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Synthesis of Tissue Nonspecific Collagen by Bovine Articular Cartilage as a Result of Aging *in Vitro* (38426)

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(Introduced by Mary A. Root)

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Normal articular cartilage synthesizes exclusively 3 α1 (II) chains that are rich in hydroxylysine and glycosidically bound hexoses, whereas osteoarthritic human cartilage is capable of synthesizing in addition a more ubiquitous form of collagen composed of 2 α1 and 1 α2 chains (1). The change in the synthetic pattern of collagen seems to disturb the structural integrity of the tissue, which is mainly dependent on the specific molecular organization of collagen, proteoglycans, and glycoproteins; the ultimate result is eburnation of the tissue and loss of function.

Increased levels of lysosomal enzymes have been reported to be in association with the degenerative process and degradation of cartilage matrix with a concomitant loss of metachromasia (2–7). We have recently reported that normal bovine articular cartilage exhibits change in collagen synthesis from 3 α1 (II) to (α1)2.α2 type as a result of pretreatment with rat liver lysosomes (8). Another agent, 5-bromodeoxyuridine (BUdR), is known to cause changes in the normal phenotypic expression of growing chondrocytes and their cell surface properties (9, 10). The altered chondrocytes show suppression of the synthesis of chondroitin sulfate and commence to synthesize fibroblast-type collagen (11). From our studies on the changes induced by lysosomal enzymes it seems apparent that the factors not directly involved in the replication process of the cells can also alter their phenotypic expression, causing them to synthesize nonspecific collagen molecules.

The present paper deals with the studies on the effects of various exogenous factors upon the synthesis of collagen by bovine articular cartilage, and the capability of the endogenous factors to produce similar effects after prolonged incubation of the tissue in culture media.

Materials and Methods. Incubation of cartilage with ^{3}H-proline. Fresh bovine articular cartilage (1 g) was cleaned, chopped into thin pieces, and incubated in 10 ml of Hank's balanced salt solution (GIBCO), containing 50 μCi of 2,3 ^{3}H-L-proline (specific activity 29.8 Ci/mmole), penicillin (50 units/ml), streptomycin (50 μg/ml), ascorbic acid (50 μg/ml), and β-aminopropionitrile (100 μg/ml) at 37° for 8 hr with gentle shaking. For *in vitro* aging studies, the incubation was carried out for longer time periods ranging between 8 and 72 hr.

Preincubation with various agents. Lysosomal preparations were made from the livers of two adult rats using the method of Ragab *et al.* (12). The final pellet obtained by centrifugation at 15,000*g* for 15 min was suspended in 5 ml saline. The mixture was frozen and thawed repeatedly before use.

Batches of cleaned and chopped cartilage (1 g each) were incubated with (a) 0.5 ml of rat liver lysosomes, (b) 50 units of β-glucosidase (Worthington), (c) 50 units of β-galactosidase (Worthington), (d) 50 units of β-glucuronidase (Worthington), and (e) 10 units of chondroitinase ABC (Seikagaku Fine Chemicals) in 0.1 *M* Na acetate buffer, pH 5, at 37° for 2–3 hr. Control cartilage was incubated with acetate buffer under identical conditions. At the end of this time period, the cartilage pieces were washed repeatedly with saline, pH 7, and incubated in Hank's solution containing ^{3}H-proline for 8 hr as described above.

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Incubation with vitamin A was done in two different ways. (a) Vitamin A (alcohol) was dissolved in a small amount of ethanol and was diluted to 10 ml with 0.1 *M* Na acetate buffer, pH 5. Cartilage was preincubated with vitamin A (100 IU) for 2–3 hr at 37° and then in Hank's solution with ^{3}H-proline for 8 hr, or (b) it was incubated directly in Hank's solution with ^{3}H-proline and vitamin A for various time periods.

In each case, after incubation the media was removed, cartilage washed with saline, homogenized in 0.45 *M* NaCl, pH 7, and kept shaking at 4° overnight. The mixture was centrifuged and the residual cartilage was treated with papain (13). The enzyme was inactivated by iodoacetic acid, the cartilage was washed thoroughly with saline and reextracted with 0.45 *M* NaCl, pH 7, at 4° overnight. Details of the extraction procedure are given in an earlier communication (8). The extracts were dialyzed against 0.05 *M* tris buffer, pH 7, and separated into collagen and proteoglycans on DEAE cellulose column by elution with the same buffer containing 0.2 *M* NaCl and then raising the salt concentration to 1 *M*. The radioactive collagen fraction was pooled, mixed with 2–3 mg of rat skin acid-soluble collagen, and dialyzed against 0.06 *M* Na acetate buffer, pH 4.8. The mixture was separated into the subunits on CM cellulose column (0.8 × 5 cm), equilibrated with the same buffer, at 40° using a linear gradient between 0 and 0.1 *M* NaCl. The eluate was monitored at 230 nm, and the distribution of radioactivity under each peak was measured by counting the fractions.

