunogold method. Gold particles are seen in the mineralization nodules (**a**) and other parts of the osteoid (**b**) and bone matrix. (× 33000)

tains all the noncollagenous bone proteins including the matrix Gla protein (Price et al. 1983).

The cellular components in which BGP was localized were osteoblasts and osteocytes in all the bone tissues examined in the present study. Chondrocytes, osteoclasts, and fibroblasts were constantly negative for BGP as consistent with the observations of Bronckers et al. (Bronckers et al. 1985). BGP was demonstrated in the rough endoplasmic reticulum of these cells by immunoelectron microscopy, indicating that the cells responsible for the production of BGP are osteoblasts and osteocytes in the bone tissue.

In the early fetus (12 weeks), BGP was localized predominantly in the osteoblasts. In the 17 week fetus, BGP was distributed predominantly in the osteoid and bone matrix, and less dominantly in osteoblasts. The latter kept constant throughout the late fetal, newborn and adult stages. Hauschka et al. (1978) reported that BGP was first measurable in an 8 day chick embryo, and continue to increase until well beyond hatching and leveled off about 3–6 weeks after hatching (Hauschka et al. 1978). The rate of increase was reported as highest in the early embryonic stages. Our observation was consistent with that of Hauschka et al. (1978) indicating that the synthesis of BGP by osteoblasts is most active in the early fetal period. During this stage, when the bone matrix and osteoid were poorly developed, only minimal distribution of BGP was observed. Thus, it is probable that BGP is synthesized prior to the formation of bone matrix.

Recently, there has been a hypothesis that osteoblasts and osteoclasts cooperate in the formation of the bone matrix. That is to say, osteoclasts produce a 'coupling factor' which stimulates the proliferation and function of osteoblasts (Farley et al. 1982; Farley et al. 1982). And it has been shown that BGP has a chemotactic affinity for

blood monocytes which have a capability to differentiate into osteoclasts (Malone et al. 1982; Mundy et al. 1983). Production of BGP by osteoblasts in the early fetus is compatible with the hypothesis that BGP has a leading role in bone formation (Price et al. 1982).

BGP was most dominantly stained in the osteoid in the later stages. BGP secreted by osteoblasts is, therefore, thought to be deposited in the unmineralized matrix of the osteoid. The mineralized bone matrix also stained for BGP but less dominantly than that of the osteoid. However, when the tissue was demineralized , the bone matrix stained more intensely in agreement with the results of Bianco et al. (1985) and Bronckers et al. (1985). Bronckers et al. and Camarda et al. (1987) described that BGP was localized most dominantly in the bone matrix and weakly or negatively in the osteoid of rat bones. However, in the present study, the osteoid stained for BGP more strongly than the bone matrix in human bones. Although the reasons for such discrepancy remain obscure, it is considered that the molecular state of BGP is different in the osteoid and bone matrix, and that BGP in the osteoid is more immunoreactive in humans than the bone matrix in rats.

The immunogold method demonstrated that BGP was evenly localized on the collagen fibers of the osteoid and bone matrix. The pattern of distribution of the osteoid and bone matrix did not show any intimate relationship to the hole zone or mineralization nodules of collagen fibers, or matrix vesicles. It is therefore probable that the deposition of BGP is not immediately related to the initiation of calcification in the bone matrix. Price et al. (1982) demonstrated that continuous administration of warfarin gave rise to a marked decrease in BGP and excessive mineralization in rat bones. It is thus conceivable that BGP may have a regulatory function in calcification.

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