THE PRESENCE OF γ-CARBOXYGLUTAMIC ACID IN THE PROTEINS ASSOCIATED WITH ECTOPIC CALCIFICATION

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SUMMARY

γ-Carboxyglutamic acid (Gla) is identified in the proteins associated with several types of ectopic calcifications in which hydroxyapatite is the major mineral component. These included the calcified skin and subcutaneous plaques from a patient with dermatomyositis, the calcium containing material extruded from the skin of a patient with scleroderma, and the calcified, atheromatous plaques from aorta. Alkaline hydrolysis of biopsy material from these and from normal tissue revealed the presence of Gla only in the plaque specimens. Since a γ-carboxyglutamic acid-containing protein is normally present in bone and absent in unmineralized tissues, the presence of Gla in soft tissue calcifications is a potentially significant finding, especially in view of its known calcium and phospholipid binding properties.

INTRODUCTION

γ-Carboxyglutamic acid, discovered in the blood clotting factor prothrombin, has the specific function of binding with calcium and phospholipid for the conversion of prothrombin to thrombin (1-4). The recent studies which have demonstrated the presence of γ-carboxyglutamic acid (Gla) in an EDTA soluble, non-collagenous protein present in bone (5,6) have raised questions concerning its possible function in the regulation of tissue mineralization. It was therefore of interest to examine a number of ectopic tissue calcifications for the presence of Gla.

MATERIALS AND METHODS

Tissues: (1) Aorta: (a) Samples of normal thoracic aortas were obtained at autopsy from a premature infant and from a 21 year old male with cystic fibrosis; (b) Samples of calcified aortic plaque, carefully dissected from surrounding unmineralized aortic tissue were obtained from a 56 year old male and a 60 year old female, respectively. Samples from both of these patients of aortic tissue from other regions of the aorta which did not contain calcified atheromatous plaque were similarly analyzed as controls. (2) Scleroderma: Chalky, paste-like material which exuded from the skin of a 52 year old woman with a 30 year history of scleroderma. (3) Dermatomyositis: Normal skin as well as portions of the calcified deeper layers of skin and subcutaneous tissue from a 21 year old female were obtained by biopsy (courtesy of Dr. R. Schwarz, Tufts Medical School, Boston).

Methods: Identification of Plaque. (1) Small aliquot samples of skin and aortic tissue specimens were examined histologically by alizarin red stain for calcium. The tissues were fixed in neutral buffered formalin, imbedded in paraffin and serial sections were stained in 2% aqueous Alizarin Red stain adjusted to pH 4.2 with dilute ammonia (7). (2) X-ray diffraction powder patterns of the ectopic calcifications were obtained in a Debye-Scherrer camera having a 57.3mm radius. The exudate material from the patients with scleroderma and dermatomyositis was dried at room temperature in a vacuum dessicator over P_2O_5 for several days, and powdered under liquid N_2 to a size less than 200 mesh. The crystal type was determined by a comparison of the reflections obtained with a hydroxyapatite standard and in accordance with the Powder Diffraction Files, Joint Committee on Powder Diffraction Standards, 1973.

Procedure for Analysis of Gla: Tissue samples were washed with saline to remove any blood and either hydrolyzed directly in 2N KOH, 22 hours or extracted in 0.5M EDTA (pH 8.0). The EDTA extracted samples from the scleroderma exudate and from the atheromatous plaque were dialyzed exhaustively against water and lyophilized before hydrolysis.

Detection of Gla: Analysis for protein bound Gla was carried out on alkaline hydrolysates of tissues and extracts using a Beckman 121-M automatic amino acid analyzer, as previously described (5) with the exception that pH 2.6 citrate was used for starting buffer in order to resolve Gla from interfering compounds in whole tissues. The color factor for Gla was obtained using synthetic Gla (a gift from Dr. R. Hisky, University of North Carolina, Department of Chemistry). The total glutamic acid content of the samples was determined on 6N HCl acid in hydrolysates using the same amino acid analyzer. From these data, the ratio or percentage of the total number of glutamic acid residues which had been converted to γ-carboxyglutamic acid could be calculated.

