Synthesis of glycosaminoglycans by human skin fibroblasts cultured on collagen gels

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A comparison has been made of the synthesis of glycosaminoglycans by human skin fibroblasts cultured on plastic or collagen gel substrata. Confluent cultures were incubated with [^3H]glucosamine and $\text{Na}_2^{35}\text{SO}_4$ for 48 h. Radiolabelled glycosaminoglycans were then analysed in the spent media and trypsin extracts from cells on plastic and in the medium, trypsin and collagenase extracts from cells on collagen gels. All enzyme extracts and spent media contained hyaluronic acid, heparan sulphate and dermatan sulphate. Hyaluronic acid was the main ^3H -labelled component in media and enzyme extracts from cells on both substrata, although it was distributed mainly to the media fractions. Heparan sulphate was the major [^{35}S]sulphated glycosaminoglycan in trypsin extracts of cells on plastic, and dermatan sulphate was the minor component. In contrast, dermatan sulphate was the principal [^{35}S]sulphated glycosaminoglycan in trypsin and collagenase extracts of cells on collagen gels. The culture substratum also influenced the amounts of [^{35}S]sulphated glycosaminoglycans in media and enzyme extracts. With cells on plastic, the medium contained most of the heparan sulphate (75%) and dermatan sulphate (>90%), whereas the collagenase extract was the main source of heparan sulphate (60%) and dermatan sulphate (80%) from cells on collagen gels; when cells were grown on collagen, the medium contained only 5–20% of the total [^{35}S]sulphated glycosaminoglycans. Depletion of the medium pool was probably caused by binding of [^{35}S]sulphated glycosaminoglycans to the network of native collagen fibres that formed the insoluble fraction of the collagen gel. Furthermore, cells on collagen showed a 3-fold increase in dermatan sulphate synthesis, which could be due to a positive-feedback mechanism activated by the accumulation of dermatan sulphate in the microenvironment of the cultured cells. For comparative structural analyses of glycosaminoglycans synthesized on different substrata labelling experiments were carried out by incubating cells on plastic with [^3H]glucosamine, and cells on collagen gels with [^{14}C]glucosamine. Co-chromatography on DEAE-cellulose of mixed media and enzyme extracts showed that heparan sulphate from cells on collagen gels eluted at a lower salt concentration than did heparan sulphate from cells on plastic, whereas with dermatan sulphate the opposite result was obtained, with dermatan sulphate from cells on collagen eluting at a higher salt concentration than dermatan sulphate from cells on plastic. These differences did not correspond to changes in the molecular size of the glycosaminoglycan chains, but they may be caused by alterations in polymer sulphation.

Hyaluronic acid, heparan sulphate and dermatan sulphate–chondroitin sulphate co-polymers are the three main types of glycosaminoglycan produced by cell cultures of human skin fibroblasts (Malmstrom *et al.*, 1975; Matalon & Dorfman, 1966; Hopwood & Dorfman, 1977). Glycosaminoglycan biosynthesis in these cells is usually studied by incubation for 1–2 days with suitable radioisotopically labelled

precursors, e.g. radioactive sulphate and glucosamine, and then examining radiolabelled glycosaminoglycans in the cell culture media, in trypsin and chemical extracts of the cell layer (the pericellular domain) and the residual glycosaminoglycans located mainly inside the cultured cells (Sjoberg *et al.*, 1979; Kleinmann *et al.*, 1975). In all the work published so far, the cells were maintained on a

plastic substratum, and under these conditions radioactive glycosaminoglycans accumulated in the culture medium, with the cell-associated fractions representing only a small proportion of the radioactive products.

However, experiments *in vitro* have shown that sulphated glycosaminoglycans both as free chains and in their native, proteoglycan, form will bind to collagen (Obrink & Wasteson, 1971; Toole, 1976). Collagen is a major constituent of the extracellular matrix of connective tissues, with types-I and -III collagen being the predominant forms in the dermis (Fessler & Fessler, 1978), from which skin fibroblasts are derived. Collagenous substrata promoted the expression of a differentiated phenotype in cultured cells (Hay, 1977; Konigsberg & Hauschka, 1965), and also stimulated the production of glycosaminoglycans by both corneal epithelial cells from chick embryos (Meier & Hay, 1974) and chick embryonic somites (Kosher & Church, 1975). On the basis of these observations we decided to study the synthesis and distribution of glycosaminoglycans of human skin fibroblasts cultured on native collagen fibres polymerized under conditions that lead to the formation of a collagen gel (Schor & Court, 1979; Elsdale & Bard, 1972). Results were compared with similar studies on skin fibroblasts maintained on surfaces of commercially available plastic Petri dishes manufactured for use in cell-culture work.

