Reversible inhibition of chondrogenic expression by certain hyaluronidase preparations

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SUMMARY

Embryonic chick chondrocytes were cultured in the presence or absence of different preparations of testicular hyaluronidase. This treatment inhibited the accumulation of cartilage matrix as indicated by phase-contrast microscopy, by Alcian green staining, and by accumulation of 35S-labeled material. In addition, most preparations of testicular hyaluronidase caused a conversion of the cells to a fibroblastic phenotype characterized by a faster growth rate and the synthesis of type-I collagen. This effect was found to be concentration-dependent and was not observed at the minimum concentration of hyaluronidase required to inhibit matrix accumulation. Since two more highly purified hyaluronidase preparations prevented matrix accumulation but did not cause the fibroblastic transformation, it is likely that the conversion to a fibroblastic phenotype is caused by a contaminant in the other hyaluronidase preparations.

INTRODUCTION

The extracellular matrix of cartilage is composed of three major macromolecular species: proteoglycan, hyaluronic acid, and a unique type of collagen (Miller & Matukas, 1969; Trelstad, Kang, Igarashi & Gross, 1970; Hascall & Heinegard, 1974). The proteoglycans of the matrix contain chondroitin-4-sulfate and chondroitin-6-sulfate with lesser amounts of keratin sulfate linked to a common core-protein (Hascall & Heinegard, 1975; Rosenberg et al. 1975; Hascall, Oegema, Brown & Caplan (1976). The organization of the cartilage matrix involves all of these components and together they provide cartilage with its unique structural resilience.

More recent studies have suggested that matrix constituents may play other active roles during chondrogenesis. For example, Kosher, Lash & Minor (1973) have demonstrated that, in culture, somite chondrogenesis is greatly enhanced by the addition of proteoglycans to the medium. Furthermore, there have been several reports that proteoglycan synthesis by cultured chondrocytes is stimu-
lated by exogenous proteoglycans (Nevo & Dorfman, 1972; Huang, 1974; Schwartz & Dorfman, 1975). Hyaluronic acid has been found to inhibit proteoglycan synthesis when added to chondrocytes in suspension culture (Solursh, Vaerewyck & Reiter, 1974; Wiebkin, Hardingham & Muir, 1975). Both the inhibition by hyaluronic acid and the stimulation by proteoglycans are abolished when the cells are pre-treated with trypsin, suggesting that these interactions involve trypsin-labile binding sites on the cell surface.

In the experiments to be reported here, the relationship between chondrogenic expression and the proteoglycan-rich matrix is further explored. Testicular hyaluronidase, an enzyme which degrades chondroitin-6-sulfate, chondroitin-4-sulfate, and hyaluronic acid, was added to the medium of cultured chondrocytes. The resulting effects on chondrogenic expression were characterized. A preliminary report of this study has been published (Pennypacker & Goetinck, 1976b; Goetinck & Pennypacker, 1977).

**METHODS AND MATERIALS**

**Cell culture.** Sterna of 14-day White Leghorn chick embryos were dissected under sterile conditions, stripped of their perichondrium and incubated at 37 °C with gentle stirring in 0.1% pronase, 0.2% collagenase and 10% chicken serum in Tyrode's solution. After incubation for 45 min, the enzyme solution was removed after centrifugation, and single cells were obtained by passing the suspension through two layers of 20 μm mesh Nitex monofilament screen-cloth. In all experiments, the chondrocytes were plated at a density of 250 cells per plate in 60 mm Falcon plastic culture dishes. The cultures were maintained at 37 °C in an atmosphere of 5% CO₂:95% air, and the medium was replaced on the 4th, 7th, 9th and 11th days in culture with 50% Ham's F-10 (Grand Island Biological Co., GIBCO) with 2× the vitamins and 4× the amino acid concentration; 38% Hanks' balanced salt solution (HBSS); 10% fetal calf serum (GIBCO); 0.5% (w/v) bovine serum albumin; 50 μg/ml ascorbic acid (Fisher); and antibiotics (Cahn, Coon & Cahn, 1967). Cultures were usually terminated after 13 days of incubation and, in some cases, were stained with Alcian green and metanil yellow (Ham & Sattler, 1968).

