Interaction of Cartilage Proteoglycans with Hyaluronic Acid

THE ROLE OF THE HYALURONIC ACID CARBOXYL GROUPS

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(Received 31 May 1977)

Hyaluronic acid-derived oligomers of five to fifteen repeat disaccharides effectively bind to bovine nasal-cartilage proteoglycan and inhibit the interaction between proteoglycans and high-molecular-weight hyaluronic acid. If, however, the hyaluronic acid oligosaccharides are modified by reaction with diazomethane to form the carboxyl methyl esters of the glucuronic acid residues, their inhibitory activity is abolished. The binding capacity can be fully restored by saponification. The amide derivative, which is formed by condensation of the oligosaccharide carboxyl groups with glycine methyl ester, is also ineffective in blocking the proteoglycan–hyaluronic acid interaction. In this case, binding activity is not restored when the amidated oligomers are subjected to saponification to yield the free carboxylate groups on the glycine residues. Thus the displacement of the carboxylate groups on the polysaccharide chain by the interposition of a glycine residue blocks the interaction between the proteoglycans and the hyaluronic acid oligomers. When the oligosaccharide methyl ester is reduced with NaBH_4 , the resultant glucose-containing oligomers exhibit decreased binding to proteoglycans. Thus it appears that the hyaluronic acid carboxylate anion in a specific spatial orientation is required for hyaluronic acid–proteoglycan interaction.

The extracellular proteoglycans of hyaline cartilage exist largely in aggregated form (Anderson & Sajdera, 1971). These aggregates can be extracted under dissociating conditions from connective tissue with concentrated solutions of electrolytes or urea (Sajdera & Hascall, 1969). Proteoglycan reaggregation takes place spontaneously *in vitro* on removal of the dissociative solute (Sajdera & Hascall, 1969; Hascall & Sajdera, 1969). Aggregated or monomeric proteoglycan respectively can be isolated and purified by density-gradient centrifugation under either associative or dissociative conditions (Hascall & Sajdera, 1969; Heinegård, 1972). Monomer obtained from bovine nasal-septum cartilage consists of a protein core with chains of chondroitin sulphate and keratan sulphate covalently attached (Hascall & Riolo, 1972; Hopwood & Robinson, 1974).

Many of the molecular aspects of the aggregation process have been elucidated (Gregory *et al.*, 1970; Hardingham & Muir, 1972, 1973; Gregory, 1973; Hascall & Heinegård, 1974). Hardingham & Muir (1972) demonstrated that cartilage proteoglycans interact specifically with hyaluronic acid. A large number of proteoglycan molecules can bind, each through a single binding site, to a single hyaluronic acid chain. The hyaluronic acid-binding site is located on the protein core of the proteoglycan (Heinegård & Hascall, 1974). A small link protein(s) is required to stabilize the aggregates (Heinegård &

Hascall, 1974; Gregory, 1973). Such a link protein has been isolated and characterized (Oegema *et al.*, 1977). Evidence indicates that the proteoglycan–hyaluronic acid–link-protein complex may exist *in vivo* (Franek & Dunstone, 1967; Sajdera & Hascall, 1969; Gregory *et al.*, 1970; Pita & Müller, 1973).

The interaction between proteoglycan and hyaluronic acid is specific. Interaction between proteoglycan and polyanions such as dextran sulphate, sodium alginate, DNA and chondroitin sulphate does not occur (Hardingham & Muir, 1972); further, chondroitin, in which galactosamine replaces the glucosamine found in hyaluronic acid, does not bind to bovine nasal-cartilage proteoglycan (Hascall & Heinegård, 1974). The binding site of the proteoglycan exhibits specificity for hyaluronic acid chain length, since it has been shown that the deca-saccharide derived from hyaluronic acid by the action of testicular hyaluronidase is much more effective than the octasaccharide in displacing the intact hyaluronic acid chain from the proteoglycan (Hardingham & Muir, 1973; Hascall & Heinegård, 1974).

The present report describes the results of experiments in which the carboxyl groups of hyaluronic acid oligosaccharides are modified in order to determine additional details of the interaction between the proteoglycan monomer from bovine nasal-septum cartilage and hyaluronic acid.

