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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 mot) for 7 days with warfarin. Ultrathin frozen sections were incubated with goat antiserum against rat osteocalcin at high dilutions (2×10^{-4} to 2×10^{-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 (2×10^{-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 D_3 (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-

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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 postfixed 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 ethylenediaminetetra-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 antisera, 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%. Gla 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 antiserum 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 antiserum against chicken osteocalcin in rabbit. In a radioimmunossay, 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 Ultrotome 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 2×10^{-2} to 2×10^{-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 2×10^{-4}); (d) 4-chloro-1-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.

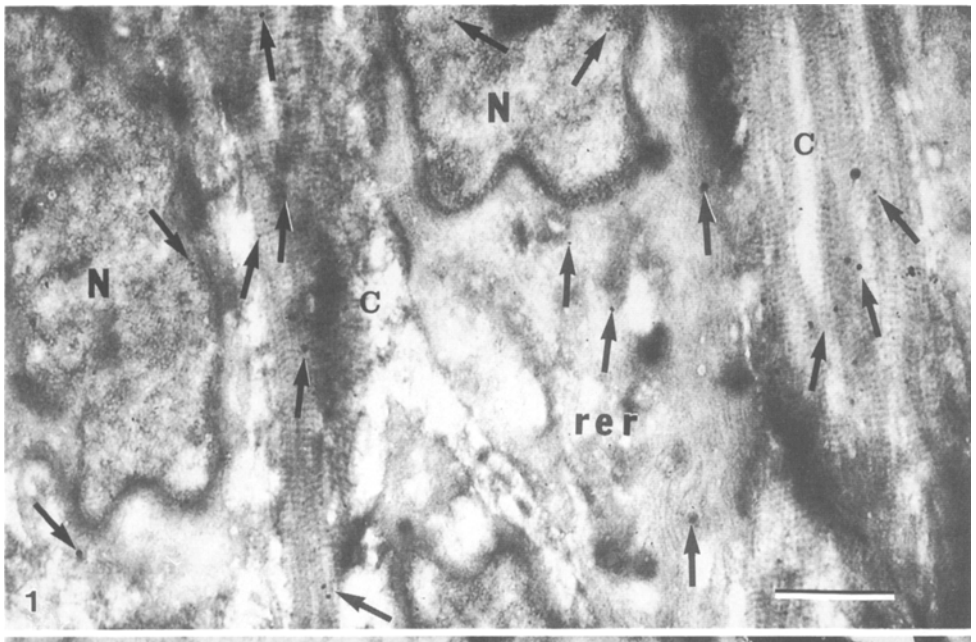
The specificity of immunocytological reaction was checked as follows: (a) using normal non-immune goat serum instead of anti-osteocalcin serum; (b) omitting the anti-osteocalcin serum; (c) using the anti-osteocalcin serum incubated overnight at 4° C with HPLC-purified rat osteocalcin; (d) using the anti-osteocalcin 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 anti-osteocalcin 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 immunocytological reactions were observed (Fig. 2). If antiserum had been incubated overnight with heterologous antigens, products of the immunocytological 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



