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Studies on Lysozyme in Articular Cartilage

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Lysozyme is a highly basic, relatively small molecular size (mol. wt. 14,300) protein which is widely distributed in animal tissues and secretions. A high concentration of lysozyme in joint tissues was noted by ALEXANDER FLEMING as early as in 1922. Besides its classical bacteriolytic function, biological roles of lysozyme in the articular cartilage still remains undecided. This study aims to reveal the localization of this enzyme in the matrix of articular cartilage, to demonstrate transfer of exogenous lysozyme in articular cartilage and also to present the alterations of lysozyme activity in various disorders of joint cartilage, comparing with those of other lysosomal enzymes. Morphological studies were also carried out.

Materials and Methods

(1) Localization of Lysozyme in Articular Cartilage

The femoral condyles of a 22-year-old male who received an above-knee amputation were used for immunohistochemical observations. The specimens were immediately stored at -70°C after operation. The cartilage was cut into serial sections 8-10μ thick, which were fixed in 95% ethanol. After washing with 0.1 M phosphate-buffered saline (PBS) of pH 7.2, anti-human lysozyme rabbit serum (DAKOPATTS, Copenhagen) was applied to the sections for 30 minutes at room temperature. Sections applied to normal rabbit serum were used as control. After washing with PBS, sections were immersed in anti-rabbit γ-globulin swine serum labeled with fluorescein isothiocyanate (FITC) (DAKOPATTS) or horseradish peroxidase (HRPO) (DAKOPATTS) for 4 hours at room temperature. After washing with PBS, sections immersed in FITC-labeled serum were examined by fluorescent microscopy.

The peroxidase-labeled sections were made to react with GRAHAM-KARNOVSKY[7] reagent including diazoaminobenze pigment for 10 minutes to develop a color, and then examined

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under a light microscope. Next, specimens were post-fixed in 1% osmic acid, dried with ethanol, and embedded in Epon 812. The thin sections were either not stained or slightly stained with lead color before examination, and then obseraved by electron microscopy.

(2) Transfer of Exogenous Lysozyme into Articular Cartilage

^{125}I-labeled hen egg lysozyme was injected into the vein and the knee joint cavity of rabbits. Distribution of exogenous lysozyme was detected by autoradiography. ^{125}I-labeled hen egg lysozyme with a specific activity of 275-352 μCi/mg was prepared according to the modified method of GREENWOOD et al. (1963[10]). A dose of 0.4 mg per kg of body weight of ^{125}I-labeled lysozyme was injected into rabbits intravenously and intraarticularly. The knee joints were harvested 10 minutes after the injection. Cryosections 30-50μ thick were prepared for contact autoradiography. Exposure time was from 1 to 14 days.

To determine the stability of radioactive lysozyme in rabbit serum, preliminary experiments were carried out as follows. Japanese white rabbits weighing 2-2.5 kg were given by intravenous ear injection 0.4 mg of ^{125}I-labeled lysozyme per kg body weight. Blood was collected at intervals from 1.7 to 60 minutes after the injection. The lysozyme activity was measured by two methods : the trichloroacetic acid (TCA) method and immunochemical method.

For the TCA method, TCA was added to the serum to a final concentration of 5%. Then the mixture was centrifuged for 10 minutes at 3,000 rpm. The precipitate was washed twice with 0.5 ml of 5% TCA and centrifuged again for 10 minutes at 3,000 rpm. The radioactivity of precipitate was measured in a well type scintillation counter (Aloka JDC-209) and the values were expressed as the TCA precipitate ^{125}I. The recovery rate was over 99%.

The immunochemical method was based on a quantitative sedimentation reaction using the dextran-charcol method described by HERBERT et al (1965[11]). To 0.1 ml of sample serum were added 0.1 ml of anti-hen lysozyme serum (×100) and 0.1 ml of 0.05 M phosp hate buffer (pH 7.4) containing 0.2% bovine serum albumin and 0.15 M NaCl. After 2 hours of incubation at 37°C, 0.5 ml each of 10% charcol and 1% dextran were added. The mixture was centrifuged for 30 minutes at 3,000 rpm. The precipitates were washed once with the buffer of pH 7.4 and the washings were combined with the original supernatant. The radioactivity of the combined mixture was measured in a well type scintillation counter (Aloka JDC-209) and was expressed as the immunoassay supernatant ^{125}I.

