Localization of endogenous osteocalcin in neonatal rat bone and its absence in articular cartilage: effect of warfarin treatment

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Summary. Immunocytochemistry after cryoultramicrotomy was used to localize endogenous osteocalcin in bone (calvaria, femoral diaphysis) and epiphyseal femoral cartilage from 8-day-old rats treated (or not) for 7 days with warfarin. Ultrathin frozen sections were incubated with goat antiserum against rat osteocalcin at high dilutions (2 x 10^-4 to 2 x 10^-6). In calvaria and femur of untreated rats, endogenous osteocalcin was observed in osteoblasts (cytoplasm and nucleus) and in the collagenous matrix. Osteocalcin appeared progressively in osteoblasts and bone matrix in the mineralization front, then increased in the regions of extended calcification. Osteocalcin was also detected in osteocytes but was not as abundant as in osteoblasts. In bone samples of warfarin-treated rats, endogenous osteocalcin was only detected in bone matrix but not in osteoblasts. Furthermore, osteocalcin was only observed if antiserum was not very dilute (2 x 10^-2). In cartilage (hypertrophied and degenerative zones), osteocalcin was not observed in matrix and chondrocytes. However, it was found in the vicinity of matrix vesicles at the initial loci of calcification. Osteocalcin was never detected in the cartilage of warfarin-treated rats. Our results provide ultrastructural immunocytological evidence for the localization of endogenous osteocalcin in osteoblasts, the presence of osteocalcin in bone matrix and a direct gradient between the presence of osteocalcin and the calcification process. Osteocalcin is absent from cartilage, except possibly close to calcifying matrix vesicles. Warfarin inhibits the formation of osteocalcin.

Key words: Osteocalcin – Osteoblast – Osteocyte – Ultrathin frozen sections – Warfarin treatment

Introduction

In adult bone, the most abundant of the non-collagenous proteins is the vitamin K dependently synthesized protein osteocalcin, also referred to as bone gamma-carboxyglutamic acid (Gla) protein (Hauschka et al. 1975; Price et al. 1976). Osteocalcin extracted from the extracellular matrix of bone has a molecular weight of 5700 and is characterized by its content of three residues of the vitamin K dependently synthesized calcium binding amino acid, Gla. To date, osteocalcin appears to be a bone-specific protein, a product of osteoblasts whose biosynthesis is stimulated by 1,25 dihydroxyvitamin D3 (Price and Baukol 1980; Lian et al. 1985). While the majority of synthesized osteocalcin accumulates in the bone matrix, a small fraction is also present in serum and can be quantified using a radioimmunoassay (Price and Nishimoto 1980). Currently, serum osteocalcin values are used as a parameter of bone turnover. It is generally accepted that serum osteocalcin measurements reflect bone formation activity rather than bone resorption (Brown et al. 1984; Lian and Gundberg 1988).

Osteocalcin shows a very high affinity for hydroxyapatite crystals, the mineral component of bone and calcified cartilage. The precise function of osteocalcin in bone physiology is still unknown. Recent studies have suggested as a chemoattractant for mononuclear cells (Malone et al. 1982), osteocalcin may function in the recruitment and differentiation of osteoclast progenitor cells (Lian et al. 1984, 1986; Glowacki and Lian 1987). The anticoagulant warfarin inhibits Gla formation in bone (Hauschka and Reid 1978) and also decreases the bone concentration of osteocalcin (Price and Williamson 1981). The appearance of osteocalcin in warfarin treated bone is of interest as warfarin exposure during fetal development may produce bone abnormalities in humans (Hall et al. 1980) and rats (Feteih et al. 1990). In addition, in the human disorder of genetic deficiency of vitamin K reductase (the enzyme inhibited by warfarin) the same abnormalities are seen as in the fetal warfarin embryopathy and Gla synthesis in osteocalcin has been shown to be inhibited in this disorder (Pauli et al. 1987). To date, most of the data concerning the localization and onset of appearance of osteocalcin in bone and cartilage are derived from biochemical investigations, histo-
logical studies (Bronckers et al. 1985; Groot et al. 1985, 1986; Mark et al. 1987) and also from a few ultrastructural observations reported (Bianco et al. 1985; Groot et al. 1986; Carmada et al. 1987; Mark et al. 1987; Ohta et al. 1989) in normal bone.

Recently, a combined method using immunocytochemistry on ultrathin frozen sections obtained by cryoultramicrotomy has been applied to bone (Boivin et al. 1983) in order to investigate the localization of endogenous hormones and receptors in bone cells (Boivin et al. 1985, 1987; Morel et al. 1985). In order to obtain more detailed information regarding the location and onset of osteocalcin in osteoblasts, osteocytes and bone matrix, this combined method was applied to calvaria and femoral diaphysis of neonatal rats previously treated (or not) during 7 days with warfarin. Observations concerning the epiphyseal growth plate cartilage in the vicinity of mineralization zone were also obtained.

Materials and methods

Three groups of 8-day-old rats were studied. The first was composed of normal rats without any treatment before sacrifice. The second consisted of control rats receiving daily subcutaneous injections of a saline solution from birth to day 7. In the third group the rats were treated from birth to day 7 with daily subcutaneous injections of 7.7 mg sodium warfarin/100 g body weight and 1.5 mg vitamin K/100 g body weight. The maintenance of animals in second and third groups was exactly as described by Price and Williamson (1981).

