Physical and Chemical Analyses of the Mineral Substance during the Development of Two Experimental Cutaneous Calcifications in Rats: Topical Calciphylaxis and Topical Calcergy

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In subcutaneous calcinoses induced in rats by topical calciphylaxis and topical calcergy, the ultrastructural aspects related to the evolution of calcified deposits were previously described. In the present study by means of X-ray diffraction, infrared spectrometry and electron spin resonance, different biophysical analyses are performed on the mineral substance deposited during development of calciphylaxis and calcergy. A rapid evolution of the calcium phosphate deposits into hydroxyapatite was noticed along the first 20 days of the calcinoses; from then, there is no important modification even at the later stages, and the characteristics of the mineral substance are mostly similar to that of bone tissue. The concentration of trace elements such as Mg^{2+} and Fe^{2+}, is found higher in cutaneous calcinoses than in bone tissue.

Introduction

The histological aspects of the experimental cutaneous calcinoses induced in rats either by topical calciphylaxis or by topical calcergy [1] have been studied as a function of their evolution in time. The principal ultrastructural stages of this evolution are also well known [2–4]. It is important to emphasize that the two experimental models are different: the topical calciphylaxis is induced in rats previously sensitized by a treatment inducing an hypercalcaemia, and in the topical calcergy the challenging is done without sensitization. In this paper we compare and analyse these two models of calcification. After the first biophysical results obtained in topical calciphylaxis [5, 6], the present study shows the chemical and biophysical modifications of the mineral substance in the two types of calcinoses and during their chronological evolution. The variations with time of the inorganic constituents, carbonate and iron, as well as the evolution of the crystalline phase in the mineral deposit, were observed and discussed. The physico-chemical analysis of these different calcified tissues were performed by means of X-ray diffraction, infrared spectrometry and electron spin resonance.

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Experimental Data

Materials

In this study 100 white female rats, 4 weeks old and weighing about 100 g each, were used. According to the procedure described by Boivin [2], for the induction of topical calciphylaxis of the skin, the animals were sensitized by gastric intubation of 1 mg dihydrotachysterol (AT 10, Bayer, Germany) per 100 g body weight. After 24 h each animal was given a dorsal subcutaneous injection of ferrous chloride ($FeCl_2 \cdot 4\,H_2O$, Merck) at a challenging dose of 50 µg per 100 g of body weight. For the induction of topical calcergy of the skin, each rat was directly injected subcutaneously in the dorsal region with 100 µg of $KMnO_4$ per 100 g of body weight [4]. Femoral diaphyses taken from five untreated rats served as bone mineral controls. The subcutaneous calcified connective tissues were taken, under ether anesthesia, at different time intervals during the life span of each calcinosis (see Fig. 1). Calcified tissue samples were defatted and pulverized for 15 min at 0 °C in a mixer mill containing a 1/1 mixture of alcohol/trichlorethylene. The powder was then dried at 60 °C and passed through a 200 mesh sieve.

Analytical procedure

The inorganic constituents of the subcutaneous calcinoses were calculated on a dry weight basis. The carbonate was measured in dry fat free samples by

means of the titrimetric method of Dallemagne [7] and calculated in terms of percentage of dry weight. The iron determinations were performed, on samples heated 4 h at 800 °C, following the CETAMA colorimetric method [8].

X-ray diffraction

X-ray diffraction analyses were performed on powder samples using a Chesley camera with a 100 μm collimator. Times of exposure were about 12 h under the nickel-filtered copper $k\alpha$ radiation. A microdensitometer Joyce Loebl gives the recording of patterns and permits to measure the width at half-maximum intensity (β index) of two main peaks; one is the portion containing the (00.2) reflection and the other the (21.1) and (11.2) reflections. The β index of the diffraction peak is inversely proportional to the average crystal size.

Infrared spectrometry (IR)

Infrared spectra in the region from 700 to 500 cm^{-1} were obtained with a Perkin-Elmer model 357 IR spectrophotometer. Each analysis was performed twice using KBr pellets containing 1 mg of calcified tissue. An empiric determination of the calcium-phosphate crystallization state was measured as an infrared absorption splitting according to the method of Blumenthal *et al.* [9]. In order to ensure satisfactory reproducibility, IR information was treated and the results optimized by an HP 21 MX computer. The crystallinity index, expressed in arbitrary units, has only a significance for comparison.

