Specific association of iduronic acid-rich dermatan sulphate with the extracellular matrix of human skin fibroblasts cultured on collagen gels

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Human skin fibroblasts cultured on collagen gels produced two dermatan sulphate species, one, enriched in iduronic acid residues, that bound specifically to the collagenous fibres of the gel, the other, enriched in glucuronic acid, that accumulated in the culture medium. Collagen-binding and collagen-non-binding dermatan sulphates were also produced by cells grown on plastic surfaces, but in these cultures each constituent was released into the growth medium. Net synthesis of dermatan sulphate was 3-fold higher in cells maintained on collagen gels. In contrast, heparan sulphate synthesis was not influenced by the nature of the culture surface. The concentration of heparan sulphate in surface-membrane extracts was similar for cells grown on plastic and on collagen gels, but cells cultured on collagen showed a notable increase in the content of surface-membrane dermatan sulphate. The patterns of synthesis and distribution of sulphated glycosaminoglycans observed in skin fibroblasts maintained on collagen gels may reflect differentiated cellular functions.

The extracellular matrix plays an important role in the growth and development of cells and tissues (for review see Hay, 1981). In order to understand the mechanisms and pathways of informational flow between cells and their environment, it is essential that we learn more about the composition and metabolism of cell surfaces and the surrounding stromal elements. Cell-culture systems have been widely used to study the biochemistry of the pericellular domain, but, in view of the importance of the extracellular matrix *in vivo*, it seems desirable to provide cells *in vitro* with a biologically relevant substratum rather than conventional plastic surfaces. Three-dimensional gels of native collagen fibres have been used for this purpose and shown to exert a profound effect on cell proliferation (Liotta *et al.*, 1978; Schor, 1980), differentiation (Emerman & Pitelka, 1977) and migration (Stenn *et al.*, 1979). Also notable are the observations that sulphated glycosaminoglycan synthesis by chick-embryonic somites (Kosher & Church, 1975), breast epithelial cells (David & Bernfield, 1979) and abdominal skin fibroblasts (Gallagher *et al.*, 1980) is stimulated by culture on collagen gels.

Specific interactions between fibrous proteins and sulphated proteoglycans are an essential feature of the molecular organization of the extracellular

matrix (Comper & Laurent, 1978; Lindahl & Höök, 1978). Dermatan sulphate chains bind strongly to collagen, whereas chondroitin sulphate shows a low collagen affinity (Öbrink, 1973; Öbrink *et al.*, 1975). However, since iduronic acid and glucuronic acid, traditionally considered to be the hexuronic acid constituents of dermatan sulphate and chondroitin sulphate respectively, may be found in the same saccharide chain, it is conceivable that these collagen-binding glycans may express a distinctive 'co-polymeric' structure. Characteristic orthogonal arrays of dermatan sulphate proteoglycans around collagen fibres have been detected *in situ* by electron-microscopic studies of rat tail tendon (Scott *et al.*, 1981), the precise arrangement of the proteoglycans presumably reflecting specific inter-molecular interactions with collagen. Heparan sulphate proteoglycans are also important constituents of the extracellular matrix, especially in basement membranes (David & Bernfield, 1982; Kanwar & Farquhar, 1979; Hassel *et al.*, 1980). Heparan sulphate has also attracted particular attention because of its association with the surface membranes of many cell types (Kraemer, 1979). The core protein of heparan sulphate proteoglycan may contain a hydrophobic region that spans the lipid bilayer of the plasma membrane (Kjellen *et al.*,

1981; Norling *et al.*, 1981) in a manner analogous to that described for several other integral membrane glycoproteins (Gahmberg, 1981). Some of the recently described properties of heparan sulphate, such as its ability to bind to fibronectin and laminin (Yamada *et al.*, 1980; Del Rosso *et al.*, 1981) and the self-associating properties of cognate heparan sulphate chains (Fransson *et al.*, 1980), point to potentially important roles in extracellular-matrix organization and in mediating interactions between cells and their environment.

