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The crystal growth of hydroxyapatite in presence of collagens

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Abstract — The crystal growth of hydroxyapatite (HAP) seeds in presence of the abnormal collagen secreted by the chondrocyte grown in media containing free radical sources or fulvic acid and the type II collagen (pig cartilage) damaged by · OH and fulvic acid(FA) from potable water in endemic region of Kaschin-Beck disease (KBD) was studied. The results showed that in the former case, the abnormal collagen rich in type I collagen promoted mineralization in contrast with the type II collagen. The oxidatively damaged type II collagen became less inhibitive or even promotive. In all cases, the reaction followed a second order kinetics. The powder X-diffraction analysis and SEM observation indicated that the damaged type II collagen lowers the crystalinity in the beginning and made the final crystal more agglomerated. All the results showed that the abnormal and the damaged cartilage matrix affected the bimineralization and was likely to have played an important role in the development of KBD.

Keywords : collagen; hydroxyapatite; free redical; fulvic acid.

In normal cases, the prodominant collagen from cartilage matrix is identified with type I. It is well known that the type II collagen plays an important roles in the normal function of cartilage and the ossification (bimineralizaton) process. In the previous report (Zhang, 1990) we reported the action of oxy free radicals and fulvic acid (FA) on the cultivated chondrocytes led to alteration in collagen synthesis, i.e. the formation of an abnormal cartilage matrix rich in type I collagen. Besides, the direct damage to type II collagen (pig cartilage) caused its oxidative degradation and changes in its ordered structure.

In the pathological process of KBD, following the initiation by free radical and the formation of abnormal matrix, an important event was considered to be the abnormality in the mineralization reaction and the accumulation of crystals. These effects are likely to be the cause of early ossification, low ossification degree and bone deformation in KBD. To make certain this postulation, in the present work, the kinetic studies of HAP crystal growth in presence of the abnormal and free radical damaged collagens as well as the crystaline properties were studied.

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MATERIALS AND METHODS

Materials

Collagen was isolated and purified from chondrocyte culture media containing $\cdot O_2^-$ or $\cdot OH$ generating systems as well as fulvic acid (Zhang, 1990). Type II collagen was obtained from pig cartilage as cited before (Wang, 1989). The standard type I and type II collagens were purchased from sigma. The collagen samples from pig cartilage were dissolved in 10 mmol/L HCl to prepare a 4 mg/ml collagen solution. The solution was then dialysed against the solution of 0.1 mol/L NaCl−0.05 mol/L Tris-HCl (pH 7.40) at 4 ℃ until the pH of inner solution attained 7.4. Then the solution was divided into two aliquots. To one of them $FeSO_4$/EDTA (mole ratio 1:1) was added by 10 mmol/L, and to the other FA was added by 400ppm. After keeping at 4 ℃ for 10 h, the solutions were dialysed against 0.1% acetic acid to remove Cl^- and were then lyophilized.

HAP seed crystals were prepared according to Nancollas (Nancollas, 1970). Characterization of the solid was made by IR, X-ray powder diffraction methods.

Fulvic acid was isolated from potable water in KBD endemic region by method cited previously (Peng, 1981).

All other reagents were of AR grade.

Methods

The effect of normal and abnormal collagens on the kinetics of crystalline growth of hydroxyapatite seeds were studied by pH-static method in a thermostatic cell at 37 ± 0.1 ℃, with continuous stirring in nitrogen atmosphere. The solutions supersaturated with respect to HAP were prepared by mixing Na_2HPO_4 solution with $CaCl_2$ solution and contained various samples of collagen, type I, type II, normal and abnormal.

After the temperature reached equilibrium, the crystal growth was initiated by the addition of hydroxyapatite seeds. During the experiments, the pH was maintained constant at 7.4± 0.01 by addition of KOH solution automatically. Reaction kinetics were monitored by recording the amount of base required to maintain pH at 7.4. In the course of the reaction, a fraction of the suspensions were collected at various time intervals. They were filtered through 0.22 μm millipore filters and the filtrate was analyzed for calcium using WFX-16 atomic absorption spectrophotometer (Beijing Second Optical Instrument Factory) and for phosphate spectrophotometrically. The solid phases were examined by SEM (Hitachi) and their crystalinity and composition were analyzed by X-ray powder diffraction (Dan Dong Instrument Factory).

RESULTS

The process of crystal growth of HAP in presence of abnormal collagen

The crystal growth curves of the accumulated amount of KOH needed to maintain the pH at 7.4 were drawn as a function of time (Fig.1). The initial concentration of calcium and phosphate were 8.10×10^{-3} mol/L and 6.10×10^{-3} mol/L, respectively. For every experiment, one of various samples of collagen, standard type I, and type II, or the abnormal collagen isolated from the chondrocyte growth media containing fulvic acid, XOD-HX, or Fe-EDTA was added.