Results obtained are given in Table 1. Data indicate that the ^{125}I-labeled lysozyme which was injected intravenously was stable at least for 15 minutes after the injection, while it gradually disappeared from the serum to 60% of the initial activity after 60 minutes.

(3) Estimation of Enzyme Activities in Articular Cartilage and Synovial Fluid.

Forty Japanese white rabbits weighing 2-2.5 kg were divided into 4 groups with 10 each. The first group was the non-treated group and was served as control. In the second group, the knee joint was immobilized in extension by plaster casting for 4 weeks. The

Table 1. Stability of ^{125} I-labeled hen egg white lysozyme in rabbit serum after intravenous injection.

Methods	Time (min) after intravenous injection					
	1.7	7	10	15	30	60
TCA-precipitable ^{125}I	100	100	100	98.3	85.5	56.9
Immunoassay-supernatant ^{125}I	100	100	100	99.6	86.7	60.4

TCA ; Trichloroacetic acid.
Each value is represented as % of total radioactivity.

third group animals were given 0.25 ml of croton oil into the knee joints once a week for two-weeks period and were killed 2 weeks after the first injection. Rabbits of the fourth group received 25 mg of hydrocortisone acetate by intraarticular injection once a week making a total of 4 injections. Specimens were taken 4 weeks after the first injection.

Articular cartilage of the knee and the synovial fluid were taken immediately after sacrifices. Enzyme activities were measured in duplicate. The femoral condyles were observed by histological and histochemical techniques using hematoxylin-eosin staining and safranin 0 fast green staining which is used to determine the amount of mucopolysaccharide in cartilage (ROSENBERG 1971[19]).

Lysozyme activity was assayed by the method of GREENWALD et al. (1972[9]). The cartilage was homogenized by N. K. microhomogenizer (Nippon Seiki Co., Osaka) and extracted with 1 M NaCl at 37°C for 24 hours. After centrifugation at 3,000 rpm for 15 minutes, the supernatant was aspirated and filtered. After dialysis overnight with normal saline in the cold, lysozyme activity was measured using *Micrococcus lysodeikticus* (Worthington Biochemical Co.) suspension as substrate. Cathepsin D activity was measured by the microassay method of SAPOLSKY et al. (1973[20]). β-Glucuronidase activity was measured by the p-nitrophenylglucuronide method[5], and acid and alkaline phosphatases were assayed using phenyl phosphate as substrate[21][8].

Results

(1) Localization of Lysozyme in Articular Cartilage

As control, the articular cartilage which was treated with normal rabbit serum and anti-rabbit γ-globulin swine serum labeled with FITC was non-fluorescent. However, the cartilage, to which anti-human lysozyme rabbit serum and subsequently FITC-labeled serum were applied, showed marked fluorescence around the rim of chondrocytes (Fig. 1). Sections immersed in anti-rabbit γ-globulin swine serum labeled with HRPO depicted diffuse staining with pigment, the finding being more intensive in the territorial region of the cartilage matrix (Fig. 2). Fig. 3 is an enlarged image of the same sample. Staining is marked in the territorial region.

Electron microscopic findings of HRPO treated sections revealed localization of the

Fig. 1.

Fig. 2.

