Journal of Environmental Sciences (China) Vol. 3, No. 4, pp. 48 –54 , 1991 ISSN 1001-0742

The damage effects of oxy free radicals and fulvic acid on chondrocytes

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(Received July 11, 1991)

Abstract — The damage effects of oxy free radical and fulvic acid on cultivated chicken embryo chondrocytes were studied. The results show that the growrth of chondrocytes is inhibited and the morphology of the cells altered. The collagen synthesizing capability of the damaged cell changes somewhat. A noteworthy change of the type of collagen synthesized by the abnormal cells was observed by CMC-chromatography and amino acid analysis. The results indicated that the abnormal cells tend to synthesize type I instead of type II collagen, which is synthesized and secreted by the intact chondrocyte.

Keywords: free radical; fulvic acid; chondrocyte; collagen; Kaschin-Beck disease.

Kaschin-Beck disease (KBD) is featured by pathological change of limb's articular and epiphyseal plate cartilages. It is an endemic disease in North and Northwest China. The early etiological and endemiological studies have led to a number of postulations to interprete the development of KBD, but as these investigations give only the relationship between a certain factor in a certain region and the occurrence of KBD, they are not generally acceptable and still facing arguments. In recent years, several groups (Peng, 1987; Hou, 1982) postulated that the oxy free radicals may be the general pathogenic factor of KBD, and the fulvic acids in endemic region are actually the free radical sources or carriers. The supporting evidences include the ESR signals in potable water in endemic regions and in blood of children with KBD, the decreased activity of GSH-Px and tocopherol content and the increased lipid peroxidation. But all the observation say no more on the mechanism of the effect of free radical.

In our earlier works (Wang, 1989) we found that both the oxy free radical and the fulvic acid isolated from the potable water in endemic regions can cause collagen oxidative degradation. The result gives us a hint that the matrix change may be the missing link of the pathological process of KBD.

As a specialized connective tissue, the functions of cartilage depend on the structure and conformation of a vast extracellular matrix. Chondrocyte, as a functional unit of cartilage, is responsible for producing the structural macromolecules and the turnover of the extracellular matrix of the entire tissue. Because for KBD, the lesion situated at cartilage, the damage of chondrocyte as well as the extracellular matrix may play a very important role in

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the development of KBD. However, in the earlier studies on the development of KBD, little attention was paid to the abnormalities of the chondrocytes and extracellular matrix. In the following works the chicken embryo chondrocytes were cultivated for studying of the effects of oxy free radicals and fulvic acid on morphology, proliferation and the synthesis of extracellular matrix, i. e. collagen and proteoglycan. The relationship between free radicals and KBD are further discussed.

MATERIALS AND METHODS

Materials

Ham's F-10 medium was purchased from Gibco Laboratories, xanthine oxidase, Type I and Type II collagens from Sigma, trypsin from Shanghai Biochemical Center. Fulvic acid was isolated from the potable water in KBD region by means of ion exchange (Peng, 1981). All other reagents were AR grade.

Growth of chondrocytes

The chondrocytes were obtained by dissociating the limb cartilage from 12-day old white leghorn embryonic chicks. After treatment with 0.25% trypsin for 30 min at 37°C, the released chondrocytes were inoculated at a density of 40×10^4 cells/ml into plastic culture plate containing 1—10 ml of Ham's F-10 medium, 10% fetal bovine serum and streptomycin-penicillin (10 μg/ml and 10 units, respectively). The plates were incubated for 16 days at 37°C in moist 95% air and 5% CO_2. The medium was renewed every 3 days.

Growth rate determination and morphological study

Chondrocytes were randomly seeded into 60 mm plastic petridishes containing a sterile 24×40 mm glass coverslip. The chondrocyte morphology and the growth state were observed under a microscope at various time intervals of 6 to 12 h. The CSPG level in matrix was estimated by Toluidin Blue method.