Experimental

Materials

Twice-crystallized trypsin (Type III), twice-crystallized papain, a mixed collagenase preparation and soya-bean (*Glycine max*) trypsin inhibitor were purchased from Sigma. Chondroitinase AC (from *Arthrobacter aureus*) and chondroitinase ABC (from *Proteus vulgaris*) were supplied by Miles Laboratories. Plastic Petri dishes (60mm diameter) were from Nunclon. Hanks' balanced salt solution, Eagle's medium, amino acids and antibiotics were obtained from Gibco Biocult. Phosphate-buffered saline (Dulbecco 'A') was obtained from Oxoid Ltd. Foetal-calf serum was from Flow Laboratories. D-[1-¹⁴C]glucosamine (sp. radioactivity 50–60 Ci/mol), D-[1-³H]glucosamine (sp. radioactivity 2–5 Ci/mol) and Na₂³⁵SO₄ (100 Ci/mol) were purchased from The Radiochemical Centre. Aquasol II was supplied by New England Nuclear Corp. DEAE-cellulose DE-52 was purchased from Whatman, and Sephadex G-50 and Sepharose CL-4B were obtained from Pharmacia Ltd.

Cell cultures

Diploid human skin fibroblasts (passages 3–10) were grown at 37°C in 4ml of Eagle's Minimal

Essential Medium supplemented with 15% (v/v) heat-inactivated foetal-calf serum, 2mM-glutamine, 1mM-sodium pyruvate, non-essential amino acids (Gibco Biocult), 100 units of penicillin/ml and 100µg of streptomycin/ml. Cells to be used for measuring glycosaminoglycan biosynthesis were plated on 60mm-diameter plastic tissue-culture dishes with or without a collagen-gel substratum, at an initial density of 1×10^5 cells/dish. The medium was changed three times during the next 7 days as cells grew to confluency at a saturation density of 0.8×10^6 – 2×10^6 cells/dish, depending on the culture. Confluent cultures were then incubated with [³H]glucosamine (5µCi/ml) and Na₂³⁵SO₄ (10µCi/ml) for 48h. An alternative labelling procedure was also used in which cells on plastic were incubated with [³H]glucosamine (5µCi/ml) and cells on collagen gels with [¹⁴C]glucosamine (2µCi/ml).

Trypsin and collagenase extracts of cell cultures

After a 48h incubation with radiolabels the medium was removed and the cell layer washed twice with warm (37°C) Hanks' balanced salt solution. The first wash was added to the culture media. The cells were then incubated for 20min at 37°C with 3ml of trypsin solution [0.05% (w/v) in phosphate-buffered saline]. Cells on plastic were removed by treatment with trypsin from the culture surface and yielded a single-cell suspension. Soya-bean trypsin inhibitor [1ml of a 0.2% (w/v) solution in phosphate-buffered saline] was added, the trypsin solution centrifuged at 800g for 5min, the supernatant removed and the cell pellet washed once with phosphate-buffered saline. The wash solution was added to the trypsin extract. Cell viability was about 95% as judged by Trypan Blue exclusion.

Cells on collagen gels were not detached from the substratum during the 20min treatment with trypsin. The trypsin solution was therefore removed, the cell layer washed twice with warm Hanks' balanced salt solution (37°C) and 3ml of collagenase [0.2% (w/v) in Hanks' balanced salt solution] was added to each culture. The gel was solubilized by collagenase after 1–2h at 37°C. The collagenase solution was centrifuged (800g for 5min), and the cell pellet was resuspended with Hanks' balanced salt solution and centrifuged again. The wash solution was added to the collagenase extract. The cell product was finally resuspended in trypsin solution (0.05%, w/v) and incubated for a further 10min at 37°C. The cell suspension was centrifuged and this supernatant was also pooled with the collagenase extract; this mixed extract is referred to as the 'collagenase/trypsin' extract. About 80–85% of the cells excluded Trypan Blue. All media and enzyme extracts were then dialysed against distilled water (containing a little non-radioactive glucosamine and Na₂SO₄) to remove most of the non-incorporated radiolabels

and then stored at -20°C . On some occasions dialysed samples were freeze-dried and digested with papain (0.1 mg of enzyme/ml in 20 mM-sodium phosphate buffer, pH 6.8, containing 1 mM-EDTA and 5 mM-cysteine) for 18 h at 60°C . The digest was applied directly to the DEAE-cellulose column (see below).

Preparation of the collagen substrata

Type-I collagen was extracted from rat tail tendons in 0.5 M-acetic acid for 2 days at 4°C (Elsdale & Bard, 1972). Debris was removed by centrifugation at 2000 g for 3 h. The supernatant was mixed with an equal volume of 20% (w/v) NaCl to precipitate the collagen. The precipitate was centrifuged (2000 g for 3 h), the pellet dissolved in 0.1 M-acetic acid and then dialysed against 10 vol. of distilled water for 2 days at 4°C , with two changes of water per day. The aqueous solution of collagen was then centrifuged overnight at 2000 g (8°C) and the clear supernatant stored at 4°C . Three-dimensional gels of native collagen fibres were prepared by mixing 8.5 ml of collagen solution in water (2–2.5 mg/ml) with 1 ml of ten-times-concentrated Eagle's Minimal Essential Medium and 0.5 ml of 4.4% (w/v) NaHCO_3 . Samples (5 ml) of this solution were pipetted into 60 mm-diameter plastic Petri dishes and the gel formed rapidly (10–20 min) in a 37°C incubator in an atmosphere of CO_2/air (1:19) (Schor & Court, 1979; Elsdale & Bard, 1972).