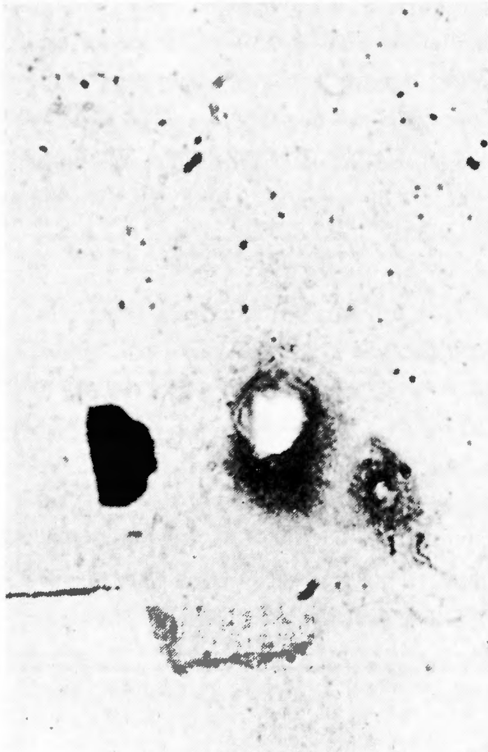


Fig. 3.

Fig. 1. Immunofluorescene examination of the articular cartilage of the knee.

Control : articular cartilage of the knee (×200).
Sample . articular cartilage of the knee given anti-human lysozyme rabbit antiserum (×200).

Fig. 2. Immunoenzyme examination of the articular cartilage of the knee.

Control : articular cartilage of the knee (×200).
Sample : articular cartilage of the knee given anti-humanlysozyme rabbit antiserum (×200).

Fig. 3. Magnification of a part of Fig. 2 (×500).

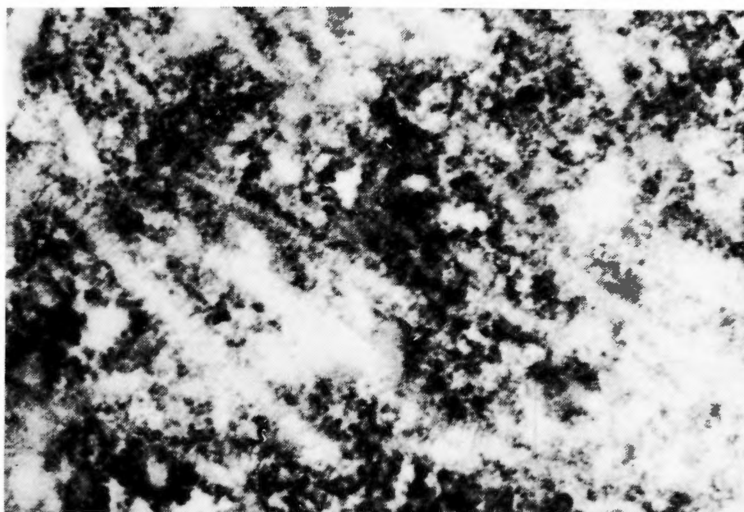
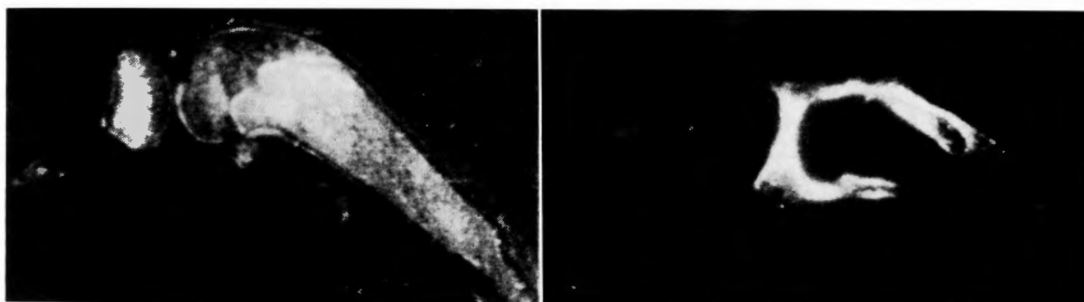


Fig. 4. Electron micrograph showing localization of the lysozyme in the articular cartilage of the knee (×45,000).



Intravenous injection (10 min. later) Intraarticular injection (10 min. later)

Fig. 5. Autoradiograms of rabbit knee joint given 0.4mg per kg body weight of ^{125}I -labeled hen egg white lysozyme.

enzyme at the chondrocyte lacunae and its close relation to collagen fibers (Fig. 4).