The foregoing evidence suggests that the localization of sulphated glycosaminoglycans in specific regions of the pericellular domain is an important feature in relation to their function. It will therefore be essential to understand structural and environmental factors that influence glycosaminoglycan compartmentalization. In the present investigation we have grown human foreskin fibroblasts on plastic and collagen-gel substrata and examined the distribution and structure of glycosaminoglycans in the extracellular matrix and the cell-surface membrane. These two compartments were identified experimentally by using two different procedures: either the cultures were extracted first with collagenase, to prepare material bound to the collagenous matrix, followed by trypsin treatment of the derived cell suspension to yield cell-surface-associated glycosaminoglycans, or cultures were treated first with a solution of Triton X-100 to extract the membrane fraction and then with 4.0 M-guanidinium chloride to extract glycosaminoglycans in the residual extracellular matrix. The results demonstrate a specific association of iduronic acid-rich co-polymeric dermatan sulphate with collagen. Furthermore, growth on a collagen-gel substratum leads to a significant stimulation of dermatan sulphate synthesis, together with an alteration in the sulphated glycosaminoglycan composition of the cell-surface membrane.

Materials and methods

Materials

D-[1-¹⁴C]Glucosamine (sp. radioactivity 50–60 mCi/mmol), D-[1-³H]glucosamine (sp. radioactivity 2–5 Ci/mmol) and Na₂³⁵SO₄ (in aqueous solution, 25–40 Ci/mg) were obtained from Amer sham International. High-purity collagenase (type VII), papain (2× crystallized) and trypsin (3× crystallized) were from the Sigma Chemical Co., and chondroitin ABC lyase and chondroitin AC lyase were purchased from Miles Chemicals. DEAE-cellulose (DE-52) was from the Whatman Chemical Co., and Bio-Gel P-10 (–400 mesh) and Bio-Gel P-4 (200–400 mesh) were from Bio-Rad Laboratories. Chemicals were AnalaR or AristaR grade from BDH Chemicals.

Cell culture

We have previously described methods for the preparation of native collagen gels and for the establishment and maintenance of cultures of human skin fibroblasts (Gallagher *et al.*, 1980; Schor, 1980). Cell cultures were initiated from explants of human foreskin from donors aged from 2 to 8 years.

Extraction of cell-surface and extracellular-matrix glycosaminoglycans

Confluent cultures of human skin fibroblasts on plastic or collagen-gel substrates were incubated for 48 h with [³H]glucosamine (5 μCi/ml) and Na₂³⁵SO₄ (10 μCi/ml). On occasions cells on plastic were incubated with [³H]glucosamine only (5 μCi/ml) and cells on collagen with [¹⁴C]glucosamine (1 μCi/ml). After incubation the medium was removed and, for cells grown on collagen, a Pasteur pipette was carefully inserted between the collagen gel and the culture-vessel wall. The gel was separated from the culture vessel by moving the pipette tip around the gel circumference. Interstitial fluid of the gel entrapped within the three-dimensional lattice of hydrated collagen fibres was then expressed by squeezing the gel with a spatula. This caused the gel to collapse to a thin film of collagen with cells still attached. The expressed fluid (essentially equivalent to culture medium) was pooled with the spent medium overlying the gel surface. The collapsed gel was washed twice in 3 ml of Hanks BSS balanced salt solution (GIBCO), and each wash was pooled with the spent medium. The gel was then extracted with either enzymes or detergent and denaturing solutions to prepare extracellular and membrane-bound glycosaminoglycans.