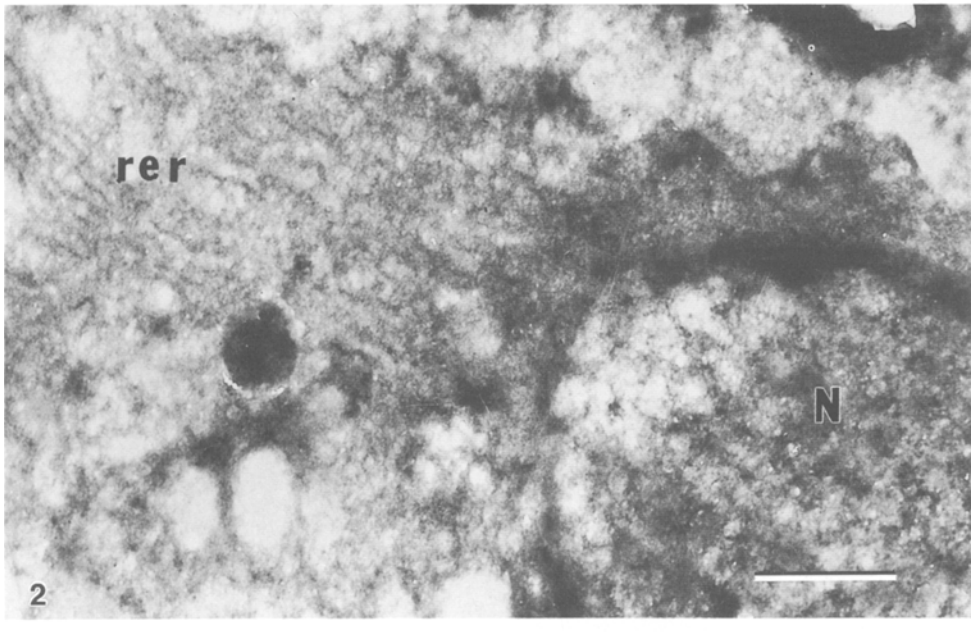


Fig. 1. Ultrastructural localization of osteocalcin immunoreactivity (IR) (*arrows*) around collagen fibrils (*C*) and in osteoblasts from femoral diaphysis of normal rats (anti-serum diluted to 2×10^{-6}). In the cells, the immunoreaction products are localized mainly in cytoplasm close to profiles of rough endoplasmic reticulum (*rer*) but are very rare in the nucleus (*N*). *Bar*: 1 μm; ×21250

Fig. 2. Control of the immunocytological reaction. When the antibody is incubated with purified osteocalcin, no immunocytological reaction is observed (*N*, nucleus; *rer*, rough endoplasmic reticulum). *Bar*: 0.5 μm; ×50000

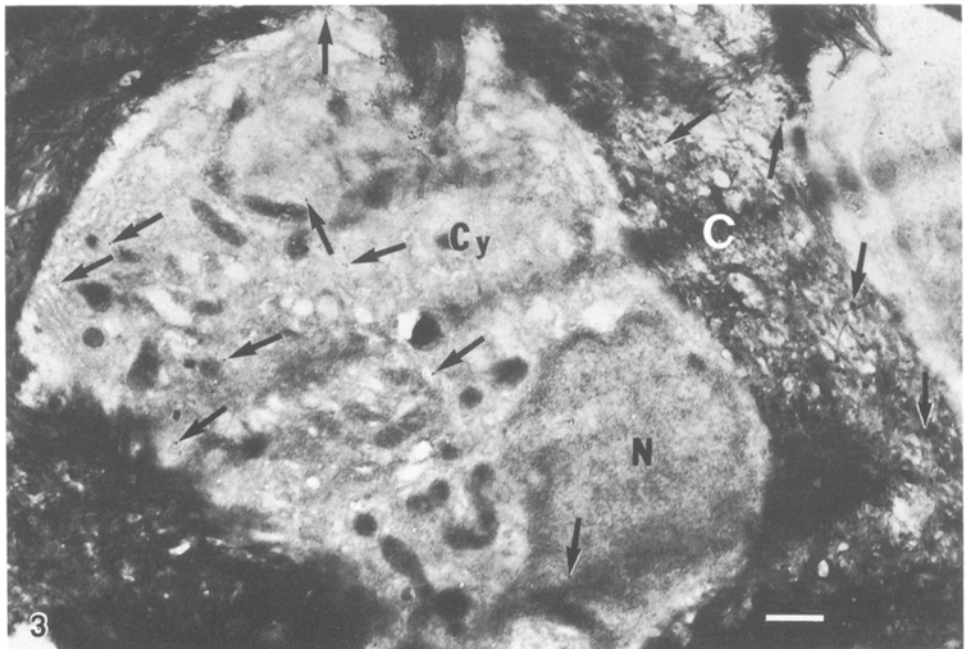


Fig. 3. Ultrastructural localization of osteocalcin-IR (*arrows*) in calcified bone matrix (*C*) and in an osteocyte of normal rat calvaria (anti-serum diluted to 2×10^{-4}). The immunoreaction products are clearly seen in the cytoplasm (*Cy*), sometimes close to profiles resembling rough endoplasmic reticulum (*rer*), and rarely in the nucleus (*N*). *Bar*: 1 μm; ×10000