(2) Transfer of Lysozyme in Articular Cartilage

The radioisotope labeled lysozyme injected intravenously was dominantly incorporated by the epiphyseal cartilage and slightly by articular cartilage within 10 minutes after the injection. Autoradiograms taken after intraarticular injection with ^{125}I-labeled lysozyme revealed the uptake exclusively in the articular cartilage. Accumulation in the epiphyseal cartilage was not found even after long exposure (Fig. 5).

(3) Enzyme Activities in Articular Cartilage

Lysozyme activity in the cartilage and synovial fluid of the immobilized and croton oil groups was higher than that in the control group, while the increase was slight in the glucocorticoid group (Fig. 6). Cathepsin D activity was higher in the immobilized, croton oil and glucocorticoid groups than in the control. β-Glucuronidase, acid phosphatase and

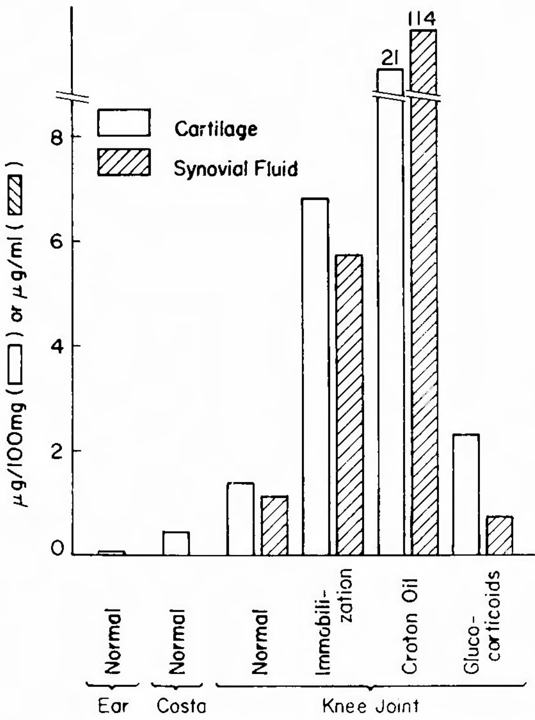


Fig 6. Extraction of lysozyme from ear and costal cartilage of normal rabbits and changes in the activity of lysozyme in synovial fluid and articular cartilage of normal and abnormal rabbit knee joints. The lysozyme content is expressed as μg per 100 mg wet weight cartilage or per ml synovial fluid.

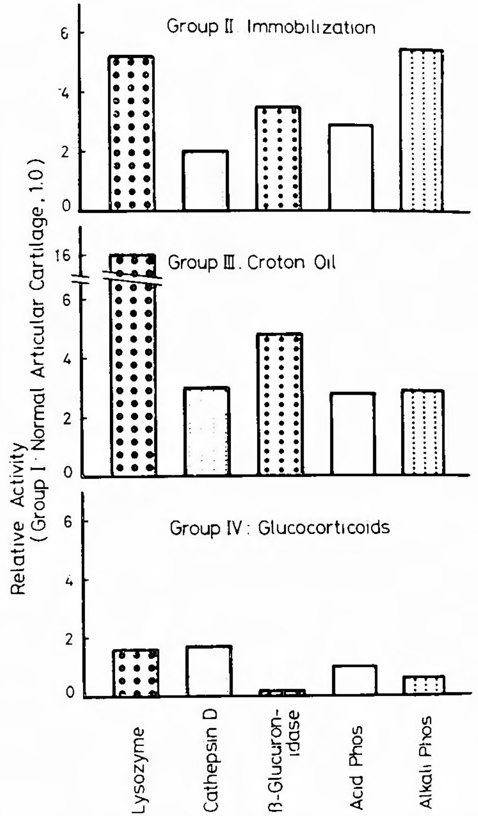


Fig. 7. Changes in the activities of various lysosomal enzymes in the articular cartilage of normal and abnormal rabbit knee joints. Relative activities in test animals are shown, taking the activity in control animal as unity.