Estimation of enzyme activities in normal and osteoarthritic human articular cartilage. Osteoarthritic cartilage was obtained from patients undergoing surgical resection of femoral heads during replacement with prosthetic devices. Normal cartilage was obtained from patients subjected to corrective surgery with no indication of osteoarthritis.

β-Glucuronidase and acid phosphatase activities were measured using *p*-nitrophenyl-β-D-glucuronide (Calbiochem) and *p*-nitrophenyl phosphate (Calbiochem) as the substrates, respectively. The assay was carried out as described by Thompson and Clark (14). Cathepsin D activity was measured by the method of Ali *et al.* (15) using denatured hemoglobin as the substrate.

Results and Discussion. Figure 1 represents the chromatographic elution pattern of ^{3}H-proline labeled normal cartilage collagen mixed with rat skin acid-soluble collagen on small CM cellulose column. Normal cartilage collagen, which is composed of 3 α1 (II) chains, elutes as a single peak in the same area where α1 chain of rat skin collagen appears, while that synthesized after the treatment with rat liver lysosomes or the other agents elutes as α1 and α2 chains (Fig. 2).

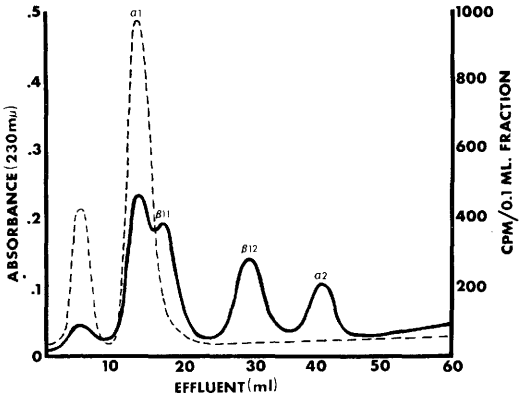

FIG. 1. Chromatographic elution pattern of ^{3}H-proline-labeled normal bovine cartilage collagen mixed with rat skin acid-soluble collagen on CM cellulose column. Elution was carried out at 40° with 0.06 *M* Na acetate buffer, pH 4.8, with a linear gradient between 0 and 0.1 *M* NaCl.

The change in the synthetic pattern of collagen due to the action of various agents is given in Table I. The extent of synthesis of α2 chains can be considered as a direct measure of fibroblastic (α1)2.α2-type collagen. It is evident that the individual enzymes such as β-galactosidase, β-glucuronidase, and chondroitinase ABC are able to bring about an effect similar to that of rat liver lysosomal enzymes, although the effect of individual enzymes is less pronounced. β-Glucosidase failed to exhibit these changes. It is important to note that the results obtained with the NaCl extracts prior to and after the treatment of cartilage with papain during the extraction procedure were essentially similar. As would be expected, the extracts prior to the treatment with papain were much more radioactive.

Studies on various enzymes in fresh frozen osteoarthritic and normal human cartilage showed that acid phosphatase and β-glucuronidase activities in osteoarthritic tissue were 2.8 and 3.3 times higher than normal, while that of cathepsin D was four times as high. Increase in the lysosomal enzyme activity in the synovial fluid and tissue involved in the de-

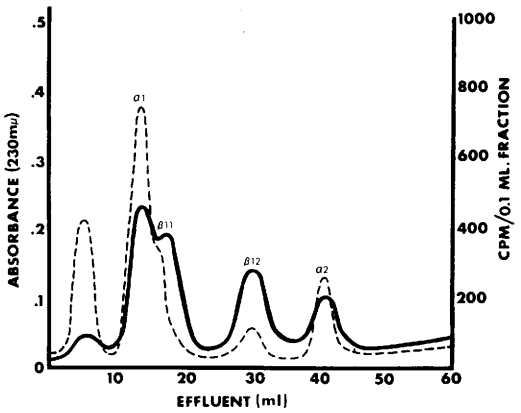

FIG. 2. Elution pattern of radioactive collagen from bovine cartilage, pretreated with rat liver lysosomes or other agents, mixed with rat skin acid-soluble collagen.

generative diseases has been reported (14, 16). Sapolsky *et al.* (4) observed a two- to threefold increase in a cathepsin D-type enzyme activity in early osteoarthritic and discolored human articular cartilage over that of normal.