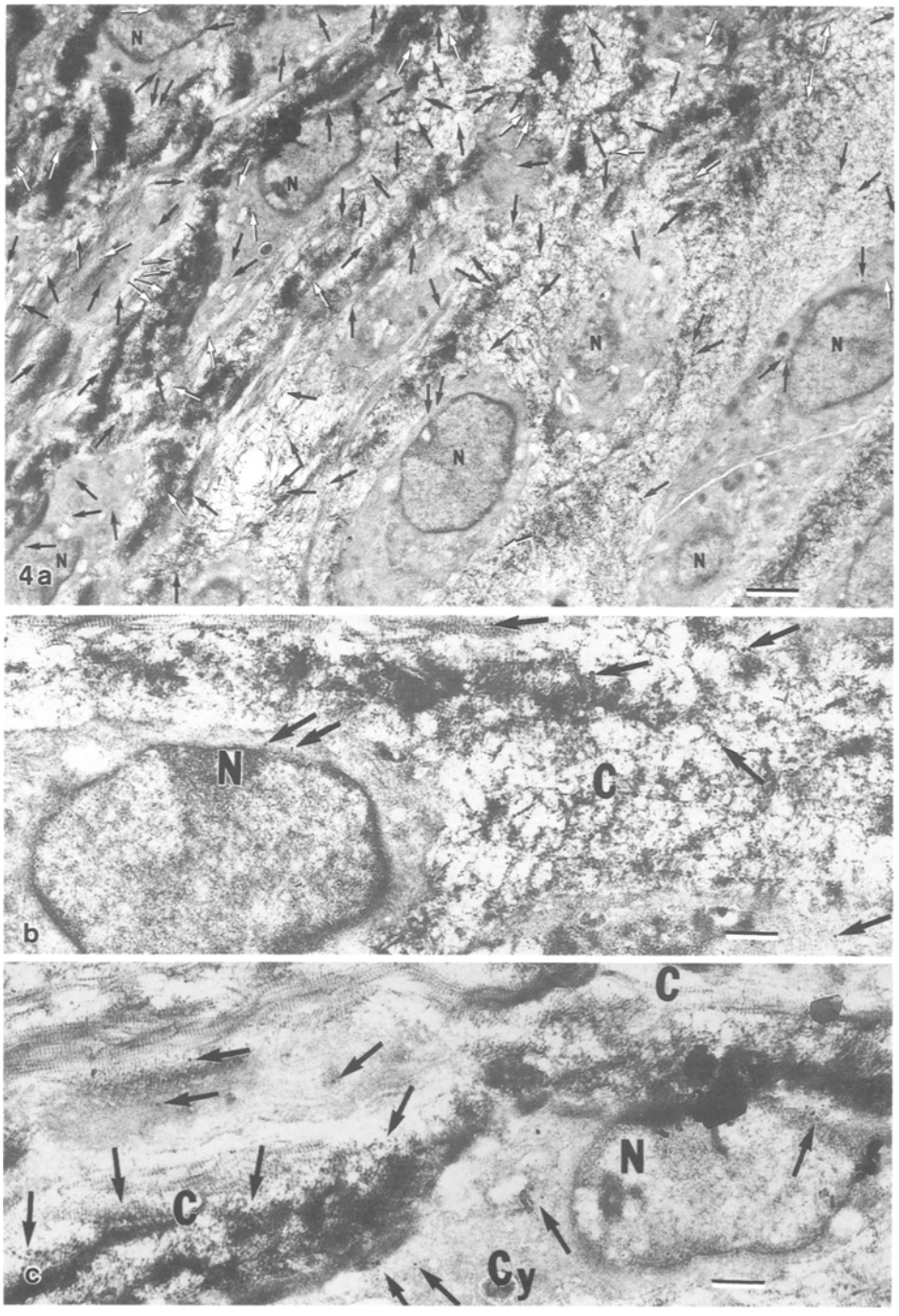


Fig. 4a–c. Electron micrographs of a frozen section from normal rat femoral diaphysis.
a Low magnification illustrating the gradient of osteocalcin-IR (*arrows*; anti-serum diluted to 2×10^{-6}) in organic matrix and osteoblasts, from regions at a distance from (lower right corner) to regions close to calcified areas (upper left corner). *Bar*: 2 μm; × 5000.
b, c Details of these two regions shown at higher magnifications (*N*, nucleus; *Cy*, cytoplasm; *C*, collagenous bone matrix). *Bars*: 1 μm; × 10500