**Proteoglycan analysis.** The total amount of cell-associated sulfated proteoglycan was determined by adding 1 μCi of Na³⁵SO₄ (New England Nuclear: NEN) per ml to the culture medium on days 7, 9, and 11. Day 7 was chosen as the time for the initial exposure as it precedes the period of cartilage matrix deposition. The isotope incorporated into the cell-associated proteoglycans was determined by measuring hyaluronidase-sensitive radioactivity. For this purpose, the ³⁵S-labeled 13-day cultures were cooled to 4 °C, the medium was aspirated off, and the cultures were washed twice with cold HBSS. The cell material was then removed from the culture dish into a known volume of cold HBSS using a Teflon scraper. The samples were placed in a boiling water bath for 5 min to
destroy any enzymic activity and homogenized by sonication. From this sample an aliquot was taken for DNA determination (Burton, 1956). Two additional aliquots were taken. Testicular hyaluronidase (Calbiochem) was added to one aliquot at a final concentration of 1 mg/ml in 0.1 M sodium acetate, 0.1 M-NaCl, pH 5.0, and buffer alone was added to the other. After incubation for 24 h at 37 °C, pronase (Calbiochem) was added to each sample to a final concentration of 1 mg/ml in 0.2 M Tris-HCl, pH 8.2. The samples were incubated 16 h at 55 °C, and then the radioactive glycosaminoglycans were precipitated by addition of three volumes of 80 % ethanol containing 5 % potassium acetate. The precipitates were collected by filtration, dissolved in water, and the radioactivity was measured by liquid scintillation spectrometry. The hyaluronidase-sensitive CPM, representing chondroitin-6-sulfate and chondroitin-4-sulfate, were obtained by subtraction of the CPM after the hyaluronidase treatment from the total CPM.

**Collagen analysis.** The relative concentration of collagen in the cultures was estimated by measuring hydroxyproline content according to the procedure of Woessner (1961). In addition, the cultures were incubated with 15 µCi/ml of 2,3-[3H]proline (specific activity 33.8 Ci/m-mole; NEN) or 2-[3H]glycine (specific activity 11.4 Ci/m-mole; NEN) to label newly synthesized protein. When [3H]proline was used, the culture medium was Dulbecco-modified Eagle’s medium supplemented with 10 % fetal calf serum (GIBCO), 50 µg/ml β-aminopropionitrile (Aldrich Chemical Co.) and 100 µg/ml ascorbic acid (Fisher Chemical). With [3H]glycine, Eagle’s minimal essential medium was supplemented with 10 % fetal calf serum (GIBCO), 50 µg/ml β-aminopropionitrile and 100 µg/ml ascorbic acid. In both cases, cultures were labeled on day-11 for 48 h. Extraction, purification and carboxymethylcellulose (CMC; CM 32, Reeve Angel) chromatography of the labeled collagen was carried out on a 1.6 x 7.0 cm jacketed column as described by Linsenmeyer, Trelstad, Toole & Gross (1973). Carrier collagen was prepared from lathyritic chicks as described by Kang, Nagai, Piez & Gross (1966).

**Liquid scintillation spectrometry.** All measurements of radioactive samples were made by liquid scintillation counting with a Nuclear Chicago (Searle Analytic) Mark I scintillation spectrometer. The counting solution used was prepared by mixing two volumes of toluene containing 4.0 g/l of 2,5-diphenyl-oxazole and 0.1 g/l of 1,4-di[2-phenyloxazoly]l benzene with one volume of Triton X-100.

**RESULTS**

The effect of testicular hyaluronidase preparations on chondrogenic expression was determined by replacing the normal medium with medium plus enzyme on days 7, 9, and 11 of culture. Different results were obtained, depending upon the source of the enzyme employed. In all cases, the presence of added hyaluronidase prevented the accumulation of a cartilage matrix as judged both by phase
contrast microscopy and staining with Alcian green. Most preparations of testicular hyaluronidase also altered the morphology of the cells. Under these conditions, the chondrocytes became fibroblast-like (Fig. 1a, b) and formed dense clones which were two to three times the diameter of the control clones. However, two preparations of enzyme (Sigma, bovine type IV and VI) did not affect the chondrocyte morphology (Fig. 1c), although the matrix was eliminated. The hyaluronidase preparations tested in this study did not cause detachment of the chondrocytes from the substratum. A list of the enzyme preparations tested is shown in Table 1.