Calvaria, compact bone of femoral diaphyses and cartilage from the femoral growth plate were taken from the three groups of rats, then cut into small pieces at the beginning of fixation. Samples were fixed for 1 h by immersion in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, at 4°C. After washing (1 h in 0.1 M sodium cacodylate buffer), tissues were postfixied for 1 h in 1% buffered osmium tetroxide, and washed again for 1 h. After washing, tissues were incubated in 0.4 M buffered sucrose as cryoprotectant (30 min). Freezing was performed in a cold gradient of fuming nitrogen (Biogel, CFPO, France) to --4°C before total immersion in liquid nitrogen, as described elsewhere (Hemming et al. 1983). All tissues were observed without previous decalcification.

For biochemical measurements of osteocalcin and gamma-carboxyglutamic acid contents, calvaria, femoral diaphyses and growth plates were taken from rats not used for electron microscope study. These samples, cleaned free of adherent tissues, were frozen by immersion in liquid nitrogen, then lyophilized.

Osteocalcin concentrations were measured in 0.5 M ethylene-diaminetetra-acetic acid, pH 8.0 (and containing proteolytic inhibitors) extracts of powdered bone and cartilage samples of less than 100 μm particle size by an established rat osteocalcin radioimmunoassay previously detailed (Gundberg et al. 1984). All reagents, goat anti-rat osteocalcin antiserum, standard and iodinated tracer were prepared in the Children's Hospital Laboratory of Skeletal Diseases, Boston, Mass. (Gundberg et al. 1984). All samples were assayed in triplicate with an interassay variation of 5%. Glu content of alkaline hydrolysed samples was measured by amino acid analysis employing a Beckmann 121 M analyzer (Palo Alto, Calif.) with a sensitivity to 1 nm/ml. From 5 to 10 mg of sample was hydrolysed in 2 N KOH 24 h, 105°C and prepared as previously described (Gundberg et al. 1984).

The antisem directed against rat osteocalcin was raised in a goat and prepared according to the method described by Gundberg et al. (1984) for the development of antisem against chicken osteocalcin in rabbit. In a radioimmunoassay, no cross-reactivity with osteocalcins from several other species could be detected. A slight cross-reactivity with precursors of rat osteocalcin was possible. Also, no cross-reactivity exists with the matrix Gla protein (Otawara and Price 1986), as demonstrated by Hauschka (personal communication). Recently, Price (1989) has reported that matrix Gla protein is synthesized by bone, cartilage and numerous soft tissues mainly lung, kidney, heart. Thus, to confirm that our osteocalcin antibody did not cross-react with matrix Gla protein, it was tested with soft tissues of normal rats (first group previously described under protocols). Finally, the absence of immunoreaction products corresponding to osteocalcin detection in soft tissues (uncalcified cartilage, lung and kidney) known to contain a lot of matrix Gla protein confirmed the specificity of our antibody directed against rat osteocalcin.

For immunocytochemistry ultrathin sections were cut at --140°C on an Ultronite III (LKB, Stockholm, Sweden) fitted with a cryokit, as described by Tokuyasu (1973). The ultrathin sections, collected on collodion-coated nickel grids, were incubated consecutively for 10-min periods with (a) anti-osteocalcin serum (dilution from 2 x 10^-2 to 2 x 10^-6); (b) rabbit anti-immunoglobulin serum against goat gamma-globulin; (c) either goat anti-rabbit gamma-globulin labelled with peroxidase, or goat anti-rabbit gamma-globulin then peroxidase-antiperoxidase complex (dilution 2 x 10^-4); (d) 4-chloro-l-naphthol (ICN Pharmaceutical, Plainview, N.Y., USA), solution in Tris-buffered saline as previously described (Hemming et al. 1983) for 3 min, this solution being used with 0.01% hydrogen peroxide as enzymatic substrate; (e) 1% phosphate-buffered osmium tetroxide. After steps (a), (b) and (c), the grids were washed in Tris buffer, and after steps (d) and (e), the grids were washed with distilled water. All washing steps were performed for 10 min.

The specificity of immunocytochemical reaction was checked as follows: (a) using normal non-immune goat serum instead of anti-osteocalcin serum; (b) omitting the anti-osteocalcin serum; (c) using the anti-osteocalcin serum incubated overnight at 4°C with HPLC-purified rat osteocalcin; (d) using the anti-osteocalcin serum incubated overnight at 4°C with heterologous antigens (blood coagulation proteins including prothrombin) at a concentration 40-fold greater than homologous antigen.

Finally, ultrathin sections, never stained with lead citrate and/or uranyl acetate, were observed with a JEOL 1200 EX transmission electron microscope operating at 80 kV.

Results

The antigen-antibody complexes appeared as small dense granules at least 30 nm in diameter (Figs. 1, 3, 4a-c, 5, 7). These granules, always round, were easily distinguishable from artefactual black dots without precise shape and generally angular. Furthermore, the granules were always underfocussed. In the absence of antiosteocalcin serum, or when serum was replaced by non-immune goat serum, or when anti-osteocalcin serum had been previously incubated with corresponding purified antigen, no immunocytochemical reactions were observed (Fig. 2). If antisem had been incubated overnight with heterologous antigens, products of the immunocytochemical reaction were observed as usual.

In bone tissue of calvaria and femoral diaphysis from rats not treated or having just received saline solution, endogenous osteocalcin-immunoreactivity (osteocalcin-IR) was detected in organic matrix i.e. all around collagen fibrils (Figs. 1, 4a-c). Osteocalcin-IR appeared more abundant in calcified zones (Fig. 4c) than in areas where collagen fibrils were not yet mineralized (Fig. 4b). The abundance of osteocalcin-IR in bone matrix constituted