Isolation of radiolabelled glycosaminoglycans

(1) *Cells labelled with [^3H]glucosamine and $\text{Na}_2^{35}\text{SO}_4$.* DEAE-cellulose chromatography was used to separate glycosaminoglycans from other non-diffusible components in media and enzyme extracts of fibroblast cell cultures. DEAE-cellulose DE-52 was equilibrated with buffer (5 mM-sodium phosphate, pH 6.8, containing 10 mM-NaCl) according to the manufacturer's recommendations and a column prepared of bed volume $8\text{ cm} \times 1\text{ cm}$. Equilibration buffer (1 vol. of a ten-times-concentrated solution) was added to 9 vol. of dialysed media or enzyme extracts and the samples were mixed, centrifuged and then applied to the DEAE-column. Two bed volumes of equilibration buffer were passed through the column and then elution of glycosaminoglycan components was achieved by using a linear gradient of NaCl from 10 mM- to 0.8 M-NaCl in a total volume of either 200 or 300 ml. The flow rate was 20–24 ml/h and 2–3 ml fractions were collected depending on the gradient used. Individual experimental details are given in Figure legends. Radioactivity in samples from each fraction was determined by liquid-scintillation counting (see below).

(2) *Cells labelled with [^3H]glucosamine (plastic) or [^{14}C]glucosamine (collagen gel).* With these cells, DEAE-chromatography of media and enzyme ex-

tracts was carried out as described above, but media samples from equivalent numbers of cell cultures on plastic and collagen gels were mixed before the dialysis step. In addition, the trypsin extract of cells on plastic was split 1:2 and mixed with enzyme extracts of cells on collagen so that one volume was mixed with the trypsin extract and two volumes with the collagenase/trypsin extract.

Characterization of glycosaminoglycans

Fractions corresponding to the major radioactivity peaks detected after DEAE-chromatography were pooled, dialysed and concentrated by rotary evaporation or freeze-drying. The concentrated samples were subjected to the following procedures.

(1) Digestion with chondroitinase ABC and chondroitinase AC was carried out as described by Saito *et al.* (1968). The degree of sample degradation was determined by chromatography on Sephadex G-50. Figures representing these experiments are not shown and results are described only in the text of the Results section.

(2) *N*-Sulphate groups, indicative of heparan sulphate or heparin, were determined by the HNO_2 method of Lagunoff & Warren (1962). Low-molecular-weight [^{35}S]sulphate was detected after Sephadex G-50 gel filtration. Results are not shown in graphical form.

(3) Electrophoretic mobilities with reference to standard preparations of hyaluronic acid and chondroitin sulphate were determined by cellulose acetate electrophoresis in 75 mM-sodium barbitone buffer, pH 8.6. Samples ($10\mu\text{l}$) were applied to the cellulose acetate paper and electrophoresis was carried out at a constant voltage (200 V) for 40 min. After electrophoresis, the cellulose acetate paper was cut into 0.5 cm segments and radioactivity determined in each segment. Standard glycosaminoglycans were located by staining with Alcian Blue [1% (w/v) in 2% (v/v) acetic acid].

(4) Elution profiles on Sephadex G-200 were determined for ^3H , ^{14}C -labelled glycosaminoglycans on a column of bed size $85\text{ cm} \times 1\text{ cm}$. The column was eluted with 0.2 M-NaCl containing 0.2% (w/v) NaN_3 at a flow rate of 6 ml/h.

(5) The presumed hyaluronic acid peaks (see Fig. 1 below) were fractionated on a Sepharose CL-4B column of bed size $80\text{ cm} \times 1\text{ cm}$. Elution was carried out with 0.5 M-sodium acetate buffer, pH 5.8, at a flow rate of 6 ml/h.

Liquid-scintillation counting and computer analysis

Aqueous samples were counted by mixing a maximum of 1 vol. of sample with 10 vol. of Aquasol II liquid-scintillation fluid. Radioactivity was determined in each sample with a Packard model-3385 liquid-scintillation counter. The channels-ratio

Fig. 1. DEAE-cellulose chromatography of radiolabelled glycosaminoglycans produced by human skin fibroblasts. Confluent cultures of human skin fibroblasts on plastic and collagen-gel substrata were incubated for 48 h with D-[1- ^{3} H]glucosamine and Na $_2$ ^{35} SO $_4$. Dialysed samples were applied to a DEAE-cellulose column. Glycosaminoglycans in the culture medium (a) and a trypsin extract (b) were isolated from cells on plastic, and on collagen gels the glycosaminoglycan composition was determined in the medium (c), a trypsin extract (d) and a collagenase/trypsin extract (e). Acidic glycoproteins and glycosaminoglycans were eluted from the column with a linear gradient of NaCl (for simplicity only the glycosaminoglycan portion of the chromatogram is shown). Results were from nine cultures for cells on plastic and five cultures for cells on collagen gels. Fractions were of volume 3 ml. Abbreviations used: HA, hyaluronic acid; DS, dermatan sulphate; HS, heparan sulphate.

