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CORRELATION OF THE APPEARANCE OF γ-CARBOXYGLUTAMIC ACID WITH THE ONSET OF MINERALIZATION IN DEVELOPING ENDOCHONDRAL BONE

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SUMMARY: γ-Carboxyglutamic acid (Gla) is a constituent of the non-collagenous bone protein osteocalcin. The appearance of γ-carboxyglutamic acid during de novo differentiation and development of endochondral bone has been correlated with the onset of mineralization. Discrete stages of endochondral bone development were studied by subcutaneous implantation of demineralized rat diaphyseal bone matrix. Residual Gla in acid-demineralized bone matrix was lost rapidly on implantation. Gla levels were basal during mesenchymal cell proliferation (day 3) and chondrogenesis (days 5-7). Gla and calcium levels began to increase during cartilage mineralization (day 9) and continuously increased after day 10 concomitant with bone differentiation.

INTRODUCTION: It is common knowledge that vitamins and hormones regulate the development of bone. Among other vitamins, vitamin K has been implicated in mineralization (1) although the precise mechanism of action is not clear. Prompted by the discovery of γ-carboxyglutamic acid (Gla) in vitamin K-dependent, calcium-binding blood coagulation factors, calcified tissues were examined for Gla. Bone was found to contain abundant quantities of a small (∿6000 dalton) Gla-rich protein which has been named osteocalcin (2,3). The developmental appearance of osteocalcin has been described for embryonic chicken bone (4). Further studies on the role of vitamin K in mineralization need an experimental system in which de novo mineralization of bone can be manipulated by nutritional factors. The onset of mineralization occurs in the mammalian fetus long before birth. In order to avoid the technical difficulties of nutritional experiments with prenatal fetuses we have examined the appearance of Gla during development of new bone induced by demineralized bone matrix (5-7), as a possible prelude to more extensive work on the role of vitamin K in biological mineralization.

In brief, the method consists of subcutaneously implanting demineralized powders of collagenous bone matrix in rats. This induces endochondral bone differentiation in an invariant temporal sequence that has been well characterized (5-7). On day 1 there is a transient appearance of polymorphonuclear

Abbreviations: Gla, γ-carboxyglutamic acid; EDTA, ethylenediamine tetraacetic acid

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leukocytes in the implant. Mesenchymal cells appear in close contiguity with the matrix on day 3, proliferate and then emerge as chondroblasts on days 5 and 6 and differentiate and elaborate cartilage matrix on day 7. On day 9 the hypertrophied cartilage matrix undergoes mineralization. Concomitant with vascular invasion, bone-forming osteoblasts appear on day 10. The newly-formed bone is remodeled by osteoclasts (days 12-18) and there is a concurrent dissolution of the implanted matrix. By day 21 hematopoietic bone marrow differentiation occurs in the ossicle. This paper describes the appearance of Gla in developing bone and its correlation with the onset of mineralization.

MATERIALS AND METHODS: Dehydrated diaphyseal shafts of rat femur and tibia were pulverized in a CRC Micro Mill (Technilab Instruments, Vineland, NJ) and sieved to a discrete particle size of 74-420 μm. The powders were demineralized with 0.5M HCl, extracted with water, ethanol and ether and prepared as described. Demineralized bone matrix was transplanted subcutaneously during ether anesthesia in male rats of Long-Evans strain, age 28-35 days (5). There were two sites over the thorax in each rat. On designated days, as described in Fig. 1, the rats were killed and the subcutaneous button-like plaques were dissected out, washed in cold 0.15M NaCl, followed by ice-cold distilled water, and then lyophilized.

For determination of Gla, lyophilized implants of different ages were weighed and individually placed in glass alkaline hydrolysis tubes (Corning 7280) with 0.8 ml freshly prepared 2M KOH. Implant dry weights ranged from 30 to 60 mg. Samples were hydrolyzed 22 hr at 110°C in a sealed vacuum desiccator, and then centrifuged (8). The pellets of insoluble material were reserved for calcium analysis as described below. The supernatants were neutralized with 70% $HClO_4$, centrifuged to remove the $KClO_4$ precipitate, and then diluted 5 to 60-fold in 0.2M citrate buffer, pH 2.2 for amino acid analysis (8). Standards of synthetic Gla were used for ninhydrin color value calibration of a Beckman 121M amino acid analyzer, and the integrated ninhydrin peak areas provided sufficient information to calculate the parameter residues Gla/1000 residues glutamic acid (8). Triplicate measurements were performed on each sample, and 3 to 6 samples of each age were studied in two separate experiments. The glutamic acid content of plaque hydrolysates is invariant over the implantation period (100 ± 3 residues glutamic acid/1000 total amino acid residues), and the total protein content of the plaques does not change appreciably.

Calcium contents of each implant were measured after alkaline hydrolysis. KOH-insoluble residue was centrifuged out of the hydrolysate and suspended in 1.0 ml of 3M HCl, 5.86% La_2O_3. After 24 hours at room temperature the samples were centrifuged and the clear supernatants diluted appropriately with 0.3M HCl, 0.586% La_2O_3 for quantitation of calcium by atomic absorption spectrophotometry. A Perkin Elmer model 603 was calibrated with calcium solutions prepared from a calcium phosphate standard (No. 120b) of the National Bureau of Standards. Calcium concentrations were sensibly linear over at least a 20-fold dilution range (0.5 to 10 ppm). Calcium contents of plaque samples are expressed as μg Ca^{2+}/mg dry weight of the lyophilized plaque.