Growth rates were determined by removing cells every other day with 0.02% EDTA solution for 20 min 37°C and counting the cells with a hemacytometer.

Determination of collagen content in culture media

Cell cultures were incubated with 10 ml hydroxyproline-free Ham's F—10 medium. The medium was collected and centrifugated to remove the insoluble substance every 2 days. After hydrolyzing with 6 mol/ L HCl for 18 h at 110°C, hydroxyproline content was determined by means of the Bojkind's method (Bojkind, 1973). Although the content of hydroxyproline was determined in hydrolyzing medium including the hydroxyproline residues, it did reflect the amount of collagen synthesized by chondrocytes, because of hydroxyproline residue was constant.

Isolation of collagen from cell culture

The culture media were adjusted to 25% ammonium sulfate and kept at 4°C for 24 h. The precipitated collagen was collected by centrifugation at 10000 r/ min for 30 min, then dissolved in 0.5 mol/L acetic acid containing 100 μg/ ml pepsin and incubated at 4°C for 48 h.

After removal of undissolved material by centrifugation,k the acetic acid solution was adjusted to 2 mol/ L NaCl and kept at 4℃ for 24 h in order to be reprecipitated for all types of collgen. The collagens were collected by centrifugation at 20000 r/ min for 30 min, dissolved in 0.5 mol/ L acetic acid, dialyzed against the same solution for 48 h at 4℃, and then lyophilized.

Chromatography and amino acid analysis of collagen

5 mg isolated collagen sample was dissolved in 5.0 ml of starting buffer (pH 4.8) and denatured by heating to 42℃ for 30 min. The solution was pumped into a CMC column (1.2 × 12 cm) at 42℃. The elution was carried out with a linear gradient between 0.1 and 0.2 mol/ L NaCl (30 ml /h, 3.4 ml/ volume). The effluent was monitored at 230 nm.

0.5 mg collagen sample was dissolved in 0.5 ml of 6 mol/ L HCl and hydrolyzed under the atmosphere of N_2 at 110℃ for 10 h. Then, the solution was evaporated slowly and the residue was dissolved in 0.02 mol/ L HCl and analyzed on an automatic amino acid analyzer.

RESULTS

Growth rate and morphology of chondrocyte

Examination of the cultivated chondrocytes by optical microscopy indicated that the normal chondrocytes were round and adhered to the growth surface within 24 h. On the 3rd day growing in culture, the cells grew rapidly, the body of cell obviously increased and was in fullness, most of cell aggregated to form clones. Confluency was reached in about one week with formation of toluidin blue metachromatic matrix. In cultures treated with experimental factors, cell grew slowly and less clones were observed. The cells changed to elongated and spindle in shape and some broken cells were also found. A small fraction of cells aggregated, and the matrix gave little or no toluidin blue metachroism.

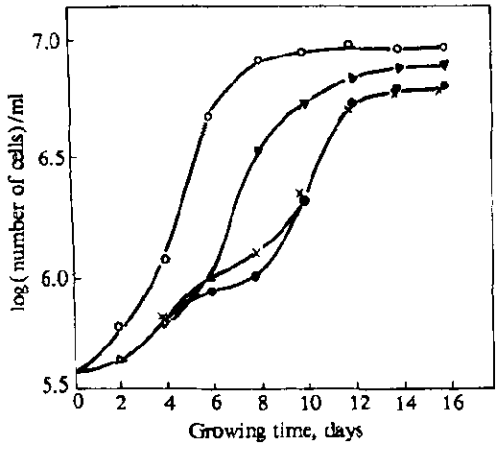

Fig. 1 Growth curves of chondrocytes

Cells grown in media: 1——normal; 2——containing 15 ppm fulvic acid; 3——containing 1.0 × 10u/ml XOD and 1.0 × 10 mol/L hypoxanthine; 4——containing 0.5 mmol/L Fe (II)-EDTA