RESULTS

Alizarin red staining showed the absence of microscopic calcification in the aortas of the premature infant, in the 21 year old patient and in certain regions of the aortas from which the calcified atheromatous plaques were removed. Calcium deposition was apparent in the scleroderma exudate, in the dermatomyositis plaque, and in the atheromatous, cal-

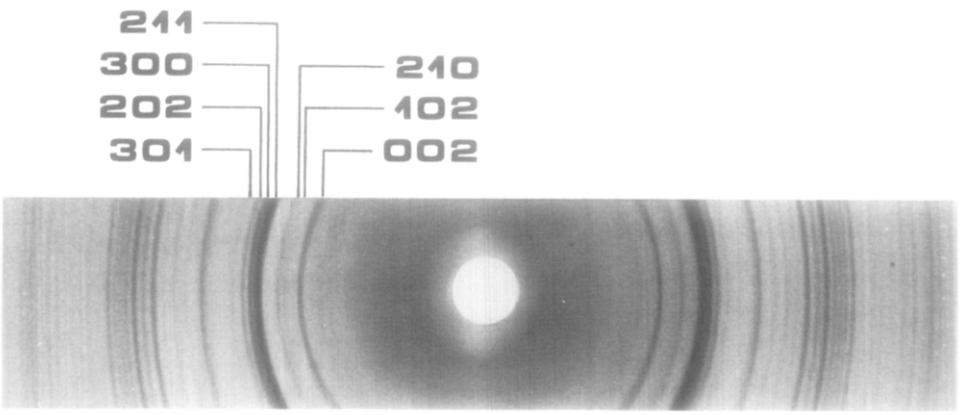

Figure 1. X-ray diffraction powder pattern of the chalky material exuded from the skin of a patient with scleroderma. The reflections characteristic of hydroxyapatite are clearly seen at 002, 102, 210, 211, 300, 202, and 301.

TABLE I

PATHOLOGICAL CALCIFICATIONS	RESIDUES GLA/1000 AMINO ACIDS
Aorta	
Normal--21 yr. old	0
Normal--Premature infant	0
Normal--Piece of tissue from aorta which had calcified elsewhere (56 yr. old man)	0
Calcified atheromatous plaque	
(a) 56 yr. old man	1.5
(b) 70 yr. old woman	1.7
(c) EDTA extract of plaque (70 yr. old tissue)	8.2
Scleroderma	
Hydroxyapatite containing skin exudate	6.0
EDTA extract of skin exudate	20.5
Dermatomyositis	
Calcified skin and subcutaneous plaque*	1.8
Normal skin and subcutaneous tissue*	0