Until recently, it was believed that lysosomal enzymes act only on the proteoglycan matrix and cause loss of metachromasia. Our findings suggest that these enzymes as a group, or some individually, alter the cell behavior either by modifying the environment around the chondrocyte or by reacting directly with the cell surface components. The phenotypic expression encompassed by synthesis of $(\alpha 1)2.\alpha 2$-type collagen may result from dedifferentiation of chondrocytes to a more primitive fibroblastic form. Holtzer and Abbott (10) have described various experiments to show that the chondroblasts replicating in the presence of BUdR rapidly transform into motile cells that are morphologically indistinguishable from authentic fibroblasts. The marked changes in morphology were associated with the inhibition of chondroitin sulfate synthesis. Schiltz *et al.* (11) reported dedifferentiation of chondrocytes, grown in the media containing BUdR or embryo extract, with the synthesis of fibroblast-type collagen. Thus the expression of chondrogenic phenotype *in vitro* seems to be susceptible to various environmental factors. Mammalian cartilage is avascular in structure and therefore should exclusively contain chondrocytes, although some microheterogeneity cannot be completely ruled out. The observations described above suggest a definite change in the function and morphology of chondrocytes, and our results indicate that similar changes occur in the explants grown in the media.

TABLE I. Effects of Various Agents on the Biosynthetic Pattern of Collagen.

Treatment[a]	% Synthesis of $\alpha 2$ chains	$\alpha 1$:$\alpha 2$ Ratio
None	0	∞
Rat Liver Lysosomes	22	3.5
β-glucosidase	0	∞
β-galactosidase	18	4.6
β-glucuronidase	16	5.3
Chondroitinase ABC	15	6.6
Vitamin A	2	49.0

[a] The batches of cartilage were pretreated with these various agents at pH 5 and 37° for 2–3 hr and then incubated with balanced salt solution containing ^{3}H-proline for 8 hr at 37°.

Vitamin A did not induce the formation of $\alpha 2$ chains by preincubation of cartilage for 2–3 hr. Nevertheless, it had a remarkable effect with prolonged incubation periods (Table II). Vitamin A is among those agents which promote the release of lysosomal enzymes *in vitro* or *in vivo*. The major effect of vitamin A in organ culture seems to be the breakdown of cartilage matrix (17). This effect could be reversed partially with a protease inhibitor or a lysosomal membrane stabilizer (18). Vitamin A treatment of cultured chick chondroblasts at a level of 3 μg/ml for 24 hr causes inhibition of mucopolysaccharide synthesis and transformation of cells into fibroblasts (19).

Prolonged incubation of bovine articular cartilage without addition of any exogenous factors seems to alter the behavior of chondrocytes, which commence to synthesize $(\alpha 1)2.\alpha 2$-type collagen (Table II). Although the effect could be observed in 16 hr, a remarkable number of cells seems to have changed function after 48 hr. Lyman *et al.* (20) have reported the synthesis of $\alpha 2$ chains of collagen in cell culture of rabbit articular cartilage without addition of any exogenous factors. The same tissue, however, failed to do so in organ culture. In the present experiments, bovine cartilage explants grown in the media exhibit an *in vitro* aging phenomenon.

Lysosomal activity has been reported to increase with age in the tissue (21). The experiments of Lucy *et al.* (22) to measure an increased release of proteolytic enzymes as an effect of vitamin A showed that control cultures also released the activity, thereby raising the possibility

TABLE II. Synthesis of $\alpha 2$ Chains by Articular Cartilage After Aging *in Vitro* (Expressed as $\alpha 1$:$\alpha 2$ Ratio).

	Incubation time (hr)				
Type	8	16	24	48	72
Untreated control	∞	48.0	10.0	5.2	4.8
Vitamin A-treated	13.0	6.2	4.5	4.0	4.0

of pathological as well as physiological role of lysosomal enzymes. It therefore seems likely that in the cartilage explants release of endogenous lysosomal enzymes could bring about the same changes in chondrocyte behavior as those seen under the effect of various exogenous factors described above, although the process of dedifferentiation is slow due to the lower levels of lysosomal enzymes in the normal cartilage.

Summary. Bovine articular cartilage synthesizes collagen containing three identical chains, $\alpha 1$(II). Treatment with rat liver lysosomes or certain individual enzymes changes this synthetic pattern to a fibroblast-type ($\alpha 1$)2.$\alpha 2$ collagen. Similar alteration can also be observed after prolonged incubation of cartilage *in vitro* without addition of any exogenous factors or in the presence of vitamin A, which is known to be a lysosomal labilizer. It is possible that the endogenous lysosomal activity is released by aging *in vitro*, leading to the dedifferentiation of chondrocytes.

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