In the enzyme procedure the collapsed gels plus attached cells were first incubated with collagenase to prepare collagen-bound (i.e. extracellular-matrix) glycosaminoglycans, followed by trypsin to extract surface-membrane-associated material. Collagenase was added to the gels at a concentration of 0.05 mg/ml in Eagle's minimal essential medium (GIBCO) containing serum albumin (0.1 mg/ml) and incubated at 37°C in CO₂/air (1:19) with occasional agitation; 3 ml of collagenase solution was added for each 60 mm-diameter gel. After 2½–3 h the collagen gel had dissolved to yield a suspension of skin fibroblasts. The cells were pelleted by centrifugation (1000 rev./min for 5 min in an MSE bench centrifuge), the supernatant was removed and the cell pellet was washed by suspension in phosphate-buffered saline (10 mM-sodium phosphate buffer, pH 7.2, containing 0.15 M-NaCl) and re-centrifugation. The supernatant was pooled with the first collagenase supernatant and the cells resuspended in phosphate-buffered saline containing 50 μg of trypsin/ml and 2 mM-EGTA, at a density of about

2×10^6 cells/ml. After 15 min incubation at 37°C , 0.25 vol. of soya-bean trypsin inhibitor (0.25 mg/ml) was added and the solution was centrifuged. The supernatant was removed, the cell pellet was washed once with phosphate-buffered saline and after a further centrifugation the supernatant was pooled with the trypsin extract. On occasions the cell pellet was then treated with papain, as described below.

In some experiments portions of radiolabelled culture medium that had been incubated for 48 h with cells growing on plastic were transferred to collagen gels. After a 24 h incubation medium plus interstitial fluid was removed and the washed collagen gels were dissolved with collagenase as described above.

In the chemical extraction method the collapsed washed gels were suspended in phosphate-buffered saline containing Triton X-100 (1%, w/v), bovine serum albumin (0.1 mg/ml), soya-bean trypsin inhibitor (0.1 mg/ml) and 5 mM-EDTA (3 ml of solution per gel) and incubated with gentle stirring at 37°C for 2 h. After this time, microscopic examination indicated complete cell lysis, although the gel structure was apparently unaffected. The Triton solution, containing cell-surface membrane and intracellular glycosaminoglycans, was removed and the gels were washed three times, once with Triton solution and then two further times with phosphate-buffered saline. Finally, the intact gels were treated either with 4.0 M-guanidinium chloride in 20 mM-sodium phosphate buffer, pH 6.8 (3 ml per gel), overnight at 4°C , or with collagenase as described above. Both procedures solubilized the collagen gel and the associated glycosaminoglycans.

Only enzyme extraction was used for harvesting glycosaminoglycans from cells grown on plastic. After incubation with labelled precursors, the medium was removed and the cell monolayer was washed twice with phosphate-buffered saline. Each dish was then incubated for 20 min at 37°C with 3 ml of collagenase solution. The enzyme solution was removed and the cells, all of which remained attached to the substratum after collagenase treatment, were washed twice with phosphate-buffered saline; the first wash was pooled with the collagenase extract. The cell monolayer was then incubated with 3 ml of trypsin solution for 20 min at 37°C . This procedure yielded a single-cell suspension, and the trypsin-extracted material was harvested as described for cells grown on collagen gels.

Papain digestion

The culture medium, the collagenase extract and the trypsin-extracted fractions were dialysed exhaustively against distilled water and concentrated by rotary evaporation to 5–10 ml. These samples

were then dialysed against the papain digestion buffer (50 mM-sodium phosphate buffer, pH 6.8, containing 1 M-NaCl, 5 mM-cysteine and 1 mM-EDTA). The cell pellet was also suspended in this buffer, and both of the extracts were incubated with papain ($2 \times$ crystallized; Sigma type III; 0.1 mg/ml) overnight at 60°C . A further $50 \mu\text{g}$ of papain/ml was then added, and the incubation was continued for a further 4 h. The digest was cooled on ice, and the reaction was terminated by addition of 0.1 vol. of ice-cold 50% (w/v) trichloroacetic acid. After standing on ice for 2 h, the precipitate was removed by centrifugation and washed once with 5 ml of ice-cold 5% (w/v) trichloroacetic acid. The wash was added to the first supernatant. The supernatants were then dialysed exhaustively against distilled water, concentrated to about 5 ml by rotary evaporation and then fractionated on a DEAE-cellulose column.

