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Proteoglycans from Adult Human Gingival Epithelium

By Ole W. WIEBKIN, P. Mark BARTOLD and John C. THONARD
Department of Oral Biology, University of Adelaide, Adelaide, South Australia 5001, *Australia*

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Proteoglycans extracted from human gingival epithelium appear to contain a proportion of molecules that will interact with hyaluronic acid to form macromolecular aggregates. In contrast, proteoglycans from underlying connective tissue behaved differently. The interactions of hyaluronic acid with proteoglycans from either epithelium or cartilage may be similar, but not necessarily identical.

Biochemical analyses of digests of suspension cultures of gingival epithelium have revealed uronic acid-containing macromolecules, some of which are sulphated to varying degrees (Wiebkin & Thonard, 1968, 1969; Wiebkin, 1970). Quantitatively, the proteoglycans of epithelium appear to be the major species of macromolecules, in the intercellular substance (Schultz-Haudt, 1958). Since this intercellular substance plays an integral part in tissue integrity (Thonard & Scherp, 1962; Page & Schroeder, 1976), further studies have been carried out to characterize some of the properties of these molecules. The present results show that epithelium dissected from human gingivae excised at operation and incubated in nutrient media without serum can synthesize proteoglycans, some of which will interact with hyaluronic acid. In so doing, they become large enough to be excluded by gel-exclusion chromatography on Sepharose 2B-CL.

Experimental

Materials

Powdered ingredients of Hanks' balanced salt solution, Leibovitz L-15 nutrient medium and gentamycin were obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. Isotopically labelled compounds were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Sepharose 2B and Blue Dextran 2000 were from Pharmacia (South Seas) Pty. Ltd., North Ryde, New South Wales, Australia. Hyaluronic acid from umbilical cord was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Scintillation fluid (toluene/2-methoxyethanol, 3:2, v/v) contained (per litre) 80g of naphthalene and 4g of 2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophen. Trasylol was purchased from Bayer Pharmaceuticals, Haywards Heath, Sussex, U.K., and the leech hyaluronidase from Biotrics Inc., Arlington, MA, U.S.A.

Methods

Small pieces of human gingival tissue, 2–3mm thick, were obtained from gingivectomy specimens. The gingivae had been subjected to pre-surgical preparation and were clinically free from inflammation. The tissue was dissected under a microscope (×10) into small pieces containing predominantly either epithelium or connective tissue. The pieces from these two tissue types were initially washed in Hanks' balanced salt solution and incubated at 37°C in Leibovitz L-15 nutrient medium without serum supplements but with 50μg of gentamycin/ml for 2–3h. They were then transferred to nutrient medium containing both 740kBq (20μCi) of $Na_2{}^{35}SO_4$/ml and 370kBq (10μCi) of [^{3}H]acetate/ml, and incubated further. After 24h, the tissue pieces were separated from the incubation media by low-speed centrifugation (150*g*, 7min) and washed once. The tissue pieces were extracted in 4M-guanidinium chloride in 0.5M-sodium acetate (pH5.8) by gently stirring for 48h at 4°C. A mixture of proteinase inhibitors was added to this extraction step, i.e. 1mg of soya-bean trypsin inhibitor and 0.1ml of Trasylol (1000 Kallikrein Inactivation Units) per litre of a solution of 0.01M-EDTA/0.1M-6-aminohexanoic acid/0.005M-benzamidine hydrochloride. The macromolecular material thus extracted from gingival epithelium and separated on Sepharose 2B-CL (30cm×0.9cm) in 0.5M-sodium acetate contained uronic acid, was sulphated and could be precipitated by 1% cetylpyridinium chloride in 0.05M-NaCl. The guanidinium chloride extracts were then dialysed against two changes of 19vol. of distilled water at 4°C. Where slight precipitation occurred, supernatants were separated by centrifugation at approx. 3000*g* for 10min. The supernatants were concentrated by vacuum dialysis and adjusted to 0.5M-sodium acetate (pH6.8), or were freeze-dried. Solutions of these extracts in sodium acetate (0.2ml) (containing up to 4500d.p.m. of [^{35}S]sulphate) were applied to the tops of columns (30cm×0.9cm) of Sepharose 2B-CL. The columns were eluted with 0.5M-sodium acetate at flow rates of about 4ml/h. Equal volume fractions (0.12ml) were collected. Samples (either 10μl or 40μl) of each fraction were taken for the determination of radioactivities. Efficiency of counting was obtained by the channels-ratio method. Yields of

recovery throughout chromatography are enumerated in Figs. 1 and 2.