From Fig. 1, we can found that the control cells reached confluency in about 8 days at a cell density of approximately 9×10^5 cells/ ml, but in presence of fulvic acid or free radicals, the cell reached confluency in about 10—12 days at a cell density of only $5-7\times10^5$ cells/ ml. These results indicated that free radicals and fulvic acid obviously inhibited chondrocyte growth and proliferating both. We also found that the cell growth became biphasic in all cases in contrast to the control group. In the first phase, the growth of chondrocytes was inhibited, but after then, during the second phase, the cell growth increased again following a sigmoid growth curve. In parallel with this effect, in the second phase, the morphologic changes of cell were observed clearly.

Analysis of collagen synthesis

The histochemical studies indicated that chondrocytes grown in media containing the factors under test tended to synthesize proteoglycans less than the control. The synthesis rate of collagen secreted by each group of chondrocytes was also determined. The data presented in Fig. 2 indicated that under action of free radicals or fulvic acid, the capacity of collagen synthesis was obvious higher than that of control cells.

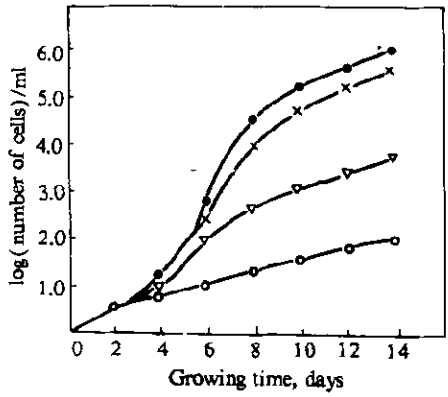

Fig. 2 Collagen synthesis by chondrocytes
The legends are the same as to Fig. 1

Type change and amino acid composition of collagens

Several types of collagens have been separated. They all consisted of three polypeptide chains. Type II collagen molecules were of triplex α-chains [$3\alpha_1$ (II)], but type I collagen contained two kinds of chains [$2\alpha_1$ (I) α_2 (I)]. In accordance with this, the CMC chromatography of type I collagen gave a pattern composed of two peaks (α_1 , α_2) (Fig. 3—1) and that of pure type II only one peak (Fig. 3—2). In normal state, chondrocytes synthesized and secreted type II collagen. In our experiments, the noteworthy results were that the chondrocytes grown in culture containing free radical sources($\cdot OH$, $\cdot O_2^-$, fulvic acid) secreted type I collagen instead of type II (Fig. 3—6, 7, 8). These effects were attributed to

the abnormal cells, because similar experiments with type II collagen incubated with free radical or fulvic acid gave the typical type II collagen chromatograph (Fig. 3－4, 5).

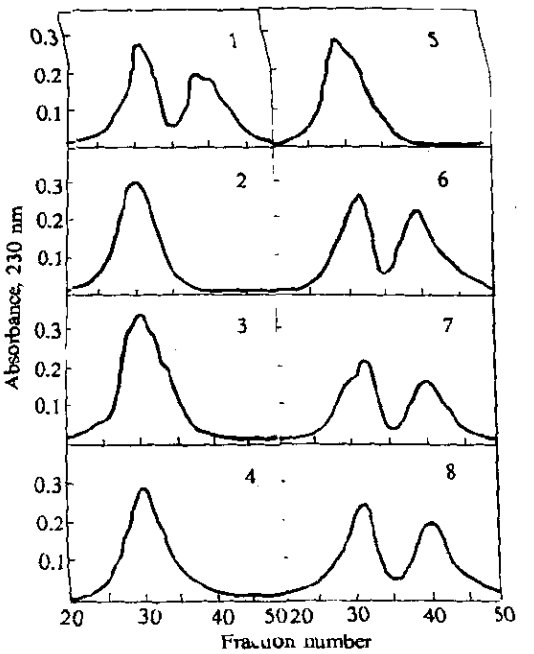

Fig. 3 CMC chromatograms of collagens

1. type I (sigma);
2. Type II (sigma);
3. isolated from normal growth media;
4. the same as 3, but treated with fulvic acid;
5. the same as 3, but treated with Fe (II)-EDTA;
6. isolated from growth media containing fulvic acid;
7. isolated from growth media containing Fe (II)-EDTA;
8. isolated from growth media containing XOD-HX