Isolation and characterization of glycosaminoglycans

Samples from papain digests were applied to a DEAE-cellulose (DE-52) column (5 cm $\times$ 1 cm) equilibrated in 20 mM-sodium phosphate buffer, pH 6.8, containing 0.15 M-NaCl. Elution was achieved with a linear gradient of 0.15–0.8 M-NaCl in 20 mM-sodium phosphate buffer, pH 6.8, at a flow rate of 20 ml/h. A total volume of 200 ml of eluting buffer was passed through the column, and 2 ml fractions were collected. Radiolabelled peaks were dialysed, concentrated by rotary evaporation and freeze-dried. Recovery of radioactivity from the DEAE-cellulose column exceeded 85% in all cases.

The Triton X-100 extracts of the fibroblast monolayers were dialysed against phosphate-buffered saline containing 0.1% (w/v) Triton X-100. The non-diffusible material was then fractionated on DEAE-cellulose as described above, except that 0.1% Triton X-100 was included in the eluting buffers. The guanidinium chloride extracts were dialysed against 6 M-urea in phosphate-buffered saline and then fractionated on DEAE-cellulose with eluting buffers prepared in 6 M-urea. Triton X-100 and guanidinium chloride extracts were not treated with papain before DEAE-cellulose chromatography.

Samples were then subjected to the following degradative procedures: chondroitin ABC lyase (0.1 unit/ml) in 50 mM-Tris/acetate buffer, pH 8.0, for 4 h at 37°C (Saito *et al.*, 1968) and chondroitin AC lyase (0.3 unit/ml) in 50 mM-sodium acetate buffer, pH 6.0, for 4 h at 37°C (Hiyama & Okada, 1975). The activity of each enzyme was checked by monitoring the increase in absorbance at 232 nm of a 1 mg/ml solution of chondroitin sulphate. The degradation products were analysed by exclusion gel chromatography on a column of Bio-Gel P-4 of bed

Fig. 1. Fractionation by DEAE-cellulose chromatography of glycosaminoglycans produced by human skin fibroblasts

Confluent monolayers of human skin fibroblasts cultured on plastic or collagen-gel substrata were incubated with [^3H]glucosamine and [^{14}C]glucosamine respectively. Radioactive glycosaminoglycans from equivalent culture compartments (medium, collagenase and trypsin extracts) from cells on each substratum were combined and fractionated on a DEAE-cellulose column eluted with a linear gradient of 0.15–0.8 M NaCl in 20 mM-sodium phosphate buffer, pH 6.8. For full experimental details see the text. In the radioactivity elution profiles, peak I is hyaluronic acid, peak II is heparan sulphate and peak III is dermatan sulphate. These assignments were made principally on the basis of the sensitivity of radiolabelled material in each peak to degradation by chondroitin ABC lyase, testicular and streptococcal hyaluronidase and

size 75 cm $\times$ 1.25 cm. The gel was equilibrated and eluted with 0.5 M-NH $_4$ HCO $_3$. Fractions of volume 2 ml were collected at a flow rate of 8–10 ml/h. Recoveries of radioactivity were 90–95%.

Heparan sulphate was identified by susceptibility to degradation by the low-pH nitrous acid procedure of Shively & Conrad (1976). The scission products were analysed by gel chromatography on Bio-Gel P-4: material described as heparan sulphate yielded di- and tetra-saccharides as the main breakdown products of nitrous acid treatment. [^{35}S]Sulphate incorporated by the cells into heparan sulphate was released by nitrous acid as free [^{35}S]sulphate derived from *N*-sulphate groups and as *O*-sulphated di- and tetra-saccharides (results not shown).

Hyaluronic acid produced by skin fibroblasts gave a characteristic elution profile on DEAE-cellulose chromatography (see Fig. 1), was sensitive to degradation by streptococcal hyaluronidase and testicular hyaluronidase and had an electrophoretic mobility identical with that of a commercial preparation of hyaluronic acid (results not shown).