method was used for quench correction of doubly labelled samples. Counting efficiencies were about 45% for ^{14}C or ^{35}S and around 20% for ^3H , depending on the degree of quenching. Computer programs were used to calculate d.p.m. values for ^3H and ^{14}C or ^3H and ^{35}S and for plotting chromatographic elution profiles. The program plots high- and low-energy radioisotopes on separate vertical axes; maximum value for each radioisotope can be chosen to correspond to the peaks with the highest d.p.m. values, or scales can be adjusted to expand a small peak into a large one. This latter facility was particularly useful when comparing ^3H - and ^{14}C -labelled glycosaminoglycans from cells on plastic and collagen gels, as it allowed a rapid comparison to be made of peak maxima for individual glycosaminoglycan components (see legend to Fig. 3 for further details) from cells grown on different substrata.

Results

In the first series of experiments, confluent cell cultures, maintained on plastic and collagen-gel substrata, were incubated for 48 h with $[^3\text{H}]$ glucosamine and $\text{Na}_2^{35}\text{SO}_4$ to radiolabel biosynthetically glycosaminoglycans for studies on glycosaminoglycan composition, distribution and relative rates of synthesis (Figs. 1, 2 and 3). The media and trypsin extracts from cells on plastic and the media, trypsin and collagenase/trypsin extracts of cells on collagen gels were first analysed by chromatography on DEAE-cellulose (Fig. 1). Two doubly labelled peaks were found in all extracts; they eluted from the ion-exchange column between 0.4 and 0.6 M-NaCl and were identified as heparan sulphate and dermatan sulphate (see below). In the trypsin extract of cells on plastic, heparan sulphate was the major sulphated glycosaminoglycan and dermatan sulphate was the minor component (Fig. 1b). In contrast, both the trypsin extract (Fig. 1d) and the collagenase/trypsin extract (Fig. 1e) of cells on collagen gel were characterized by the predominance of dermatan sulphate as the main sulphated glycosaminoglycan, with heparan sulphate the minor component. Dermatan sulphate was the main sulphated glycosaminoglycan in the media irrespective of the culture substratum (Figs. 1a and 1c).

When the quantitative distribution of sulphated glycosaminoglycans in the culture media and cell-associated extracts was calculated, it was clear that, whereas cells on plastic released most of their sulphated glycosaminoglycans into the medium (75% of heparan sulphate and 95% of dermatan sulphate) (Fig. 2a) the medium contained only a small proportion of both heparan sulphate and dermatan sulphate when cells were cultured on

collagen gels (Fig. 2b). The collagenase/trypsin extract of these cells was the most abundant source of sulphated glycosaminoglycans, containing 80% of the total dermatan sulphate and 60% of the total heparan sulphate (Fig. 2b).

Fig. 2. Distribution of glycosaminoglycans between the medium and enzyme extracts of the cell layer from (a) plastic substrata and (b) collagen-gel substrata.

The histograms were calculated from the data in Fig. 1. Results here show the percentage of the total radioisotope incorporation into each glycosaminoglycan component of the media and enzyme extracts. For example, of the total $[^3\text{H}]$ glucosamine and $\text{Na}_2^{35}\text{SO}_4$ incorporation into heparan sulphate produced by cells on plastic, about 75% was in the medium and 25% in the trypsin-extracted material (b). Abbreviations used: M, media; T, trypsin-extracted material; CT, collagenase/trypsin-extracted material; other abbreviations as in Fig. 1; $\square$, ^3H ; $\square$, ^{35}S .

Fig. 3. Total radioisotope incorporations into glycosaminoglycans produced by human skin fibroblasts. Data are calculated from Fig. 1. $[^3\text{H}]$ Glucosamine and $\text{Na}_2^{35}\text{SO}_4$ incorporations into each glycosaminoglycan in the media and enzyme extracts were added together and expressed as d.p.m./10⁶ cells. Abbreviations are as in Fig. 1. $\square$, ^3H ; $\square$, ^{35}S .