an increasing gradient from uncalcified to fully calcified parts of bone (Fig. 4a–c). Osteocalcin-IR was also observed in osteoblasts mainly in cytoplasmic matrix (Figs. 1, 4a–c). When organelles were clearly recognizable, osteocalcin-IR was present in the vicinity of rough endoplasmic reticulum (Figs. 1, 4b, c). Only some deposits of the immunocytochemical reaction were also seen in the nucleus (Figs. 1, 4c). An increasing gradient of osteocalcin-IR could also be established from cells at a distance from a calcified area (Fig. 4b) to cells in close contact with mineralized zones (Fig. 4c).

In cells totally surrounded by calcified tissue (osteocytes) endogenous osteocalcin-IR was observed mainly in the cytoplasmic matrix sometimes close to profiles of rough endoplasmic reticulum and hardly ever in the nucleus (Fig. 3). Osteocalcin-IR was very intense in calcified matrix surrounding osteocytes (Fig. 3). However, osteocalcin-IR appeared less abundant in osteocytes than in osteoblasts.

In bone tissue of calvaria and femoral diaphysis from rats treated with warfarin, endogenous osteocalcin-IR was detected in organic matrix around collagen fibrils (Fig. 5) but rarely in the cytoplasm of osteoblasts. Furthermore, it is important to emphasize that in warfarin-treated rats, osteocalcin-IR was detected when the anti-serum was diluted only at 2×10^{-2}, while in untreated

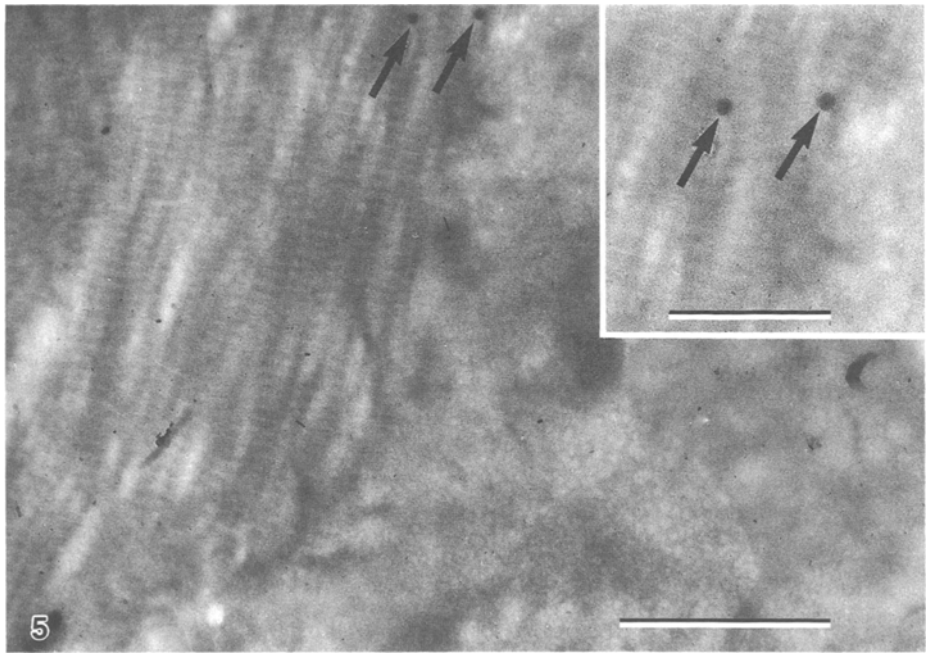


Fig. 5. Ultrastructural localization of osteocalcin-IR (*arrows*) in extracellular matrix of bone from a warfarin-treated rat (anti-serum diluted to 2×10^{-2}). The immunoreaction products, also shown in *inset*, are weak and almost exclusively observed around collagen fibrils. *Bar*: 1 μm; ×37200. *Inset:bar*: 0.5 μm; ×57350

rats, the immunoreaction products were seen even at high dilutions of 2×10^{-4} to 2×10^{-6}.