The enzyme preparations that caused no morphological changes were also the most purified hyaluronidases suggesting that the morphological changes were due to a contaminant in the preparations. This possibility was considered by testing lower concentrations of a preparation (Calbiochem, Grade B) to determine if the morphological effects could be eliminated while still preventing matrix deposition. Matrix accumulation was measured by Na$_3^{35}$SO$_4$ incorporation during the period of active proteoglycan synthesis (days 7-13) as well as by Alcian green staining. Between 0.0125-0.5 µg hyaluronidase per ml of medium there is a gradual reduction in accumulated sulfated proteoglycans, and at concentrations of 0.5 µg/ml and higher, all cell-associated sulfated proteoglycan is eliminated (Fig. 2). Similar results were obtained by staining with Alcian green and metanil yellow. In both cases, at the minimum enzyme concentration required for inhibition of matrix accumulation (0.5 µg/ml), there was no effect on the chondrocyte morphology. Concentrations greater than 0.5 µg were required to bring about the morphological transition to fibroblast-like cells.

The amount as well as type of collagen synthesized by the cells was determined at three concentrations of hyaluronidase. Table 2 shows that at 0.025 µg/ml and 0.5 µg/ml, there is no difference in hydroxyproline accumulation per µg DNA. However, in cultures treated with a concentration (125 µg/ml) sufficient to bring about morphological changes, the hydroxyproline content is reduced to less than half the amounts accumulated in control cultures. The type of collagen synthesized in hyaluronidase-treated cultures was characterized by chromatographing radioactively-labeled collagen on CMC. Collagen extracted from control cultures (Fig. 3), or from cultures treated with 0.5 µg of hyaluronidase per ml or less (not shown) co-eluted from the CMC with carrier skin α1(II). The absence of a radioactive α2 peak suggests that all of the collagen is [α1(II)]$_3$ (Miller & Matukas, 1969). Labeled collagen from cultures treated with 125 µg hyaluronidase/ml, however, co-eluted with both α1 and α2 carrier collagen peaks in a ratio of approximately 4:1 (Fig. 4). The presence of α2 indicates that the cells were synthesizing type I collagen.

Concentrations of 'hyaluronidase' that caused the fibroblastic transformation also increased the amount of DNA per plate, while lower concentrations did not change the DNA content (Table 2). Between 5-125 µg hyaluronidase/ml,
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Fig. 1. Phase contrast micrographs (× 197) of day-13 control cultures (A), and day-13 cultures treated with 100 μg/ml of Calbiochem, Grade B testicular hyaluronidase (B) or Sigma, type IV testicular hyaluronidase (C).

the DNA content per dish on day 13 increased in a linear relationship with increasing enzyme concentration (not shown). As expected from this observation the growth rate of cultures exposed to hyaluronidase (125 μg/ml) was greater than control cultures (Fig. 5).

The transforming activity in the hyaluronidase preparations caused chondrocytes to assume a fibroblastic appearance within 24 h. The ability of the cells
Table 1. *Hyaluronidase preparations tested*

<table>
<thead>
<tr>
<th>Source</th>
<th>Activity</th>
<th>Test concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calbiochem, Grade B</td>
<td>300 i.u.* (u./mg)</td>
<td>100 μg/ml</td>
</tr>
<tr>
<td>Lot 200943</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sigma, type I</td>
<td>300 n.f.† (u./mg)</td>
<td>100 μg/ml</td>
</tr>
<tr>
<td>Lot 116C-0259</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sigma, type III</td>
<td>610 n.f. (u./mg)</td>
<td>100 μg/ml (61 u./ml)</td>
</tr>
<tr>
<td>Lot 446-0890</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sigma, type IV</td>
<td>1090 n.f. (u./ml)</td>
<td>66 μg/ml (66 u./ml)</td>
</tr>
<tr>
<td>Lot 76V-0342</td>
<td></td>
<td>100 μg/ml (100 u./ml)</td>
</tr>
<tr>
<td>Sigma, type VI</td>
<td>57 n.f. (u./mg)</td>
<td>50 units/ml</td>
</tr>
<tr>
<td>Lot 16C-0176</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* i.u., international units.
† n.f., national formulary units.