Experimental

Materials

Umbilical-cord hyaluronic acid (grade I) was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Rooster comb hyaluronic acid (mol.wt. approx. 1.5×10^6) was kindly provided by Dr. Endre Balazs, Boston Biomedical Research Institute, Boston, MA, U.S.A. Purified hyaluronidase (EC 3.2.1.35; 4000 turbidity-reducing units per mg) was purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A., *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was from Aldrich Chemical Co., Milwaukee, WI, U.S.A., and 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide was from Pierce Chemical Co., Rockford, IL, U.S.A. Proteoglycans were isolated from bovine nasal-septum cartilage by a procedure described by Oegema *et al.* (1975). Hyaluronic acid oligosaccharides were prepared by partially digesting umbilical-cord hyaluronic acid with testicular hyaluronidase and isolated by gel chromatography on a standardized column of Sephadex G-50 (superfine grade) by the method of Hascall & Heinegård (1974). Mixed oligosaccharides ranging in size from five up to fifteen repeat disaccharide units, as well as dodecasaccharides and decasaccharides, were isolated, characterized and used in subsequent studies.

Analytical procedures

Uronic acid was determined by an automated carbazole method (Heinegård, 1973). Glycine was determined by the procedure of Spackman *et al.* (1958), except that TiCl_2 was used as the reducing agent in the ninhydrin reagent. Hydrolysis was carried out at 110°C in 6M-HCl for 24 h in evacuated tubes. The concentrations of hexosamines were determined with an automated amino acid analyser after hydrolysis at 100°C in 4M-HCl for 16 h as described by Hascall *et al.* (1972). The methyl ester content of esterified hyaluronic acid oligosaccharides was measured by the method of Wood & Siddiqui (1971). Glucose in reduced oligosaccharides was determined with glucose oxidase (Keleti & Lederer, 1974) after hydrolysis at 100°C in 0.5M- H_2SO_4 for 4 h.

Chemical modification of the hyaluronic acid oligosaccharides

The modifications, which were done on hyaluronic acid decasaccharides or mixed oligosaccharides, are outlined in Scheme 1.

(1) *Preparation of the methyl ester.* Methyl esters of the glucuronic acid residues were prepared by the method of Aspinall (1965). A 25 mg portion of

decasaccharides or mixed oligosaccharides was converted into the free acid form by passage over a 1 ml column of Dowex 50W (X8; H^+ form) and was then freeze-dried. The oligosaccharide(s) was moistened with anhydrous methanol and treated with diazomethane in diethyl ether at 4°C for 15 min. The diazomethane was prepared from 147 mg (1 mmol) of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described by Fales *et al.* (1973). Excess diazomethane was destroyed by the addition of $5\mu\text{l}$ of acetic acid and, after removal of the ether with a stream of N_2 gas, the product was dissolved in water and freeze-dried.

The methyl ester was saponified by maintaining an aqueous solution of the oligosaccharide(s) at pH 10 by the addition of 0.05M-NaOH. At appropriate intervals, samples of the solution containing $600\mu\text{g}$ of oligosaccharide were adjusted to pH 5 with 0.25M-acetic acid/0.28M-pyridine buffer and freeze-dried. The extent of esterification was estimated from the methyl ester/uronic acid ratio.

(2) *Reduction of the glucuronic acid residue to glucose.* Glucuronic acid residues of mixed oligosaccharides were converted into glucose by reduction of the methyl ester derivative with NaBH_4 (Aspinall, 1965). A 25 mg portion of the ester was dissolved in 2.5 ml of water and then treated with an equal volume of an aqueous solution of NaBH_4 at room temperature (25°C) for 1 h. The extent of reduction was controlled by varying the NaBH_4 concentration from 5 mM to 500 mM. NaOH was added to a final concentration of 0.1M and the solution was left at room temperature for 1 h to hydrolyse any remaining ester. Excess NaBH_4 was destroyed by the addition of acetic acid. The reduced product was desalted by gel chromatography on a column (1.5 cm $\times$ 100 cm) of Sephadex G-50; elution was with 0.25M-acetic acid/0.28M-pyridine. Effluent fractions containing the product were combined and freeze-dried.