Fig. 4. Co-chromatography on DEAE-cellulose of ^3H glucosamine and ^{14}C glucosamine-labelled glycosaminoglycans produced by cells on plastic (^3H) and collagen gels (^{14}C)

Confluent monolayers of cells on plastic and collagen gels were incubated for 48 h with ^3H glucosamine and ^{14}C glucosamine respectively. Before chromatography on DEAE-cellulose, media samples from similar numbers of cultures were mixed. The trypsin extract of cells on plastic was split 1:2 and one part mixed with the trypsin extract and two parts mixed with the collagenase/trypsin extract of cells on collagen gels. Glycosaminoglycans were eluted with a linear gradient of NaCl. Peak maxima for ^3H - and ^{14}C -labelled hyaluronic acid in media and enzyme extracts were identical, but only hyaluronic acid in the media is shown (a). (b-f) Show only the sulphated-glycosaminoglycan regions of chromatograms. For comparison of trypsin extracts (c and d), vertical axes have been adjusted to give full-scale profiles for each sulphated glycosaminoglycan in order to illustrate clearly the shifts in peak maxima for heparan sulphate and dermatan sulphate. A similar procedure was adopted for comparison of trypsin and collagenase/trypsin extracts (e and f). With glucosamine as the radioactive precursor, heparan sulphate in the medium is poorly resolved (see also Figs. 1a and 1c), and better resolution of medium heparan sulphate is obtained with $\text{Na}_2^{35}\text{SO}_4$ as precursor (Figs. 1a and 1c). Fractions of 2 ml were collected. Abbreviations are as in Fig. 1.

An increase in net synthesis of dermatan sulphate by cells on collagen gels was also evident because the incorporation of both [^3H]glucosamine and $\text{Na}_2^{35}\text{SO}_4$ into this component was about 3-fold higher than in cells cultured on plastic (Fig. 3). There was little difference in isotope incorporation into heparan sulphate between cells on plastic and collagen gel.

Hyaluronic acid was the main [^3H]glucosamine-labelled acidic component produced by the fibroblast cultures (Figs. 1 and 4). Over 90% of the hyaluronic acid was found in the medium from cells growing on plastic, and cells on collagen released about 40% of the hyaluronic acid into the medium (Fig. 2). The trypsin extracts of cells on either substratum contained quite small amounts of hyaluronic acid, but a considerable amount of this glycosaminoglycan (about 45%) was found in collagenase/trypsin extracts of cells on collagen gels. In contrast with the increased synthesis of dermatan sulphate by cells on collagen, such cells showed a 40% decrease in the incorporation of [^3H]glucosamine into hyaluronic acid (Fig. 3).

In order to identify possible differences in molecular weight of glycosaminoglycan chains or alterations in their chemical structure (e.g. small changes in charge density) double-labelling experiments were carried out in which cells on plastic were incubated with [^3H]glucosamine and cells on collagen gels with [^{14}C]glucosamine. Identical numbers of cultures were set up under the two conditions. After a 48 h incubation with the appropriate radiolabel, the media from cells on both substrata were mixed, digested with papain and then analysed by DEAE-cellulose chromatography (Fig. 4); papain digests of enzyme extracts of the cultured cells were also compared by analysis on DEAE-cellulose after pooling one-third of the trypsin-extracted material from cells on plastic with the trypsin-extracted material of cells on collagen gels and the remaining two-thirds were pooled with the collagenase/trypsin extract. Elution profiles obtained from the ion-exchange column gave peak maxima for hyaluronic acid at an identical fraction number for both radiolabels in all extracts; the hyaluronic acid peak is shown only for mixed media fractions (Fig. 4a). However, different peak maxima were observed for dermatan sulphate and heparan sulphate (Fig. 4). ^{14}C -labelled dermatan sulphate from cells on collagen gels eluted at a higher salt concentration than did ^3H -labelled dermatan sulphate from cells on plastic. In contrast, ^{14}C -labelled heparan sulphate eluted at a lower salt concentration than did ^3H -labelled heparan sulphate. These differences were noted in glycosaminoglycans released into the culture media (Figs. 4a and 4b) and in comparisons between trypsin extracts of cells on plastic with trypsin (Figs. 4c and 4d) and collagenase/trypsin

(Figs. 4e and 4f) extracts of cells on collagen gels. We have studied three separate skin fibroblast cell lines by this double-labelling technique, and they

Fig. 5. Electrophoresis of glycosaminoglycans on cellulose acetate

Radioactivity peaks (presumed to represent glycosaminoglycan component) from DEAE-cellulose chromatography (Fig. 1) were pooled, dialysed and freeze-dried. Samples were dissolved in water (0.1–0.5 ml) and 10 μl portions applied as narrow bands to the cellulose acetate paper. Standard preparations of hyaluronic acid and chondroitin sulphate (both 1 mg/ml) were run simultaneously. After electrophoresis, standard glycosaminoglycans were located by Alcian Blue staining and radioactivity distributions were determined by cutting the cellulose acetate into 0.5 cm segments and counting each segment for radioactivity in 5 ml of Aquasol II. (a) Heparan sulphate; (b) dermatan sulphate; (c) hyaluronic acid. Abbreviations used: HA, hyaluronic acid; CS, chondroitin sulphate.

all show similar shifts in peak maxima for sulphated glycosaminoglycans.