alkaline phosphatase were present in markedly high amounts in the immobilized and croton oil groups but in normal or subnormal amounts in the glucocorticoid group (Fig. 7). Compared with the control, the immobilized group contained 5.4-fold more alkaline phosphatase and 5.2-fold more lysozyme, which were the largest increases found. The lowest increasement was the 2-fold one shown by cathepsin D. This agrees with ALI's report (1973[1]) on osteoarthritis. In the croton oil groups, lysozyme was increased markedly by 16-fold in the cartilage and 95-fold in the synovial fluid. The activities of both acid and alkaline phosphatases were three times as high as those of the control group. These facts indicate that lysozyme has an important relationship with the development of inflammation. In the glucocorticoid group, the increases were small, cathepsin D by 1.7-fold and lysozyme by 1.6-fold, while activities of β-glucuronidase, acid phosphatase and alkaline phosphatase were normal or subnormal. Thus, the effect of lysosomal enzyme should be slight in steroid-

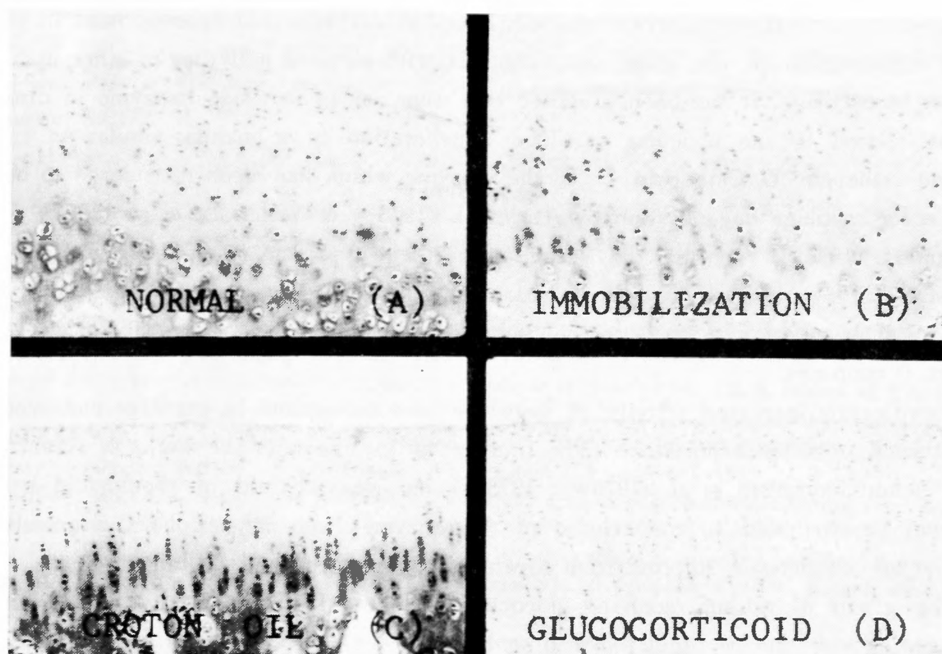


Fig. 8. A. Normal articular cartilage of rabbit knee joint (safranin O, ×100).
B. Articular cartilage after four weeks of immobilization (safranin 0, ×100).
C. Articular cartilage after two injections of croton oil (safranin 0, ×100).
D. Articular cartilage after four injections of hydrocortisone acetate (safranin 0, ×100).

induced degeneration of the articular cartilage. Diffuse safranin 0 staining was found in articular cartilage matrix of non-treated animals, the territorial regions of chondrocytes being more intensely demonstrated. However, safranin 0 stainings decreased, more or less, particularly in the interterritorial matrix of articular cartilages of each experimental groups (Fig. 8). These histological and histochemical findings apparently show that cartilage degeneration took place in any of experimental groups.