Electron spin resonance (ESR)

From each sample 2 aliquots of 10 mg powder were X-irradiated under different conditions before ESR measurement. Both powders were exposed for 2 h to a tungsten-anode X-ray tube (30 mA and 30 KV) of a Philips generator. The X-irradiation of the first sample was performed in an unsealed tube and ESR spectra were recorded at room temperature 2 h later with a Varian E-9 X band spectrometer using 100 KHz field modulation. On the second sample the X-irradiation was performed under argon atmosphere in a sealed tube and ESR spectra were recorded 7 days after the opening of the tube. For each spectrum the magnetic field was calibrated with a NMR marker, and the Klystron frequency was measured by means of a transfer oscillator (HP 540B) and a frequency counter (RACAL 9839). Intensities of the ESR signals in arbitrary units were calculated by means of an HP 21 MX computer and normalized for weight of mineral constituents of each calcified tissue sample.

Results

The results are reported separately for each experimental model, but in general they are not significantly different and both calcinoses are listed on the same graph in order to appreciate the chronological evolution. It should be emphasized that for each result, the individual variations are not due to imprecision of the measurements but reflect the individual differences between several animals.

Chemical assays

In calciphylaxis and calcergy, the ash percentage of the calcified tissue of each treated rat was determined and the evolution as a function of the time after challenging is illustrated in Fig. 1 a. No significant difference of the percentage of the mineral constituents is observed between these two cutaneous calcinoses at different times after challenging. A steady increase is observed till stage 20 days, and from then on the mineral constituents of the calcinoses are slowly decreasing. Both calciphylaxis and calcergy, even when the mineralization appears maximum in development, show percentage of mineral significantly lower than that of femoral bone tissue.

The carbonate concentration (Fig. 1 b) shows no significant correlation ($r = 0.34$; $p < 0.20$) in function of the time after challenging both for calciphylaxis and calcergy. The linear regression stays lower than that of bone mineral.

On Fig. 1 c we observe that iron concentration is as high in calcergy as in calciphylaxis, and in both is significantly higher than in femoral bone. Note that the positive slope is significant ($r = 0.76$; $p < 0.007$) in function of the time after challenging.

X-ray diffraction

The X-ray diffraction patterns show for calciphylaxis and calcergy an inorganic substance with an apatite-like structure and reveal a slight delay in the appearance of this apatitic pattern for calcergy. The microdensitometric recording of these patterns al-