(3) *Preparation of the glycine methyl ester derivative.* The carboxyl groups of the glucuronic acid residues were converted into amides by reaction of the mixed oligosaccharides with glycine methyl ester in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide as described by Danishefsky & Siskovic (1971). The reaction mixture was applied to a column (1.5 cm $\times$ 100 cm) of Sephadex G-50. Elution was with 0.25M-acetic acid/0.28M-pyridine. The fractions containing the uronic acid were combined and freeze-dried. The material was treated three times in this manner to obtain 70% conversion into the amide. The extent to which the glucuronic acid residues were amidated was reflected in a decreased reactivity in the carbazole reaction; the extent of carboxyl substitution was additionally estimated from the glycine/glucosamine ratio. Conversion of the glycine methyl ester derivative of the oligosaccharide into the glycine derivative was

Scheme 1. Modifications of the glucuronosyl residues of hyaluronic acid oligosaccharides

achieved by saponification in 0.1M-NaOH at 25°C for 1 h.

Viscosity measurements

The binding of hyaluronic acid to the proteoglycan and its displacement by hyaluronic acid oligosaccharides were measured by a viscometric method (Hascall & Heinegård, 1974). The mixtures examined contained proteoglycan in amount equivalent to 750 μg of uronic acid, hyaluronic acid in amount equivalent to 12 μg of uronic acid, various amounts of hyaluronic acid oligosaccharides and 0.5 mmol of guanidinium chloride in a total volume of 1.1 ml of 0.05M-sodium acetate, pH 5.8. After the solutions

had remained at room temperature for 3 h, the viscosities were measured at $20.4 \pm 0.1^\circ\text{C}$ in a Cannon 100 K520 Ubbelohde viscometer, which had a solvent flow time of 68.5 s. Specific viscosities were calculated by the relationship:

$$\eta_{\text{sp.}} = (t - t_0)/t_0$$

where t_0 is the outflow time of a solution of proteoglycan alone (approx. 105 s) and t is the flow time of proteoglycan/hyaluronic acid mixtures (approx. 200 s) or proteoglycan/hyaluronic acid/oligosaccharide mixtures. The coefficient of variation of the viscosity measurements was about 1.3% for replicate determinations on one sample and 1.1% for replicate samples.

Results and Discussion

Inhibition of proteoglycan-hyaluronic acid interaction as a function of hyaluronic acid oligosaccharide size

The interaction of cartilage proteoglycans with hyaluronic acid produces a large increase in hydrodynamic volume which can be detected by viscometry or gel-permeation chromatography. Using these techniques to monitor aggregate formation, Hardingham & Muir (1973) and Hascall & Heinegård (1974) demonstrated that oligosaccharides produced by digestion of hyaluronic acid with testicular hyaluronidase competitively displace the proteoglycan molecules from the large hyaluronic acid molecules and that the decasaccharide is the smallest fragment that can compete effectively for the hyaluronic acid-binding site. In the present studies, viscosity measurements were used to evaluate the effect of carboxyl-group modification on the ability of hyaluronic acid oligosaccharides to reverse the hyaluronic acid-proteoglycan interaction.

Ideally the modification reactions should be done on the decasaccharide, since it is the minimum-sized oligomer that still interacts strongly with the proteoglycan. However, the recovery of decasaccharides from enzymic digests of hyaluronic acid is quite low (5–10%), and to obtain sufficient material for subsequent modifications and analyses, inhibitory oligo-

saccharides of a range of chain lengths were used for the preparation of some of the derivatives in the present study. As shown in Fig. 1, a wide range of oligomer sizes gave a constant amount of inhibition when added in equivalent amounts to the proteoglycan/hyaluronic acid mixture. Standardization of the column with oligosaccharides of known size showed that the collected material included oligomers from 10 to 30 sugar residues in length. The latter value was obtained by extrapolation of the plot of K_{av} versus $\log(\text{mol.wt.})$ (Determann & Michel, 1966).