The amino acid composition of the collagen samples were determined and the results supported the type change (Table 1). The preferential synthesis of type I collagen in the case of abnormal chondrocyte led to higher contents of Lys, Ala, Ser, Val, His and lower content of Glu and Met.

Electrophoric method was also used to identify the type of collagen in our recent works and the results were consistent with the CMC chromatography.

Table 1 Amino acid composition of collagen, residues/ 1000 total residues

Amino acid	Control	• OH	XOD/ HX	FA	Type I	Type II
Asp	48.8	46.6	50.6	50.7	45.7	36.0
Thr	20.9	20.5	20.7	20.7	18.0	24.0
Ser	32.0	39.6	40.4	41.3	33.0	21.0
Glu	102	72.2	85.5	87.8	74.5	87.0
Gly	281	279	272	268	330	335
Ala	114	126	122	122	114	101
Val	20.0	26.9	28.3	27.6	24.3	18.0
Met	7.9	4.4	4.4	3.2	5.1	9.0
Ile	8.9	10.2	10.0	9.6	10.8	10.0
Leu	25.9	24.1	25.6	25.2	24.3	26.0
Tyr	4.6	6.0	6.0	5.7	2.5	3.0
Phe	12.2	13.3	11.9	12.3	12.4	12.0
Lys	15.6	27.8	28.3	27.7	24.7	19.0
His	3.8	5.4	6.0	6.1	6.0	4.0
Arg	53.0	52.5	52.0	51.5	49..7	52.0
Hypro	81.0	84.2	83.8	85.1	112	98.0
Pro	163	156	150	150	116	126

DISCUSSION

In relation to the etiolongical and endemiological studies of KBD, a ESR signal was observed in the cereals and potable water in endemic region (Yang, 1988), indicating that the development of KBD might be related to free radicals. The present results indicated that both fulvic acid from endemic regions and oxy free radicals ($\cdot OH$, $\cdot O_2$) could damage the chondrocytes and the abnormal cells could exhibit morphological changes, the inhibition of proliferation, and change in content of collagen and CSPG in matrix. All the factors gave same or similar effects on chondrocyte, with some difference only in intensity. Therefore, our results supported the hypotheses as mentioned above.

A notable change in the matrix secreted by the abnormal chondrocytes is that it becomes rich in type I collagen. The result was not only elucidated by CMC chromatography, but supported by the change in amino acid composition. The direct damage effect on matrix is possible, but it does not induce the type alteration of the collagen. This effect may be attributed to the abnormal given expression of the abnormal chondrocytes.

Collagen and proteoglycan as two major components in cartilage matrix play very

important roles in maintaining the intensity of bone and the elasticity of articular cartilage, regulating the growth of HAP crystal in cartilage and ensuring the normal osteogensis. Therefore, the change in these two main components in matrix, especialy the change from normal type II collagen to type I collagen, may induce the change in physiological function and cause the development of cartilage disease. The aging of cartilage and early ossification in KBD may be related to the changes in matrix.

REFERENCES

Bojkind, M., Analytical Biochemistry, 1973, 55: 288

Hou, S. F., Chinese J. Endem., 1982, 1(2): 84

Peng, A., Chinese J Control Endem. Disease, 1987, 2(3): 151

Peng, A. and Wang, W. H., J. Environ. Sci., 1981, 1: 126

Wang, C. X., Xu, S. J. and Wang, K., J. Beijing Med. Univ., 1989, 21(4): 307

Yang, C. L. and Peng, A., Environ. Chemi., 1988, 7: 10