Fig. 2. Effect of testicular hyaluronidase (Calbiochem, Grade B) on the accumulation of cell-associated sulfated proteoglycans. Each point is based upon the mean of at least four determinations.

to recover from the enzyme treatment was determined by exposing 7-day cultures to hyaluronidase (125 μg/ml) for 24, 48, 72, or 96 h, and then maintaining them in medium without hyaluronidase. After 24 or 48 h, the cells regained a polygonal morphology and stained with Alcian green at day 13. Beyond the 48 h exposure, the clones of cells were larger in diameter, and there was an increase in those that did not recover from the treatment. After 72 h, only a few clones recovered, even when the recovery period was extended. The effects of the 96 h exposure were irreversible. However, when cells from cultures exposed to hyaluronidase for at least 96 h were subcultured at low density, they regained the chondrocyte morphology and deposited an Alcian green staining...
Fig. 3. Elution profile of collagen extracted from control cultures and chromatographed on carboxymethylcellulose. The chondrocytes were labeled with [3H]-proline during the final 48 h in culture.

Table 2. Effect of hyaluronidase on collagen accumulation

<table>
<thead>
<tr>
<th>No. of cultures</th>
<th>Hypro/plate (µg)</th>
<th>DNA/plate (µg)</th>
<th>Hypro/DNA (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.025 µg hyaluronidase/ml medium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>2.729 ± 0.746</td>
<td>0.239 ± 0.057</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>9</td>
<td>2.765 ± 0.739</td>
<td>0.192 ± 0.042</td>
</tr>
<tr>
<td>0.5 µg hyaluronidase/ml medium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>4.610 ± 0.886</td>
<td>0.134 ± 0.22</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>6</td>
<td>4.414 ± 0.735</td>
<td>0.134 ± 0.035</td>
</tr>
<tr>
<td>125.0 µg hyaluronidase/ml medium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>2.316 ± 0.682</td>
<td>0.261 ± 0.071</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>10</td>
<td>8.724 ± 0.953*</td>
<td>0.124 ± 0.014*</td>
</tr>
</tbody>
</table>

Values represent mean ± s.d. * Significant difference at P < 0.001.

matrix. Furthermore, the collagen synthesized was type II, as judged by CMC chromatography (not shown).

Previous studies on various cell lines have suggested that protease treatment can stimulate cell division (Burger, 1970; Noonan & Burger, 1973). Proteases also cause the release of matrix glycosaminoglycans from cartilage rudiments (Bosmann, 1968). One ‘hyaluronidase’ preparation (Calbiochem Grade B) was tested for its ability to degrade protein and proteoglycans under normal culture conditions. For this purpose purified 35SO4-labeled proteoglycans or [3H]-tryptophan-labeled embryo protein (Peterkofsky & Diegelmann, 1971) were solubilized in culture medium and maintained in the presence or absence of testicular hyaluronidase in a CO2 incubator for 24 h. Over 90% of the 35SO4-labeled proteoglycans were degraded by the hyaluronidase treatment while there was no effect on the [3H]-tryptophan-labeled protein (Table 3). This
Fig. 4. Elution profile of collagen extracted from cultures treated with 125 µg hyaluronidase (Calbiochem, Grade B) per ml and chromatographed on carboxymethylcellulose. The chondrocytes were labeled with [³H]proline during the final 48 h in culture.

Fig. 5. Growth curves of control (□—□) and 125 µg/ml hyaluronidase (Calbiochem, Grade B)-treated cultures (■—■). In this experiment, hyaluronidase treatment was initiated on day 2 of culture. Each point represents the cell number per plate based upon the cell count of three cultures.
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Table 3. Enzymic activity in hyaluronidase preparations

<table>
<thead>
<tr>
<th></th>
<th>CPM/sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]Tryptophan embryo protein*</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1549 ± 136</td>
</tr>
<tr>
<td>Hyaluronidase (125 μg/ml)</td>
<td>1528 ± 33</td>
</tr>
<tr>
<td>35S-SO4 Proteoglycan†</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>36507</td>
</tr>
<tr>
<td>Hyaluronidase (125 μg/ml)</td>
<td>3673</td>
</tr>
</tbody>
</table>