Columns were characterized by elutions of standard hyaluronic acid, Dextran Blue 2000 and [^{35}S]sulphate and by standard preparations of proteoglycan extracted from laryngeal cartilage and purified by density-gradient centrifugation under associative and dissociative conditions (Wiebkin *et al.*, 1975).

The material represented by fractions at the void volume was pooled and concentrated by vacuum dialysis and adjusted to 4M-guanidinium chloride. This was then applied to a column (30cm×0.9cm) of Sepharose 2B-CL and eluted with 4M-guanidinium chloride in 0.5M-sodium acetate (pH5.8) at a flow rate of about 4ml/h. Since much of the [^{35}S]sulphate and a proportion of the [^{3}H]acetate radioactivity were quenched by 4M-guanidinium chloride, the determination of radioactivity profiles relied on ^{3}H values. Fractions representing partially retarded material were pooled, concentrated and re-adjusted to 0.5M-sodium acetate. This was then applied to a column equilibrated with sodium acetate. The retarded material was collected, concentrated to about 0.25ml and mixed with 10μl of hyaluronic acid (2μg/ml). The mixture was further fractionated on the same Sepharose 2B-CL column with sodium acetate. The radioactivities of the fractions eluted were measured and the resultant profiles compared with those produced from the previous separation, i.e. without hyaluronic acid.

In a further series of experiments, material extracted with 4M-guanidinium chloride from four prelabelled human gingival-epithelial specimens was pooled. By the procedure described above, the extract was dialysed against distilled water, concentrated and re-adjusted to 0.5M-sodium acetate. This material was fractionated sequentially on Sepharose 2B-CL in 0.5M-sodium acetate, and in 4M-guanidinium hydrochloride as described previously. The partially retarded material was concentrated to 1ml, and divided into two batches of 0.25ml and one of 0.5ml. One of the smaller samples was mixed with 10ml of hyaluronic acid (2μg/ml) and fractionated as described above. The largest sample (0.5ml) was applied to a Sepharose 2B-CL column (30cm×0.9cm) equilibrated and eluted with 0.5M-sodium acetate. The material representing the retarded fraction was vacuum dialysed, then dialysed against 0.5M-sodium acetate and again applied to the same column. This procedure was repeated twice.

The remaining 0.25ml sample was treated with leech hyaluronidase; 0.25ml of a 0.7μg/ml solution of the enzyme was added to the sample, followed by three further equal additions at 30min intervals to replenish the enzymic activity. The total mixture was then applied to a Sepharose 2B-CL column (30cm×0.9cm) and eluted with 0.5M-sodium acetate. Fractions (0.2ml) were collected and 10μl of each was tested for radioactivity. The retarded material was pooled, dialysed, concentrated to 0.25ml and finally chromatographed on the Sepharose 2B-CL column with 0.5M-sodium acetate. The activity of the leech hyaluronidase was established by demonstrating an abolition of interaction between hyaluronic acid and cartilage proteoglycans.

Results and Discussion

Intercellular substances in gingival epithelium

The intercellular substances of gingival epithelium consist predominantly of proteoglycans and glycoproteins (Sisca *et al.*, 1971; Thonard & Wiebkin,