The cells used for the [^3H]- and [^{14}C]-glucosamine mixed-labelling experiment (Fig. 4) were a separate cell line from those used in the experiments described in Fig. 1. Whereas the former cells grown on plastic had a much greater amount of heparan sulphate than dermatan sulphate in the trypsin-extracted material, the corresponding trypsin-extracted material from the cells described in Fig. 4 had a proportionately greater amount of dermatan sulphate. We have now studied five separate human fibroblast cell lines of low pass number and we have always found that radioactivity in heparan sulphate from the trypsin-extracted material from cells on plastic was greater than that in dermatan sulphate. All these cell lines, however, showed a characteristic shift in their cell-associated sulphated-glycosaminoglycan phenotype towards an excess of dermatan sulphate over heparan sulphate when grown on collagen gels.

Characterization of glycosaminoglycan peaks from DEAE-cellulose chromatography

The peak designated 'HA' in the elution profiles in Figs. 1 and 4 was characterized by its complete degradation to disaccharides by chondroitinase ABC, its similar electrophoretic mobility (Fig. 5c) to that of a standard preparation of hyaluronic acid and its high molecular weight (most of the radio-label was excluded from Sepharose CL-4B) (Fig. 6).

Fig. 6. Gel filtration of hyaluronic acid on Sepharose CL-4B

The hyaluronic acid peak isolated after DEAE-cellulose chromatography was dialysed, freeze-dried and dissolved in 1 ml of water before application to the column. Samples from media and enzyme extracts gave similar profiles. Fraction volumes were 0.9 ml.

Fig. 7. Gel filtration on Sephadex G-200 of $^3\text{H}/^{14}\text{C}$ -labelled sulphated glycosaminoglycans

Elution profiles are shown for papain-digested glycosaminoglycans isolated after DEAE-cellulose chromatography (Fig. 4). Profiles in (a) show the dermatan sulphate component from mixed media samples from cells on plastic (^3H]glucosamine-labelled) and cells on collagen gels (^{14}C]glucosamine-labelled). The dermatan sulphate component from mixed enzyme extracts gave similar profiles. (b) Is a composite illustration showing a comparison of heparan sulphate profiles from mixed enzyme extracts (trypsin extract from cells on plastic, collagenase/trypsin extract from cells on collagen gels) and mixed media extracts. Fraction volumes were 0.9 ml.

The heparan sulphate peak was identified by its resistance to chondroitinase ABC and by the extensive degradation caused by nitrous acid. The heparan sulphate component had a lower electrophoretic mobility than dermatan sulphate (Fig. 5a). Characterization of the dermatan sulphate component was achieved principally on the basis of its sensitivity to chondroitinase ABC and resistance to depolymerization by chondroitinase AC and nitrous acid. On electrophoresis, this component had a similar mobility to standard chondroitin sulphate, but, under the conditions used, chondroitin 4- and

6-sulphates had similar mobilities to dermatan sulphate (Fig. 5b).

The molecular sizes of the dermatan sulphate chains obtained from cells on plastic and collagen gels were similar when assessed by their elution from Sephadex G-200 (Fig. 7a). Gel-filtration profiles were similar for spent media and cell-associated dermatan sulphate, with most of the radioactivity eluting immediately after the void volume of the column.

An interesting difference in chain length was observed with heparan sulphate; heparan sulphate isolated from enzyme extracts of the cell layers eluted in or near the void volume from Sephadex G-200, and the profiles for cells on plastic and collagen gels were identical (Fig. 7b). However, in the spent medium, heparan sulphate from cells on both plastic and collagen gels was significantly retarded by Sephadex G-200 and it eluted close to the total column volume.

Discussion

The main conclusions from our results are that the net synthesis and distribution of sulphated glycosaminoglycans and hyaluronic acid were markedly altered by culturing skin fibroblasts on a collagen-gel substratum instead of a plastic substratum of the type used in conventional cell culture (Figs. 2 and 3). Below we will suggest that these changes may be interrelated and that the increase in dermatan sulphate synthesis could be a consequence of the localization of dermatan sulphate in the cellular microenvironment.

Cells on collagen gels showed about a 3-fold increase in the synthesis of dermatan sulphate (Fig. 3); this increase is particularly significant, since it demonstrates that collagen can stimulate the production by skin fibroblasts of the major sulphated glycosaminoglycan found in human skin (Toledo & Dietrich, 1977). It should be noted that we used only radiolabel incorporations as indices of glycosaminoglycan synthesis. Misleading conclusions could arise if the collagen-gel substratum evoked changes in radiolabel-uptake rates or nucleotide sugar concentrations, either of which would alter the specific radioactivities of both the precursor pools and glycosaminoglycan products without necessarily changing rates of synthesis (Gallagher, 1977). However, only dermatan sulphate synthesis was increased and the increase was evident from the incorporation of both [^3H]glucosamine and [^{35}S]-sulphate (Fig. 3). No generalized increase in glycosaminoglycan synthesis was found. By contrast, hyaluronic acid synthesis was decreased on collagen gels (Fig. 3). These findings are suggestive of a specific effect on dermatan sulphate synthesis that is probably unrelated to influences on the