* CPM/sample precipitated with 10 % trichloracetic acid.
† CPM/sample precipitated with 80 % ethanol containing 5 % potassium acetate.

indicates that hyaluronidase is active under normal culture conditions and suggests that the transforming activity may not be a protease. The latter conclusion is supported by the observation that trypsin (0·01 % or 0·001 %) or pronase (0·001 %) alone or in combination with a hyaluronidase preparation lacking the transforming activity (Sigma, type IV) do not cause the morphological changes. Higher concentrations of either protease cause the chondrocytes to detach. However, this does not rule out the possibility that a protease with a different specificity is involved.

DISCUSSION

The stability of the cartilage phenotype in culture is important because it provides a model system for developmental studies. The cell morphology, growth in culture, and extracellular matrix constituents have been well characterized and provide phenotypic markers. A variety of treatments, such as embryo extract, 5-bromo-2'-deoxyuridine, and repeated subculture cause the transition to a fibroblast-like morphology (Coon, 1966; Mayne, Vail & Miller, 1965; Mayne, Vail, Mayne & Miller, 1976; Mayne, Vail & Miller, 1976). In general this transition to a fibroblastic morphology is accompanied by decreased proteoglycan synthesis and the accumulation of [α1(I)]α2 and [α1(I)]3 collagen.

In this study, higher concentrations of certain hyaluronidase preparations inhibited chondrogenic expression. The cells became bi-polar, and in many areas of the colonies, were organized in parallel arrays. Accompanying these morphological changes, there was an increase in the growth rate, and the density to which the cells grew. Analysis of the collagen by CMC chromatography indicated a 4:1 ratio of radioactivity in the α1 and α2 peaks. Although the collagen was not further characterized in this study, a similar ratio was observed after embryo extract and 5-bromo-2'-deoxyuridine treatment, suggesting the accumulation of type I trimer (Mayne et al. 1975, 1976).

The fibroblastic transition was found to be reversible after subculture at low density. While the effect of embryo extract is also reversible after subculture
5-bromo-2'-deoxyuridine is not readily reversible (Mayne, Abbott & Holtzer, 1973). Furthermore, the morphology, organization and growth characteristics of embryo extract and 'hyaluronidase-' (125 μg/ml; Calbiochem, B grade) treated cultures are similar (unpublished observations). However, 5-bromo-2'-deoxyuridine-treated chondrocytes are more flattened and have a lower confluent density (Daniel, Kosher, Lash & Hertz, 1973). Therefore, similarities exist between the embryo extract and 'hyaluronidase' effects and their mechanism of action may be similar.

Two hyaluronidase preparations did not cause the fibroblastic transition. This finding indicates that the effect is due to a contaminant. Several studies have suggested that the growth of fibroblasts can be stimulated by mild treatment with proteases (Burger, 1970; Noonan & Burger, 1973). However, neither trypsin nor pronase alone or in combination with inactive hyaluronidase preparations caused morphological changes. Furthermore, testicular hyaluronidase (Calbiochem, B grade) had no effect on [3H]tryptophan-labeled embryo protein. This observation suggests that if the contaminant that caused the transformation is a protease, it is highly specific.

It is also of interest that at the minimum concentration of hyaluronidase required to prevent matrix accumulation, the chondrocytes retained a normal morphology, and synthesized type II collagen. This would suggest that chondrocytes can maintain their normal phenotype in the absence of cell-associated proteoglycans. This conclusion is supported by studies on chick embryos homozygous for the autosomal recessive gene, nanomelia (Laundauer, 1965). In this mutant, the proteoglycan content is 10% or less of normal amounts (Mathews, 1967). In other respects, the cartilage of the mutant is indistinguishable from normal cartilage (Pennypacker & Goetinck, 1976a). Other studies have also demonstrated that normal chondrocytes can respond to such a change in their microenvironment by enhanced synthesis of proteoglycans (Thomas, 1956; Sheldon, Robinson & Afzelius, 1960; Bosmann, 1968; Jackson, 1970). Studies are in progress to determine what further changes resulting from treatment with crude hyaluronidase preparations initiate altered cellular function by chondrocytes.

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REFERENCES


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