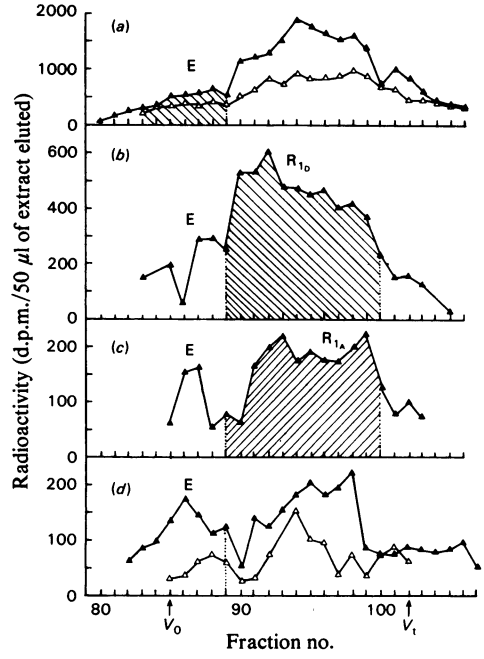

Fig. 1. *Gel chromatography of material extracted from pieces of gingival epithelium incubated for 24h in nutrient medium containing [^{3}H]acetate and [^{35}S]sulphate*

Macromolecular material extracted with 4M-guanidinium chloride was applied to a column of Sepharose 2B-CL and eluted with 0.5M-sodium acetate at pH6.8 (*a*). The excluded peak (E) was then fractionated under dissociative conditions in 4M-guanidinium chloride at pH5.8 (*b*). The partially retarded material (R_{1D}) of Fig. 1(*b*) was further separated under associative conditions in 0.5M-sodium acetate (pH6.8) (*c*) and its retarded material (R_{1A}) was mixed with hyaluronic acid and finally fractionated under associative conditions (*d*). △, [^{35}S]Sulphate; ▲, [^{3}H]acetate. Column size was 30cm×0.9cm, and fraction size 0.12ml. Abbreviation: V_t, total volume.

1973), there being little or no collagen. In the pathogenesis of periodontal disease there is a loss of connective-tissue integrity ultimately followed by the disappearance of the intercellular substances of the crevicular epithelium (Page & Schroeder, 1976). The presence of oral bacterial or tissue enzymes acting on the substrate in the intercellular location has been suggested as a possible factor in the aetiology of periodontal disease (Thonard & Scherp, 1962). Studies on suspension and monolayer cultures of either gingival or amniotic epithelium indicated that cell aggregation correlated positively with the degree of sulphation of intercellular proteoglycan and that the hyaluronic acid synthesis was limited in cultures where cell/cell contact was minimal (Wiebkin & Thonard, 1969). Radioactively labelled macromolecular material extracted from small pieces of gingivae (predominantly epithelium) under dissociative conditions was chromatographed on Sepharose 2B-CL with 0.5M-sodium acetate. The elution profile (Fig. 1*a*) shows a distribution of labelled material in which about 17% of the total ^3H and 18% of the [^{35}S]sulphate were excluded from the gel. When the fractions containing this large macromolecular material (E) were pooled, concentrated and re-separated on Sepharose 2B-CL under dissociative conditions in 4M-guanidinium chloride, 80% of its total radioactivity (^3H) was partially retarded (R_{1_D}) by the gel (Fig. 1*b*). This retarded material (R_{1_D}) behaved similarly when further eluted with 0.5M-sodium acetate from the same Sepharose 2B-CL column (Fig. 1*c*). However, a proportion (22%) of the radioactivity was eluted at the void volume (V_0) and was not included in further characterization. When hyaluronic acid was mixed with partially retarded material (R_{1_A}), up to 26% of the ^3H and 37% of the [^{35}S]sulphate appeared at the void volume on elution from Sepharose 2B-CL under the associative conditions (Fig. 1*d*). Omission of proteinase inhibitors in the extraction and elution procedures resulted in the appearance of radioactive label being distributed throughout the retarded portions of the profiles.