The concentration-dependence of the inhibition by hyaluronic acid dodecasaccharide, decasaccharide and mixed oligosaccharides was compared. On a weight-concentration basis, there is little difference among the three preparations (Fig. 2). Similar results, in experiments in which the dodecasaccharide was compared with the decasaccharide, had been obtained previously by Hardingham & Muir (1973). Thus it appears that, on a weight basis, the mixed oligosaccharides inhibit similarly to the decasaccharides and can be used in modification studies.

Inhibition of proteoglycan-hyaluronic acid interaction by oligosaccharide methyl esters

Completely esterified hyaluronic acid oligosaccharides did not block the proteoglycan-hyaluronic acid interaction (Fig. 3). Saponification of up to 40% of the methyl esters from the carboxyl groups of the glucuronic acid residues resulted in only slight

Fig. 1. *Inhibitory activity of various hyaluronic acid oligosaccharide fractions*

Hyaluronic acid (25mg) in 2.5ml of 0.1M-sodium acetate/0.15M-NaCl, pH 5.0, was digested with 800 turbidity-reducing units of testicular hyaluronidase at 37°C for 4h. The digest was fractionated on a column (1.5cm×90cm) of Sephadex G-50 (superfine grade) eluted with 0.25M-acetic acid/0.28M-pyridine, pH 5.0. The uronic acid (----) content of each fraction (3.5ml) was determined. Each fraction was freeze-dried and redissolved in water to give a uronic acid concentration of 172μg/ml, of which 50μl (8.6μg of uronic acid) was used to determine the reduction in viscosity (●—●) as described in the text. The shaded area of the peak was combined and used for modification studies. V_0 and V_t mark the void volume and the total volume of the column respectively.

Fig. 2. *Decrease in the viscosity of hyaluronic acid/proteoglycan solutions as a function of hyaluronic acid oligosaccharide concentration*

The ability of oligosaccharides at the indicated concentrations to inhibit the interaction between proteoglycan and hyaluronic acid was assayed viscometrically as described in the text. The oligosaccharides used were decasaccharide (●), dodecasaccharide (▲) and mixed oligosaccharides (○).

Fig. 3. Decrease in the viscosity of hyaluronic acid-proteoglycan solutions as a function of the extent of saponification of oligosaccharide methyl ester

The effectiveness of the methyl ester derivatives of the decasaccharide (●) and the mixed oligosaccharides (○), saponified to the indicated extents, to inhibit the interaction between proteoglycan and hyaluronic acid were assayed viscometrically as described in the text. The amount of oligosaccharide used in each case was equivalent to $12\text{ }\mu\text{g}$ ($0.068\text{ }\mu\text{mol}$) of uronic acid. The lines show the results obtained by calculation, assuming that either four specific carboxyl groups (—) or all five carboxyl groups (----) per decasaccharide unit are required for interaction with the proteoglycan, as described in the text.

increase in the inhibition. With further saponification, the inhibition increased linearly with increase in free carboxyl-group concentration. The binding capacity was nearly completely restored after complete saponification, indicating that the esterification reaction does not alter the oligosaccharides in ways other than masking of the carboxyl groups. The observation that 40% of the ester groups had to be removed before inhibition was again detected suggests that more than one free carboxyl group is required for specific binding to occur. That is, if the only requirement for binding is the presence of one free carboxyl group per decasaccharide unit with the other carboxyl groups being esterified or free, the inhibition curve would be similar to the concentration curve in Fig. 2. As shown in Fig. 3, the experimental results are most consistent with the results expected if it is assumed that the only species of oligosaccharide that binds has five free carboxyl groups per decasaccharide unit. For comparison, the results expected when the binding requires four specific free carboxyl groups out of a sequence of five are also shown. All other possibilities have higher probabilities and are therefore even less consistent with the experimental results. The probability calculations were done with the assumption that the reactivities of all the ester

Fig. 4. Decrease in the viscosity of hyaluronic acid-proteoglycan solutions as a function of the unchanged carboxyl-group content of hyaluronic acid oligosaccharides