*Obtained by punch biopsy

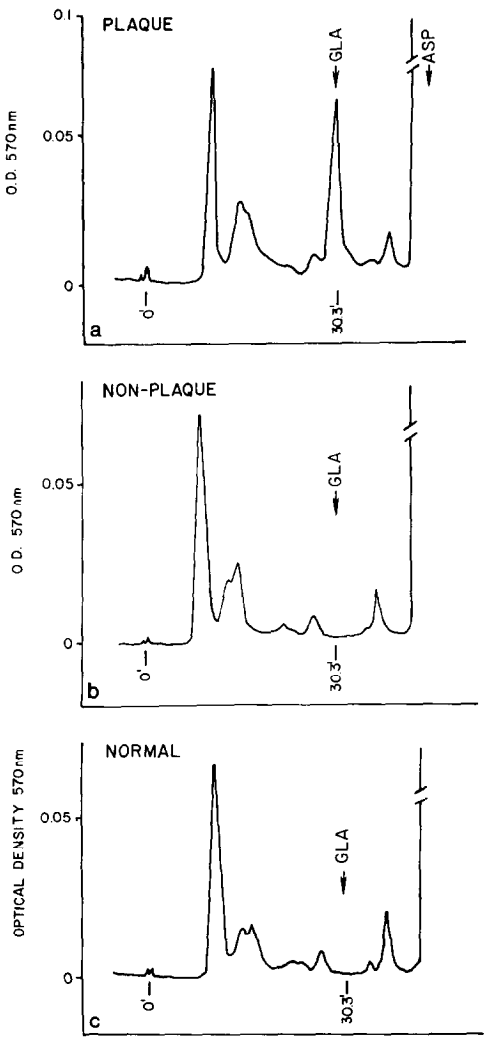

Figure 2. Elution profile of the alkaline hydrolysates from (a) atheromatous plaque; (b) aortic tissue from a non-plaque area and (c) aortic tissue from an infant with no evidence of aortic tissue calcification. Analyses were carried out on a Beckman 121-M automatic amino acid anayzer. Ninhydrin was measured at 570nm.

cified tissue plaques by alizarin red staining. The chalky paste-like material from the patient with scleroderma, the calcified skin plaque from the patient with dermatomyositis and the atheromatous calcified plaques are shown to contain hydroxyapatite by x-ray diffraction analysis (Figure 1).

Considerable amounts of Gla were detected in all of the ectopic calcifications studied (Table 1). Significantly, no Gla was detected either in the normal skin and subcutaneous tissue of the patient with dermatomyositis or in specimens of normal human skin and subcutaneous tissue from other patients. Similarly, Gla was not detected in uncalcified aortic tissue, either from patients without atheromatous plaques, or from uncalcified portions of the aortas of the patients from which the calcified atheromatous plaques were taken. The ninhydrin profiles of the alkaline hydrolyzate of these aortic tissues demonstrating the presence and absence of Gla is shown in Figure 2.

As in the case of bone, the EDTA soluble, non-dialyzable proteins were enriched in their content of Gla. For comparison, Table II is presented showing the content of Gla in normal chicken bone preparations, in bovine prothrombin and in the F_1 fragment of prothrombin.

TABLE II

GLA-CONTAINING PROTEINS	RESIDUES GLA/1000 AMINO ACIDS
Bone (Chicken, 10 week)†	
Whole long bone	0.67
EDTA extract	15.9
Pure protein	70.0
Prothrombin (Bovine)††	17
F_1-Prothrombin (Residues 1-156)	64

†Hauschka, P.V. and Lian, J.B., unpublished data

††Magnusson, S., Petersen, T.E., Sottrup-Jensen, L. and Claeys, H., in Proteases and Biological Control, Cold Spring Harbor Laboratories, 1976, pp. 123-149.

DISCUSSION

Although the biochemical and physical-chemical functions of the Gla containing protein(s) in bone are not known, the capacity of this unique amino acid residue to bine calcium has suggested that it is involved in some way with tissue mineralization (5,6). It is quite possible that the Gla-Ca^{++} complex binds the phosphoproteins present in bone, dentin and enamel (8) in a manner similar to the binding of phospholipid by the Gla-Ca^{++} complex in the conversion of prothrombin to thrombin (1-4). The absence of Gla in normally unmineralized tissues and its presence in the ectopic calcifications of several of these tissues such as skin and aorta, provides further evidence for its role in calcification. This is also supported by our findings that only those renal calculi which contain calcium have Gla as a component of their organic matrix, whereas those consisting of other crystalline material such as uric acid or struvite (ammonium-magnesium phosphate) do not contain Gla (9).

Since hydroxyapatite appears to be the major mineral component of plaque in scleroderma, dermatomyositis and atherosclerosis, the question of whether the Gla-containing protein in the plaque is similar to osteocalcin, the normal Gla-containing bone protein arises. It is also quite relevant to inquire if the plaque material contains the Gla portions of the vitamin K dependent blood clotting factors. It would be of considerable interest to establish whether or not there is an active process of *de novo* synthesis of Gla-containing protein by cells which are present at the site of calcification. This preliminary study raises potentially significant questions concerning a possible role of proteins containing the calcium-phospholipid binding amino acid, γ-carboxyglutamic acid, in the etiology of ectopic calcification and in the pathogenesis of atherosclerosis.

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