precursor pools. Furthermore, collagen gels occupied a volume of 5 ml on the surface of 60 mm plastic Petri dishes and each gel contained culture medium trapped within the interstices of the gel fibres. When radiolabels in 4 ml of culture medium were added to cells on these gels (a similar volume of the same medium was added to cells on plastic), we found, by isotope dilution, that the low-molecular-weight radiolabelled compounds equilibrated with the 'interstitial' fluid to cause a 2-fold decrease in radioactivity concentrations in the medium. For [^{35}S]-sulphate there was also a corresponding decrease in specific radioactivity, since the culture medium contained streptomycin sulphate and MgSO_4 . These changes will bias the method against demonstrating an increase in synthesis of radiolabelled products and imply that the 3-fold increase in dermatan sulphate synthesis is an underestimate of the real increase. It follows that the small increase in heparan sulphate synthesis and the decrease in hyaluronic acid synthesis by cells on collagen gels may be artefacts resulting from decreased concentrations of radiolabels.

The mechanism responsible for the increase in dermatan sulphate synthesis on collagen is not understood. Collagen may function in either an 'instructive' manner and directly influence dermatan sulphate synthesis or in a 'permissive' fashion by promoting the accumulation of matrix macromolecules, such as dermatan sulphate itself, in the pericellular domain, which then stimulate synthetic activity of the cells by positive feedback. With both mechanisms the surface membrane will play a key role in mediating the regulatory signals. In this context, it is significant that addition of exogenous chondroitin sulphate-proteoglycan to cultured chondrocytes leads to a stimulation of chondroitin sulphate-proteoglycan synthesis (Nevo & Dorfman, 1972) and the activation does not require uptake of the added proteoglycan (Huang, 1974). In our system, dermatan sulphate may accumulate around the cells through an interaction with collagen fibres, and the ensuing high localized concentration may activate dermatan sulphate synthesis. A net increase in dermatan sulphate synthesis could also arise from a decreased rate of degradation, and a recent report has indicated a slower turnover of sulphated glycosaminoglycans by mammary epithelial cells on collagen gels (David & Bernfield, 1979).

The glycosaminoglycans of the cell layer which were extracted with trypsin and collagenase were presumably located either at the surface membrane or in the extracellular matrix. It is well established that sulphated glycosaminoglycans will bind to collagen (Lindahl & Hook, 1978; see also the introduction) and to surface membranes of cultured cells (Prinz *et al.*, 1978). Trypsin treatment readily

detached cells from plastic substrata and released about 90% of the cell-associated glycosaminoglycans; these cells were not extracted with collagenase, so we cannot distinguish between membrane-bound and collagen-bound components. Cells on collagen gels were not detached by trypsin. Scanning electron microscopy has shown that fibroblasts on collagen gels do not completely cover the gel surface; they penetrate between superficial gel fibres and a small proportion of fibroblasts migrate into the gel matrix (S. Schor & T. Allen, unpublished work). Trypsin will therefore have access to glycosaminoglycans bound both to the apical surface membrane and to collagen fibres. The rate of diffusion of trypsin into the gel will determine the extent of trypsin-induced release of glycosaminoglycans of the basal surface membrane and the gel matrix beneath the cell layer. For these reasons it is reasonable to assume that the first trypsin treatment will release membrane-associated and collagen-associated glycosaminoglycans. The collagenase/trypsin extract, which was the main source of glycosaminoglycans when cells were grown on collagen (Fig. 2b), would contain radiolabelled material bound mainly, but not exclusively, to collagen-gel fibres. The collagenase used was relatively crude and contained other proteinase activities. Also, any trypsin that diffused into the gel during the first trypsin treatment would further contaminate the preparation. These proteinases could extract membrane-associated glycosaminoglycans that were not extracted by the first trypsin treatment. To ensure complete removal of proteinase-sensitive glycosaminoglycans from cell surfaces, the cell suspension obtained after collagenase had solubilized the collagen gel was given a final brief treatment with trypsin and this extract was pooled with the collagenase extract (see the Experimental section). In our results on the distribution of glycosaminoglycans between trypsin and collagenase/trypsin extracts (Fig. 2b) we found that, in four out of five cell lines studied, the amount of heparan sulphate compared with dermatan sulphate was always higher in the trypsin extract (heparan sulphate/dermatan sulphate = 1:2) than in the collagenase/trypsin extract (heparan sulphate/dermatan sulphate = 1:5). The one exception was the cell line illustrated in Fig. 4, where little change occurred in heparan sulphate/dermatan sulphate ratios. These findings may reflect disproportionate contributions made to these extracts by glycosaminoglycans from the cell surface membrane and the extracellular matrix with the trypsin fraction enriched in membrane-associated glycosaminoglycans and the collagenase/trypsin fraction containing mainly collagen-bound components.