In the three groups of rats, endogenous osteocalcin-IR was not detected in hypertrophied and degenerative zones of cartilage from femoral growth plate. Osteocalcin-IR was observed neither in cartilage matrix nor in resting and degenerative chondrocytes (Fig. 6). However, osteocalcin-IR was sometimes found in cartilage matrix only in the vicinity of calcifying zones, where structures resembling matrix vesicles were present (Fig. 7).

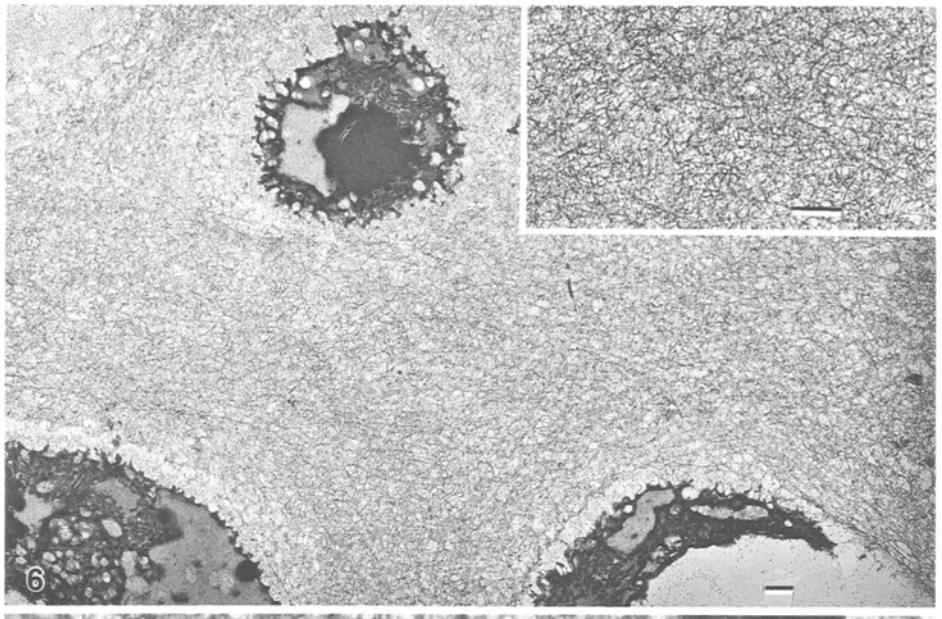
The immunocytochemical distribution of osteocalcin-IR throughout the bones of untreated normal rats and of control rats (saline-solution treated ones) corresponds to the biochemically detected levels of immunoreactive osteocalcin extracted from the various anatomical regions of the bones (Table 1). Gla and osteocalcin contents were deeply decreased in bones of warfarin-treated rats. It is noteworthy that proportionately more Gla is measured in the non-calcified region of the bones where osteocalcin is undetectable. This Gla content is probably related to matrix Gla protein (Otawara and Price 1986), which is not detected by our anti-osteocalcin serum. Thus, these data further confirm that our antibody directed against osteocalcin did not react with matrix Gla protein or it would have been detected in the cartilage region. As described in Materials and methods, the specificity of our antibody is also confirmed on soft tissues of our normal untreated rats.

Discussion

The technique employed in the present study (fixation, freezing, then cryoultramicrotomy) allows a suitable preservation of the ultrastructural aspects of bone tissue and cartilage (Boivin et al. 1983). Recent results show that the present technique may be used successfully to detect endogenous hormones and their receptors (Boivin et al. 1985, 1987; Morel et al. 1985; Groot et al. 1986). Furthermore, this method is a compromise between a suitable preservation of the ultrastructural aspects of tissues (fixation for a short time but no decalcification) and of their antigenicity (freezing). Major changes in the localization of osteocalcin during methodological procedures seem to be ruled out because of the constant absence of products of immunocytochemical reactions in some tissular and cellular compartments. Further, the histological localization of osteocalcin in bone matrix undergoing mineralization confirms previous results reported in calf bone (Bianco et al. 1985) and in developing rat bones (Bronckers et al. 1985; Groot et al. 1986; Carmada et al. 1987).