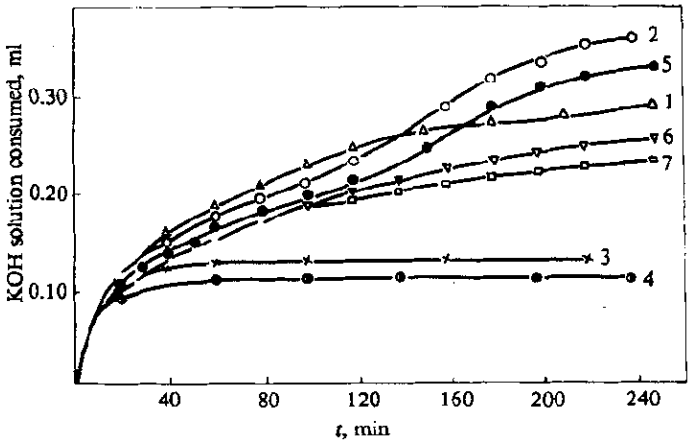

Fig.1 Crystal growth of hydroxyapatite

1. blank (no collagen added); 2. type I collagen (sigma) added;
3. type II collagen (sigma) added; 4. collagen isolated from normal growth media added;
5. collagen isolated from media containing fulvic acid;
6. collagen isolated from media containing XOD-HX;
7. collagen isolated from media containing Fe-EDTA.
calcium: 8.10×10^{-3}mol /L; phosphate: 6.10×10^{-3}mol/L;
ion strength: 0.1(KCl); pH 7.4; temperature: 37.0 ± 0.1 ℃; hydroxyapatite seeds added: 2.00 mg.

The curves in Fig.1 show that type II collagen, the main matrix component of cartilage, can significantly retard mineralization, and on the contrary, the type I collagen, the main matrix component of matured bone, promotes the mineralization. This diversity may be important in cartilage and bone formation and cartilage-bone transition. The abnormal collagens isolated from chondrocyte growth media containing oxy free radical sources promote the mineralization in different intensity, and under the influence of fulvic acid, the mineralization curves become stepped with two phases. As type II collagen, the collagen isolated from

normal growth media inhibits mineralization somewhat.

Kinetics of crystal growth and crystalinity

Considering that the crystal growth is sensible to the difference in supersaturation degree concentration of collagen and the amount of HAP seeds, the experiments were divided into two groups different in the collagen and the amount of HAP seeds: (1) 1.656×10^{-3} mol/L calcium, 8.026×10^{-4} mol/L phosphate, and collagen 15 mg, HAP seed 20 mg; (2) 3.312×10^{-3} mol/L calcium, 1.597×10^{-3} mol/L phosphate, 20 mg collagen and 10 mg HAP seeds. The Ca: P ratio in the supersaturated solutions were 2.06 and 2.10, respectively.

The alkali consumed to keep pH constant at 7.4 and the calcium and phosphate concentrations in solution were plotted against reaction time are given in Fig.2, 3 and 4.

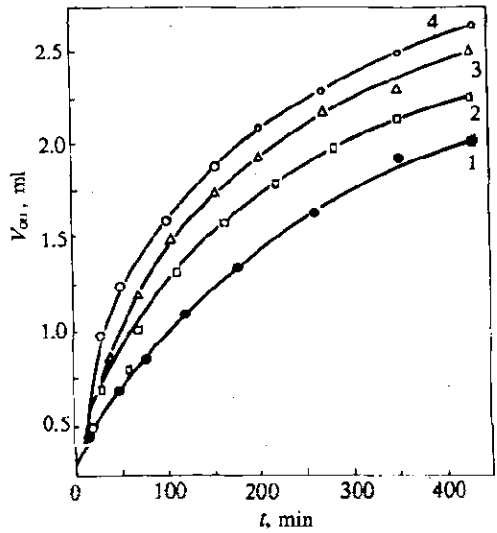

Fig.2 The amount of base required in mineralization

1. control (untreated collagen added); 2. collagen treated with FA added;
3. collagen treated with OH added;
4. blank (no collagen added); calcium: 1.656×10^{-3} mol/L; phosphate: 8.0264×10^{-4} mol/L; collagen: 15 mg; hydroxyapatite seeds added: 20mg.

During the crystal growing, crystal agglomeration may happen, thus the data were processed by Blomen method (Blomen, 1982). In this method, function of crystal growth for HAP is defined as

$$R = K \cdot s(AP_t^{1/9} - AP_\infty^{1/9})^n.$$

Where, R is the rate of crystal growth; AP_t and AP_∞, the activity product of HAP in solution at time t and ∞, respectively; K, the rate constant; s, the surface area of solid; n, reaction order.