Discussion

Immunofluorescence method employed in this study clearly demonstrated intense localization of lysozyme in the territorial region of the matrix of articular cartilage. These findings are in agreement with the results of in vitro experiments by KUETTNER et al. (1971[15]) who used embryonic chick cartilage. Autoradiographic investigation revealed that exogenous lysozyme is able to freely travel in the matrix of articular cartilage. This finding confirms the result obtained by YUZURIHA et al. (1977[23]), and further provides more precise information. The localization and intensity of lysozyme distribution in human articular cartilage was exactly coincident with those of staining with safranin 0 which is believed to

indicate quantitatively the content of proteoglycans in cartilage matrix.

Elevation of lysozyme activity was disclosed in cartilage and synovial fluid in experimental degeneration of the knee joint, together with elevated activities of other hydrolytic enzymes in cartilage. It has been proposed that the rise in cartilage lysozyme in disorders may be related to the articular cartilage degeneration in a manner similar to that in cartilage cathepsin D. Cathepsin D is the enzyme which has been considered to be responsible for cartilage degeneration by ALI et al. (1973[1]) & WOESSNER et al. (1973[22]).

HOWELL (1975[13]) reported the action of cartilage lysozyme on proteoglycans depletion in osteoarthritic cartilage. The results obtained in the present stuides indicate that the elevation of lysozyme activity is associated with decrease in proteoglycans as judged by safranin 0 stainings.

Significantly increased activity of lysozyme was recognized in cartilage and synovium in croton oil injection experiment. The finding is in line with the works of KERBY et al. (1967[14]) and PRUZANSKI et al. (1970[17] ; 1973[18]) on synovial fluid of rheumatoid arthritis. This may be attributed to the release of the enzyme from polymorpho-nuclear cells and synoviocytes which were suffered from severe disruption provoked by inflammation.

The group of rabbits receiving glucocorticoid showed higher activity of lysozyme in cartilage than in synovial fluid, the finding being reverse to those obtained for the group with immobilization or with croton oil. It is well established that glucocorticoids have a stabilizing effect on lysosomal membranes[2)4)12)16]. Thus, it can not be assumed that lysosomal enzymes caused the primary lesion observed in the case of the group with glucocorticoid, as it was pointed out by BEHRENS et al. (1975[3]). They speculated that the changes found in these specimens were due to direct mechanical damage to the chondrocytes.

Conclusion

The results obtained by this study are as follows :

1) Localization of lysozyme in human articular cartilage was defined by immunohistochemical techniques. Lysozyme was intensely found in the territorial region of chondrocyte lacunae, closely related to collagen fibers.

2) Exogenous lysozyme was able to travel through the articular cartilage, as studied by ^{125}I-labeled lysozyme.

3) Lysozyme activities of articular cartilage and synovial fluid were definitely elevated in experimentally produced degeneration of joints.

The investigations reported here suggest that lysozyme per se plays an important role in metabolism of articular cartilage and its alteration is associated with that of the matrix proteoglycans.

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和文抄録

関節軟骨における Lysozyme の研究

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正常関節軟骨の lysozyme の局在及び関節軟骨の色々な病態における lysozyme 活性と他の lysosomal enzyme 活性とを比較検討した．局在を知る為青年男子の大腿骨下端の軟骨を採取し、螢光抗体法，酵素抗体法及び電顕像を作製した．日本白色家兎40羽を使用し，膝関節伸展位にて固定した群，croton oil 及び hydrocortisone acetate を各々膝関節腔内に注入した群を作製し屠殺後直ちに膝関節軟骨を採取した．採取した sample の lysozyme, cathepsin D, β-glucuronidase, acid & alkaline phosphatase の諸酵素活性を測定すると共に H-B 及び safranin O 染色を施行した．Lysozyme は関節軟骨の matrix の territorial region に濃染され電顕像では collagen fiber に密接していた．

Lysozyme 活性は色々の病態で増加し cathepsin D 活性とほぼ平行していた．Lysozyme 活性の変動と関節軟骨の変性との間には密接な関連があることが示唆された．