We show that osteocalcin is absent from cartilage, is present in calcified cartilage only when associated with hydroxyapatite crystals in the vicinity of matrix vesicles, and increases in bone in proportion to mineralization activity. The present observations are in good agreement with the first biochemical data demonstrating the appearance of osteocalcin with the onset of mineralization (Hauschka et al. 1983).

Thus, the results of the present study which correctly reflect biochemical analyses provides a clear localization of osteocalcin in bone tissue and cartilage of neonatal rats. This is also confirmed by the controls of the immunocytological reaction. Our results showed that osteocalcin antigenicity may be detected in bone matrix and osteoblasts of calvaria and femoral diaphysis simultaneously with the beginning of bone calcification. Osteocalcin increased in matrix and bone cells in parallel with the increase in mineralization. Furthermore, these studies show a relationship between synthesis of osteocalcin and mineralization process, confirmed by our observation of a gradient of osteocalcin-IR with the pro-



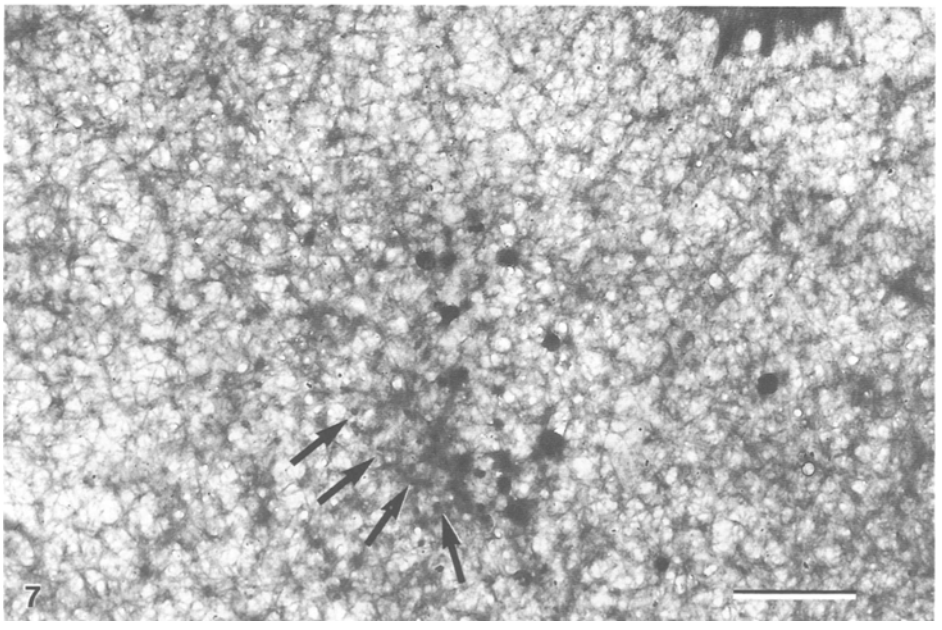


Fig. 6. Electron micrograph of a frozen section of cartilage from a normal rat femoral growth plate. No immunoreaction product (anti-serum diluted to 2×10^{-5}) is seen either in cartilage matrix, also shown in *inset*, or in chondrocytes. *Bar*: 1 μm; ×4500. *Inset:bar*: 1 μm; ×8400

Fig. 7. Ultrastructural localization of osteocalcin-IR (*arrows;* anti-serum diluted to 2×10^{-5}) in the vicinity of electron-dense matrix vesicles found in the cartilage matrix undergoing mineralization. *Bar*: 1 μm; ×21400

gress of mineralization. Recently, Andujar and Magloire (1989) have similarly shown that dentin Gla-containing proteins synthesis is independent of the mineral deposition, while their secretion and matrix deposition are closely related to the mineralization process.