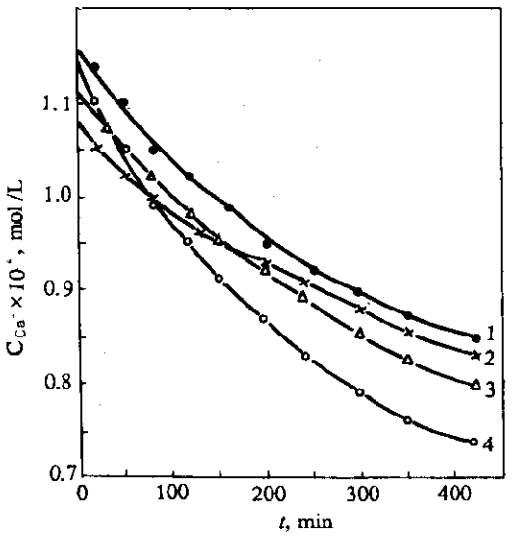

Fig.3 Concentration change of calcium in mineralization (under the same conditions as in Fig.2)

The relation may be rewritten in the following form:

$$R = AP_0^{1/9} \frac{U_\infty - U_t}{t_m - t}$$

Where U_t is defined by:

$$U_t = \frac{AP_0^{1/9} - AP_t^{1/9}}{AP_0^{1/9}}$$

U_∞ is U_t when t is ∞, t is the time required as $U_t = 1/2\ U$, AP_t and AP_∞ are obtained from experimental data. A straight line was got when plotting lg R against lg $(AP_t^{1/9} - AP_\infty^{1/9})$. From the slope and intercept of the straight line, the reaction order $-n$ and k.s. can be calculated. Following $\frac{t}{U} = \frac{t}{U} + \frac{t_m}{U}$, the U_∞ and t will be determined.

The results are tabulated as in Table 1.

Table 1 The results of kinetics

	AP_0, $\times 10^{-7}$	U_∞	t, min	k.s., $\times 10^4$	n
Blank (no collagen)	1.71	0.292	178.1	1.22	1.93
Untreated collagen	1.73	0.299	251.1	0.565	1.98
Collagen treated with OH	1.73	0.242	177.1	1.82	1.90
Collagen treated with FA	1.91	0.185	91.2	5.31	1.88

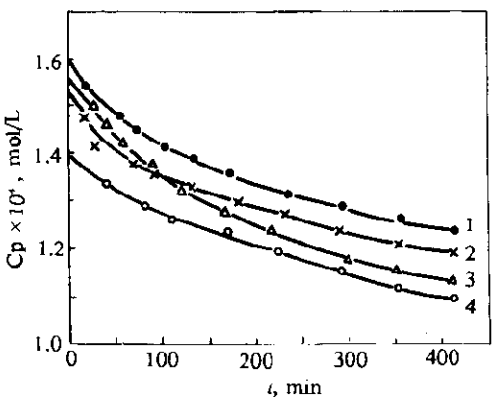

Fig.4 Concentration change of phosphate in mineralization (under the same conditions as in Fig.2)

The SEM observation of the solids indicates that in all the cases, only HAP crystal was observed, but the aggregation of the crystals are somewhat different. In initial solid phase, the HAP seeds grow significantly, but no aggregation can be observed. In the last phase, the seeds aggregated to a larger cluster. In presence of the collagen damaged by OH and FA, the aggregation is promoted but the clusters become loose.

The crystal shape changes slowly, giving better crystalinity. But in a highly supersaturated solution, the perfection of crystal shape needs a long time.

The X-ray powder diffraction analysis of solid indicates that, in all the cases, whether collagen was present or not, having grown for 3, 5, 7 h in solution, all crystals were HAP crystal with different crystalinity depending on the supersaturation of solution, the damage of collagen and the time of mineralization.

These results indicate that the abnormal matrix may induce abnormal mineralization, featured by rapid mineralization, small crystals, highly but loosely aggregation and low crystalinity. Anyhow, in all the cases, the reactions are of second order and the solid phases are consistently hydroxyapatite.

DISCUSSION

During the ossification, the collagen in cartilage matrix acts as the carrier of nucleation and growth as well as the template controlling the morphology of crystals. The results reported in this paper show that in contrast with the promotion action of type I collagen on HAP crystal growth, the type II collagen manifests inhibition action. In the presence of abnormal collagen secreted from the abnormal chondrocytes, the action of collagen turns from inhibi-

tion to promotion. This is in accordance with the alteration of collagen type from richness in type II to richness in type I under the affecting of ·OH and FA. Beside this, the ·OH and FA both cause a direct oxidation damage to type II collagen, and the damaged collagen gives rapid crystal growth and somewhat lower crystalinity. Nevertheless, the composition of the solid phases and the order of rections are not affected. Based on the results as mentioned above, we may arrive at a postulation that as the initial step the pathogenic factors generate free radical, followed by the secretion and formation of abnormal collagens rich in type I, and finally the acceleration of mineralization and the low crystalinity of crystal. These results are considered to be the main steps in KBD development.

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