High-molecular-weight material similarly extracted from the underlying connective tissue, and subjected to gel-exclusion chromatography, was not dissociated by 4M-guanidinium chloride and remained in the void volume in the absence of hyaluronic acid (Fig. 2). When the R_{1_A} fraction was again concentrated by vacuum dialysis and further fractionated on Sepharose 2B-CL under associative conditions, less than 5% of the radioactivity appeared at V_0. Subsequent chromatographic separations of the retarded material on Sepharose 2B-CL (R_{1_A}) revealed no labelled material at V_0. The initial chromatographic elution (Fig. 1*c*) separated all the self-aggregated material from the totally aggregatable fraction, which had been extracted with 4M-guanidinium hydrochloride. Similar elution profiles were obtained from chromatographic separations of material from which hyaluronic acid had been specifically removed after exhaustive digestion with leech hyaluronidase. The ratio of tissue hyaluronic acid to total proteoglycan in cultured epithelium appears to be governed by and to correspond to the microscopic development of the culture, namely the extent of cell/cell contact (Wiebkin & Thonard, 1968, 1969; Wiebkin, 1970). Control of synthesis and secretion of proteoglycan by epithelium may be regulated by a physicochemical interaction with hyaluronic acid (Hardingham & Muir, 1972, 1973), similar to, but not necessarily identical with, that described by Wiebkin & Muir (1975) and Wiebkin *et al.* (1975) for cartilage cells.

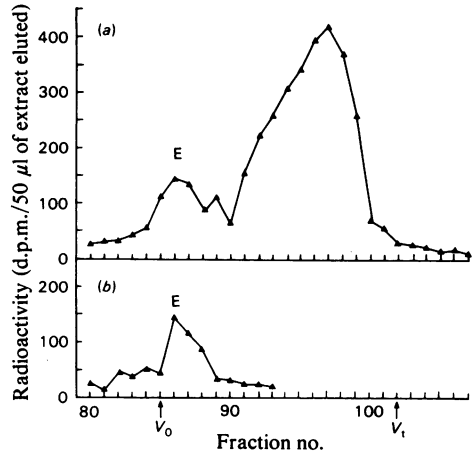

Fig. 2. *Gel chromatography of macromolecular material extracted from gingival connective tissue*
Gingival connective tissue was incubated for 24h in nutrient medium containing [^3H]acetate and extracted with 4M-guanidinium chloride. This material was applied to a column of Sepharose 2B-CL and eluted with 0.5M-sodium acetate (*a*). Material eluted at the void volume (E) was further separated in 4M-guanidinium chloride (*b*). ▲, [^3H]-Acetate. Column and fraction sizes and abbreviations are as for Fig. 1.

Aggregated proteoglycan

The present results indicate that associated proteoglycans of large molecular size (> 500000 mol.wt.) (Fig. 1*a*) can be dissociated (Fig. 1*b*) and that not more than 20% (as determined by ^3H incorporation) of them readily self-aggregate (Fig. 1*c*). Enzymic degradation of residual hyaluronic acid, or the repeated concentration of the R_{1_D} fraction (Fig. 1*b*), did not result in further self-aggregation. Nevertheless the return of those proteoglycans that do not self-

aggregate to the void volume of a Sepharose 2B-CL separation in the presence of hyaluronic acid under associative conditions indicated that these molecules had the capacity to aggregate with hyaluronic acid (Fig. 1*d*).

Further radioautographic studies by Wiebkin & Thonard (1977) and Wiebkin *et al.* (1979) have shown inhibition of both the synthesis and the localization of sulphated proteoglycan by gingival-epithelial slices incubated in the presence of hyaluronic acid. Thonard & Scherp (1962) were unable to remove all intercellular proteoglycan from fixed histological sections of gingival tissue by testicular hyaluronidase. Preliminary investigations in our laboratory have shown dramatic loss of tissue integrity when hyaluronidase was added to gingival-epithelial organ cultures. In contrast with the effects observed on fixed tissue matrix by particular enzymes, the turnover of intercellular material by metabolizing tissue would provide for cryptic enzyme-cleavage points to be intermittently exposed. Evidence that gingival-epithelial proteoglycans are capable of forming both intercellular aggregates with hyaluronic acid and self-aggregated material (Fig. 1*d*) accords with the view that, owing to the stocheiometry of these aggregates, enzyme-cleavage points remain cryptic in epithelium in the early stages of periodontal disease. Such proteoglycan aggregates have not been identified in the underlying connective tissue, which is more susceptible to loss of tissue integrity in disease (Fig. 2). Elucidation of the fundamental differences between the aggregability of various intercellular proteoglycans may also lead to a better understanding of the interaction of epithelium and connective tissue under both normal and pathological (e.g. neoplastic) conditions.

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