RESULTS AND DISCUSSION: Implantation of demineralized diaphyseal bone matrix to subcutaneous sites in the rat resulted in new endochondral bone formation as described previously (5-7). The acid-demineralized rat bone matrix used for implantation contained about 3.5 residues of Gla per 1000 residues of glutamic acid. On implantation in vivo there was a rapid loss of Gla as seen

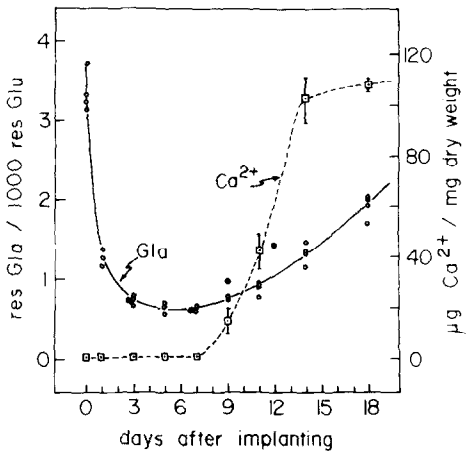

Figure 1. Changes in the Gla and Ca^{2+} content of demineralized rat bone matrix after subcutaneous implantation in 6 week rats. Gla content of plaques from two separate experiments (●,○) was determined after 2M KOH hydrolysis. Ca^{2+} was measured in the alkali-insoluble residue (⊡--⊡); the bars indicate ± standard error of the mean.

on day 1 (Fig. 1) and the levels declined even further on day 3, when mesenchymal cell proliferation was maximal (9,10). On days 5 and 7 the levels of Gla were minimal and calcium was not detectable. The basal level of Gla could reflect the background due to endogenous circulating plasma proteins. There was a slight rise in Gla content on day 9 during cartilage calcification which was promptly reflected by the swift increase in calcium. Following vascular invasion of the interior of the button-like plaques, bone formation begins on days 10-11. From this point on, there is a steady increase in Gla levels in the implants, correlated with an abrupt rise in calcium content. Gla was the only amino acid showing any significant change in the implants during the period studied.

Demineralization removes most of the non-collagenous proteins from bone matrix, serving as an initial step in the purification of such proteins as the Gla-rich protein osteocalcin (2). However, it is clear that a significant fraction of osteocalcin is not extractable from the matrix; this fraction apparently includes a higher molecular weight precursor form of osteocalcin (11). Table 1 shows the Gla (osteocalcin) contents of various rat connective tissues before and after demineralization. Bone powder retains 21% of its original Gla content after acid treatment, while EDTA removes all but 14% of the Gla. The powder used for implantation was from a different batch and retained about 39% of its original Gla content. For all of these demineralized samples, less than 0.1% of the original calcium content remains. Rat incisor dentin also contains a small but significant amount (5%) of its original Gla after acid extraction. Rat tail tendon contains no Gla (Table 1). The bone-inducing

Table 1.

γ-CARBOXYGLUTAMIC ACID CONTENT OF RAT BONE, TOOTH AND TENDON

Matrix Material	Demineralization Procedure	res Gla[a] / 1000 res Glu
Rat long bone diaphysis	none	8.67 ± 0.42
Rat long bone diaphysis	0.5M HCl	1.78 ± 0.08
Rat long bone diaphysis	0.5M EDTA[c]	1.21 ± 0.07
Rat incisor[b]	none	7.97 ± 0.24
Rat incisor	0.5M HCl	0.34 ± 0.02
Rat tail tendon	none	< 0.09
Rat tail tendon	0.5M HCl	< 0.09
Rat tail tendon	0.5M EDTA	< 0.09

[a]Mean ± standard error (n=7). The glutamic acid content of all samples was 100 ± 3 residues per 1000 amino acid residues.

[b]Predominantly dentin, after removal of enamel by scraping.

[c]Extraction by same procedure as described in Methods for 0.5M HCl, except that 0.5M EDTA, pH 8 was substituted.

activity of demineralized rat bone and incisor is high, while rat tail tendon collagen is devoid of such activity (7). Whether non-collagen proteins such as osteocalcin are involved in the specific induction process is under further investigation.

During days 1 to 3 after implantation (Fig. 1), the precipitous decrease in Gla content may be due to the transient host inflammatory response observed earlier (5-7). It is noteworthy that trypsin in vitro will release most of the residual Gla (osteocalcin) from bone which is otherwise inaccessible to extraction by demineralizing solvents (11). The upswing in the Gla content of implants after day 9 corroborates the previously observed coincidence of osteocalcin appearance with mineralization of embryonic chicken bone (4). The removal of osteocalcin and its subsequent reappearance during bone induction sheds new light on the dynamics of this complex differentiation process. This experimental system is now amenable to further studies on the biosynthesis and function of Gla-containing proteins in developing bone. Further, this experimental model may be informative in studies on vitamin K deficient rats, and such work is currently in progress.

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