A widely recognized problem in cell-culture work is the relevance of experimental findings to the conditions existing *in vivo*. The extracellular matrix

plays a particularly important role in differentiation (Meier & Hay, 1975; Grobstein, 1975) and in the maintenance of a normal cell phenotype (Tarin, 1972). In our studies on human skin fibroblasts the composition of the collagenase- and trypsin-extractable fractions of cells on collagen gels indicate an extracellular matrix with over 85% of its sulphated glycosaminoglycans as dermatan sulphate and less than 15% as heparan sulphate (Fig. 1). This corresponds to published data on intact human skin, which showed that dermatan sulphate represented over 90% of the total sulphated glycosaminoglycans and heparan sulphate less than 5% (Toledo & Dietrich, 1977). Our results and the results of other studies (Sjoberg *et al.*, 1979; Kleinman *et al.*, 1975) show that, when human skin fibroblasts are cultured on a plastic substratum, heparan sulphate is the main sulphated glycosaminoglycan in enzyme extracts of the cell layer. Therefore, the dermatan sulphate-rich-sulphated-glycosaminoglycan composition of the cellular environment on collagen gels compares favourably with that found in whole skin; in contrast, the heparan sulphate-rich trypsin extract of cells on plastic is indicative of significant, and possibly unfavourable, changes in matrix composition.

We have not so far carried out a detailed analysis of the polymeric structure of the designated 'DS' peak (Figs. 1 and 4). This assignment was based on the resistance to degradation of this component by chondroitinase AC and the complete degradation by chondroitinase ABC. However, Sjoberg *et al.* (1979) reported that the galactosaminoglycan component from human embryonic skin fibroblasts contained a high proportion of chondroitinase AC-sensitive GlcUA-GalNAc disaccharides. Our biopsies were taken from adults over 50 years old and so the differences observed may relate to the age of the donor.

The accumulation of sulphated glycosaminoglycans in the medium from cells on plastic (Fig. 2a) may reflect a deficiency in collagen synthesis and/or fibrillogenesis, so that appropriate binding sites for dermatan sulphate and heparan sulphate in the extracellular matrix are unavailable. Indeed, collagen fibrillogenesis may be defective in some cell cultures (Prockop *et al.*, 1977; Uitto & Prockop, 1974). From our findings we predict that the composition of trypsin and collagenase extracts of cultured cells on plastic will be strongly influenced by their capacity for constructing a collagenous matrix. In comparison with sulphated glycosaminoglycans, a much smaller proportion of the total hyaluronic acid produced by cells on collagen (45%) was found in the collagenase/trypsin extract (Fig. 2b), and about 40% of the hyaluronic acid was in the culture medium. This finding presumably reflects the low affinity of hyaluronic acid for collagen, in contrast

with the relatively high affinity demonstrated for sulphated glycosaminoglycans (Obrink, 1973; Greenwald, *et al.*, 1975).

The production, by cells on collagen gels, of heparan sulphate and dermatan sulphate species that differed in their elution characteristics on DEAE-cellulose from heparan sulphate and dermatan sulphate synthesized by cells on plastic was an unexpected finding (Fig. 4). Alterations in glycosaminoglycan chain length were not a contributing factor to these changes (Fig. 7). Since cells on collagen gels produced a heparan sulphate species that eluted from a DEAE-column at a lower salt concentration than cells cultured on plastic, whereas the dermatan sulphate peak from cells on collagen eluted at a higher salt concentration (Fig. 4) than cells on plastic, it seems that the changes were not the result of a modification in a metabolic process common to both glycosaminoglycans, such as an increase in the concentration of the endogenous sulphate pool or a decrease in rates of desulphation. Possibly plastic and collagen-gel substrata exert different selection pressures for subpopulations of sulphated glycosaminoglycans. DEAE-cellulose fractionates according to charge density, so these differences in chromatographic behaviour may arise through changes in the degree of sulphation of glycosaminoglycan chains. Decreased sulphation of heparan sulphate and chondroitin sulphate has been observed in SV40-virus-transformed AL/N mouse embryo cells (Winterbourne & Mora, 1978), and a decrease in sulphation probably accounts for the altered elution from DEAE-cellulose of heparan sulphate produced by SV40-virus-transformed 3T3 mouse cells (Underhill & Keller, 1975, 1977). Our results indicate that the environment of cultured cells can also influence glycosaminoglycan sulphation; the diverse nature of the changes observed on collagen (decreased sulphation of heparan sulphate, increased dermatan sulphate sulphation) suggest different regulatory mechanisms are involved from those operating after transformation with SV40 virus.

The difference observed in molecular size between heparan sulphate in the media (low molecular weight) and heparan sulphate of the pericellular domain (high molecular weight; Fig. 7b) has been reported previously for embryonic (Malmstrom *et al.*, 1975) and adult human skin fibroblasts (Hopwood & Dorfman, 1977). This difference is intriguing, since, in our studies, cells on both substrata produced low-molecular-weight heparan sulphate but the medium from cells on plastic contained 75% of the total heparan sulphate, whereas the medium contained only 15% of the heparan sulphate produced by cells on collagen gels. The low molecular weight of heparan sulphate in the medium suggests that it is a degradation product and the low con-

centration of heparan sulphate in the collagen-gel medium may indicate that heparan sulphate breakdown is inhibited by the binding of heparan sulphate to collagen fibres.

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