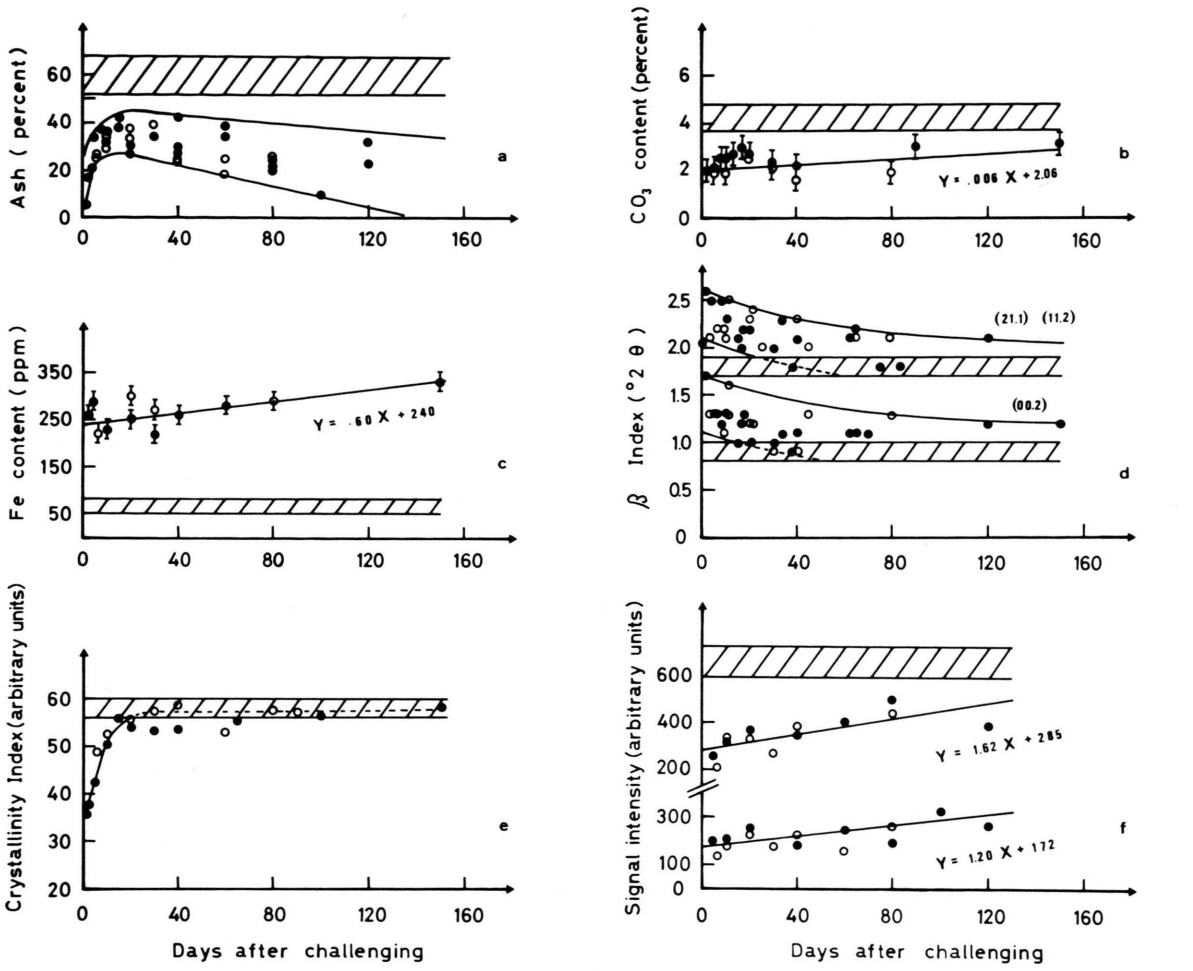

Fig. 1. Chemical and physical analyses in calciphylaxis (●), calcergy (○) and bone tissue (////), as a function of the time after challenging of the cutaneous calcinoses: (a) percent of inorganic constituents, (b) carbonate content, (c) iron content, (d) width at half-maximum intensity of (21.1), (11.2) and (00.2) reflections, (e) crystallinity index measured as an infrared absorption splitting, (f) quantity of stabilized radicals, upper: ESR spectra after irradiation in air atmosphere, lower: ESR spectra after irradiation under argon atmosphere and storage 7 days in air atmosphere.

lows us to measure the width at half-maximum intensity and gives information on the crystal size (Fig. 1 d). Evolution of the crystal length is indicated by modifications of the β index of the (00.2) reflections and that of the crystal width by those of the β index of the (21.1) and (11.2) reflections. During the first 40 days the crystal size increases rapidly as revealed by the decrease of the two β indexes. After these first stages of the development of both experimental models, the crystal size appears very similar to that of the mineral substance of bone tissue of the rats.

Infrared spectrometry

In Fig. 1 e, the evolution of the crystallinity index of the cutaneous calcinoses is given from the IR spectra taken at different days after challenging. For calciphylaxis, as early as stage 1 day, it is possible to measure a crystallinity index of 36% while for calcergy only from stage 5 days onwards it is possible to determine an IR-value. After about 5 days, both calciphylaxis and calcergy are similar and 20 days after challenging the crystallinity index is comparable to that of the bone mineral substance.

Electron spin resonance

Stabilization of paramagnetic species is simultaneously depending on the nature of the radical and on the physico-chemical properties of the crystal lattice. X-irradiated calcified tissue gives rise to two groups of radical species appearing in the $g = 2$ region of the ESR spectrum: i) inorganic radicals (CO_3^{3-} or CO_2^-) stabilized in the apatite crystals – ii) organic radicals (doublet of the protein) which are not stable in air atmosphere. In this work we plan to obtain informations on hydroxyapatite, this is why only the inorganic radicals were considered and in order to take out the organic signal perturbation, two independent measurements were performed. The quantities of carbon-centered radicals trapped in different calcinoses samples are normalized for weight of calcium phosphate deposit and are illustrated on the Fig. 1 f. Both of these under different condition of X-irradiation show a significant ($r = 0.74$ and 0.61; $p < 0.005$ and 0.02) increase of the radicals trapped in the crystalline matrix as a function of the time. No significant difference is found between calciphylaxis and calcergy, but these values never reach the values obtained for bone mineral.

Discussion

A chronological delay is observed in the initiation of the calcergy if compared to calciphylaxis and can be explained as follows: i) only calciphylaxis is sensitized by a treatment inducing an hypercalcaemia, and the high level of calcium in the extracellular fluid could play a role in the early stage of mineralization – ii) two different subcutaneous injections, $FeCl_2$ and $KMnO_4$, are respectively used for calciphylaxis and calcergy; these two chemical compounds probably have different properties of nucleation and can explain this chronological delay [4].

The ultrastructural aspects observed during the long term evolution of topical calciphylaxis and calcergy [3] are in agreement with the present results (Fig. 1 a). The ultrastructural observations show a continuous mineralization of the calcinoses during the first 20 days then an appearance of newly formed and unmineralized bundles of collagen fibrils. The ash percentage analyses in the later stages of both subcutaneous calcinoses confirm the macroscopic observations which still show a calcified tissue later than 150 days in calciphylaxis but never later than 100 days after challenging in calcergy. Ash percentage values of the topical calcinoses are always found lower than those of bone tissue. It appears important to emphasize that in bone tissue it is a normal skeletal calcification and ossification while in calcinoses it is a pathological phenomenon without subsequent ossification provoking a skin reaction against the foreign body constituted by the calcified tissue.