The ultrastructural localization of osteocalcin in osteoblasts is consistent with studies showing that osteoblasts are the site of synthesis of osteocalcin (Nishimoto and Price 1980; Groot et al. 1985; Thavarajah et al. 1986; Carmada et al. 1987; Mark et al. 1987; Lian et al. 1989), and that the post-translational synthesis of Gla occurs within the rough endoplasmic reticulum (Lian and Friedman 1978). Osteocalcin was mainly localized in the cytoplasmic matrix of osteoblasts often in the vicinity of rough endoplasmic reticulum but in fact there was no accumulation of osteocalcin in bone cells and this protein appears to be secreted in bone matrix as soon as it is formed. An accumulation of osteocalcin in the Golgi was not observed in the present study and this fact appears consistent with our present knowledge that there is no evidence of glycosylation of pro-osteocalcin or osteocalcin. This is concordant with the absence of osteocalcin storage in bone-derived cells (Thavarajah et al. 1986), as well as the absence of immunostained secretory vesicles in rat osteoblasts (Mark et al. 1987). The presence of a weak osteocalcin antigenicity in the nucleus of osteoblasts and osteocytes appears very difficult to explain. The existence of osteocalcin in osteocytes confirmed histological data from Bronckers et al. (1985).

In bone of warfarin-treated rats, and using an antibody 100- to 10000-fold more concentrated than for untreated rats, only a small amount of osteocalcin was found and localized in the extracellular matrix. These

Table 1. Gamma-carboxyglutamic acid (Gla) and osteocalcin concentrations in bones of 8-day-old rats treated (or not) with warfarin (mean ± SE)

		Gla (residues/ 10^3 Glu)	Osteocalcin (ng/ mg dry wt.)
Untreated rats			
Calvaria		2.75 ± 0.40	483 ± 51
Femur	diaphysis	2.80 ± 0.32	582 ± 65
	metaphysis	1.15 ± 0.08	115 ± 20
	cartilage	0.083 ± 0.05	5 ± 3
Saline solution-treated rats			
Calvaria		3.15 ± 0.30	450 ± 37
Femur	diaphysis	2.95 ± 0.45	543 ± 25
	metaphysis	0.98 ± 0.13	125 ± 30
	cartilage	0.09 ± 0.05	7 ± 5
Warfarin-treated rats			
Calvaria		0.02 ± 0.01	47 ± 2
Femur	diaphysis	0.02 ± 0.01	31 ± 3
	metaphysis	ND[a]	3.5 ± 1
	cartilage	ND[a]	1.0 ± 0.4

[a] ND, Not detectable in 5 mg tissue dry weight

ultrastructural results are in agreement with biochemical data and both confirm studies reporting the inhibition of Gla formation in bone (Hauschka and Reid 1978; Price and Williamson 1981). The absence of osteocalcin-IR in the cytoplasm of osteoblasts seems to us to be confirmation that warfarin inhibits carboxylation of glutamic acid residues. However, the osteocalcin-IR noted in the extracellular matrix was probably due to osteocalcin synthesized before the beginning of warfarin treatment.

The ultrastructural absence of osteocalcin antigenicity in degenerative and hypertrophied zones of cartilage from femoral growth plates confirmed histological observations in rat bones (Bronckers et al. 1985; Groot et al. 1986) and is in good agreement with biochemical findings (Glimcher et al. 1979). It is now established that only osteoblasts and osteoblast-like cells and odontoblasts are capable of osteocalcin synthesis (Bronckers et al. 1985; Groot et al. 1985, 1986; Thavarajah et al. 1986; Carmada et al. 1987; Gorter de Vries et al. 1987; Mark et al. 1987). The observation of osteocalcin in close proximity to matrix vesicles in calcifying cartilage may result from the affinity of osteocalcin for the hydroxyapatite formed in matrix vesicles (Hauschka 1986). However, the possibility exists of an induction of osteocalcin synthesis in hypertrophic chondrocytes in the calcifying zone analogous to the induction of mRNA that occurs in osteoblasts in vitro (Lian et al. 1989) during mineralization of the extracellular matrix.

In conclusion, observations in neonatal rats treated or not with warfarin showed that osteocalcin is present in bone matrix and osteoblasts as soon as calcification is initiated and appears to increase with the development of mineralization. Osteocalcin clearly appears to be synthesized in osteoblasts and osteocytes then secreted in bone matrix without previous storage in these bone cells. However, the formation of osteocalcin is deeply inhibited by a treatment with warfarin. Osteocalcin is not detected in cartilage matrix and resting and/or degenerative chondrocytes except in the close vicinity of matrix vesicles.

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