Mixed hyaluronic acid oligosaccharides, treated to give the indicated extent of conversion of the glucuronosyl residue into the glycine methyl ester derivative (□) or into glucose (△), were tested for inhibitory activity by the viscometric method. The amount of glycine methyl ester derivative added in each experiment was equivalent to $0.068\text{ }\mu\text{mol}$ of uronic acid; for the carboxyl-reduced oligosaccharide, the sum of the glucose plus uronic acid content was $0.068\text{ }\mu\text{mol}$. The line shows the results obtained by calculation, assuming that five free carboxyl groups per decasaccharide unit are required for inhibition, as described in the text.

groups are similar. However, the results could be explained by hydrolysis rates which are dependent on the position of the ester in the oligosaccharide chain. For instance, the ester groups on the glucuronic acid residue of the non-reducing and/or the penultimate position at the reducing end of the decasaccharide might differ in reactivity from those on the interior residues. This interpretation is made less tenable by the similar results obtained with mixed oligosaccharides, where the fraction of end residues is not as great.

Inhibitory activity of glycine methyl ester derivatives

Mixed oligosaccharides substituted to various degrees (20–70%) with glycine methyl ester were also tested for inhibitory activity. The results (Fig. 4) are similar to those obtained with the methyl ester derivative. Binding activity of the oligosaccharides was essentially eliminated when 60% of the carboxyl groups were amidated. The glycine derivatives of the oligosaccharides were prepared by saponification of the glycine methyl ester derivative (Scheme 1). On extensive saponification of the most highly substituted oligosaccharide preparation, the methyl ester/glycine ratio was decreased from 1.0 to 0.07; concomitantly the inhibition increased from 1.6% to

3.2%. The results can be interpreted to mean that precise positioning of the anionic charges on the hyaluronic acid chain is required for hyaluronic acid-proteoglycan interaction.

Inhibition of proteoglycan-hyaluronic acid interaction by carboxyl-reduced hyaluronic acid oligosaccharides

The loss of inhibitory capacity of the hyaluronic acid oligomers after esterification or amidation of the carboxyl groups is consistent with steric interference and/or loss of ionic charge. Reduction of the carboxyl group to the alcohol will eliminate the ionic charge, but will not result in the formation of a bulky methyl ester or glycine methyl ester group which might sterically interfere with the binding. Reduction of a portion of the glucuronic acid residues to glucose residues diminished the inhibition in a manner consistent with the results obtained with the substituted oligomers (Fig. 4). These results suggest that, although steric interference may play a part in the loss of binding after carboxyl modification, the loss of ionic charge is more important. When the reduction procedure was done on non-esterified oligosaccharide, no glucose was formed and no decrease in inhibition was noted. Therefore the reduction procedure abolishes binding by formation of glucose and not by some other modification.

Hardingham & Muir (1972) have shown that proteoglycan-hyaluronic acid complexes are dissociated at pH 3, which suggests the involvement of an ionized carboxyl group in the binding. However, the pH studies did not differentiate between carboxyl groups on the proteoglycan and the hyaluronic acid. The results of the present study suggest that the carboxyl groups on hyaluronic acid are required for interaction with proteoglycan. Also it appears that five consecutive unmodified carboxyl groups are required for the binding and that, as shown by the results with the glycine derivatives, the precise positioning of the ionic charge is important. The elimination in binding after modification of the carboxyl group may be interpreted to mean that for binding to occur, the anionic charge is required for interaction with specific groups, such as cationic amino acid side chains, in the binding site of the proteoglycan molecule. Alternatively, the ionized carboxyl group may be required for the polysaccharide to assume the proper conformation. In support of the first interpretation, Hardingham *et al.* (1976) demonstrated that selective modification of the arginine and lysine residues of the proteoglycan abolishes aggregation with hyaluronic acid.

Thus it appears that the minimal requirements for the strong binding of hyaluronate to proteoglycan are a sequence of five disaccharide units (Hardingham & Muir, 1973; Hascall & Heinegård, 1974) containing at least one *N*-acetylglucosamine residue, since

chondroitin does not interact (Hascall & Heinegård, 1974), and, as suggested by the present work, five unmodified carboxylate groups.

This work was supported by the National Institutes of Health (grant no. DE-02731). We are grateful to Dr. V. C. Hascall, Dr. G. W. Jourdan and Dr. C. F. Nawrot for helpful discussion.

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