Measurements of the cabonate ion concentrations are made on dry fat-free samples, and it is difficult to compare the results because the samples have a different proportion of mineral/organic constituents. The incorporation of carbonate slowly increases till it reaches the level of the concentration in bone mineral (Fig. 1 b).

The normal mean value for iron in bone tissue is low (about 60 ppm) and in a previous article [6] we found that iron value is five times higher in calciphylactic tissue than in bone tissue. Gabbiani *et al.* [10] have demonstrated that the iron plays a role during the initial stages of topical calciphylaxis. These authors have showed by means of microprobe analysis that iron, calcium and phosphorus increased simultaneously in the area where $FeCl_2$ was injected. It is important to know if the high concentration level of iron is due to the 50 µg of $FeCl_2$ (14 µg Fe^{2+}) injected subcutaneously in topical calciphylaxis. In the present study we show that the iron concentration is as high in calciphylaxis where $FeCl_2$ was injected as in calcergy where only $KMnO_4$ was injected (Fig. 1 c). It clearly appears from this result that increase of iron during the inflammatory process in calcinoses is independent of the chemical compound used for challenging. The challenging dose of iron injected in calciphylaxis plays a role only during the initial stages of calcinoses like the challenging dose of manganese in calcergy. Indeed when the Mn content is determined in calcergy, its concentration appears very high during the first hours of the reaction but rapidly decreases at the stages 18 – 24 h (Walzer, unpublished results).

In calciphylaxis, a high concentration of magnesium was found only during the early stages and then a rapid decrease was noticed [6]. In calcergy, preliminar observations show a similar evolution with an increase of Mg content at stages 1 – 2 days and a subsequent important decrease. The role of magnesium in the mechanisms of crystallization and the inhibitory effect on hydroxyapatite formation,

has been reported [11 – 13]. The inhibitory role of magnesium may be important in the first hours of the calcium phosphate precipitation which forms the calcinoses.

The macroscopical and ultrastructural observations show that the cutaneous calcinoses progressively disappears with time. This phenomenon is due to a fragmentation process without important modifications of the calcified fragments; mechanisms of demineralization and/or resorption are very restricted in terms of time and area [3]. Thus it seems important to verify if, in the absence of histological modifications, biophysical variations in the mineral substance occur or not.

The X-ray diffraction study of mineral substance in the two models, reveals the evolution of the crystal size with time: it shows an increase during the first 40 days, then a stabilization at a size similar to that of bone crystals (Fig. 1 d). Kato and Ogura [14] reported comparable results in calcergy challenged with lead acetate. In fact only a few data consider the evolution of the β index in the mineral deposits. Aoba *et al.* [15] found in pathological calcification of the dental pulp, a β index identical to that of bone tissue. The present X-ray results prove the absence of crystallographic perturbation during the later stages of both cutaneous calcinoses.

IR results show (Fig. 1 e) a marked evolution as a function of the stage of calcinoses with no significant difference between the two calcinoses. In the early stages a steady increase is observed and corresponds to a crystallization into apatite of the calcium phosphate newly deposited. The precipitation of calcium phosphate goes on till stage 20 days, and up to time of the disappearance of the mineral deposits, no modifications in the apatite crystallization are observed. On the other hand, the stabilization of the crystallinity index appears at a level comparable to that of bone tissue. Our IR analyses are in agreement with the absence of modification of the mineral substance during the long term evolution of both calcinoses.

Radiogenic free radicals are widely used as paramagnetic probes in biological calcified tissues [16–23]. In a previous work [24] we have discussed the origin of the signals appearing in the $g = 2$ region by studying the ^{13}C hyperfine coupling. Two carbon containing molecules are certainly involved in the mechanism giving rise to the observed radicals: i) the carbonate which substitutes the phosphate group in the hydroxyapatite lattice – ii) the carbon dioxide molecules adsorbed on the hydroxyapatite surface. Whatever the real description of the trapping process, it is established that it depends upon the presence of hydroxyapatite (these signals are not observed in a non apatitic calcium phosphate). In the present work only the intensity of these ESR lines is used in order to get an estimation of the amount of hydroxyapatite present in the sample. From the Fig. 1 f it clearly appears that the proportion of hydroxyapatite does not decrease in the mineral deposit with the age of the calcinoses.

Finally this ESR result is totally in accordance with those found by X-ray diffraction and infrared spectrometry, which demonstrate the absence of modification of the apatitic mineral deposit during the fragmentation process and the disappearance of subcutaneous calcinoses.

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