Radioactivity determinations

Samples (maximum volume 0.5 ml) were mixed with 5 ml of Aquasol II and their radioactivities counted on a Beckman series 7500 with an external-standard 'H' number quench correction. The d.p.m. values were plotted and stored by using a Hewlett Packard 1845 computer (Gallagher *et al.*, 1980).

Results

Distribution of glycosaminoglycans

Collagenase and trypsin extracts. Fractionation of radiolabelled material by NaCl-gradient elution from DEAE-cellulose produced three glycosaminoglycan components. In Fig. 1 profiles are shown for admixtures of [^3H]glucosamine-labelled glycosaminoglycans from cells grown on plastic with [^{14}C]glucosamine-labelled material from cells grown on collagen gels. In the chromatograms (Fig. 1) peak I was identified as hyaluronic acid, peak II was

nitrous acid. Full details are given in the Materials and methods section. The profiles show glycosaminoglycans in the medium (a), in the collagenase extract (b) and in the trypsin extract (c). The scales on the vertical axes are different in each panel. Identical proportions of material in each compartment were fractionated, so the d.p.m. values for the individual compartments are directly comparable. Hyaluronic acid peak maximum values in panel (a) were 220×10^4 for the ^3H label and 80×10^3 for ^{14}C . —, ^3H radioactivity, cells grown on plastic; ---, ^{14}C radioactivity, cells grown on collagen gels.

mainly heparan sulphate and in peak III dermatan sulphate was the major constituent (see the legend to Fig. 1). When cells were grown on plastic the medium was the main source of sulphated glycosaminoglycans (Fig. 1a), although the trypsin extracts of these cells contained a considerable proportion of heparan sulphate (Fig. 1c), which was the major sulphated species found in association with the cell surface. Relatively low concentrations of glycosaminoglycans were found in the collagenase extract of cells grown on plastic (Fig. 1b). However, when cells were grown on collagen gels the collagenase extract was the richest source of dermatan sulphate, and this extract also contained about one-third of the heparan sulphate (Fig. 1b). In the trypsin extract of cells grown on collagen about equal amounts of heparan sulphate and dermatan sulphate were found, in contrast with the corresponding fraction from cells grown on plastic; this fraction, as already noted above, was considerably enriched in heparan sulphate (Fig. 1c). It is also notable that heparan sulphate was the major sulphated glycosaminoglycan produced by skin fibroblasts growing on plastic, whereas dermatan sulphate was the predominant sulphated species when cells were grown on collagen (Fig. 4). This point is emphasized by the fact that most of the sulphated glycosaminoglycans produced by cells grown on collagen were found in the collagenase extract (considerably enriched in dermatan sulphate), whereas cells grown on plastic released most of their sulphated glycosaminoglycans into the culture medium, which was enriched in heparan sulphate (Fig. 4). Hyaluronic acid was found predominantly in the culture medium irrespective of the type of culture surface (Figs. 1 and 4).

In the DEAE-cellulose chromatograms hyaluronic acid (peak I) was eluted at an identical NaCl concentration (0.3 M-NaCl) in each extract. In the collagenase and trypsin extracts the NaCl concentrations required for peak maximum elutions of heparan sulphate (peak II) and dermatan sulphate (peak III) were identical, at 0.42 M-NaCl and 0.5 M-NaCl respectively (Figs. 1b and 1c). Heparan sulphate and dermatan sulphate in the culture medium (Fig. 1a) were eluted at slightly higher NaCl concentrations than corresponding components in the enzymic extracts. All extracts were treated with papain before DEAE-chromatography, in order to study glycosaminoglycans rather than proteoglycans. However, it is possible that, in the culture medium, high concentration of serum glycoproteins, which would include known proteinase inhibitors such as α_2 -macroglobulin, may have resulted in incomplete proteolysis of the protein core of the proteoglycans. Partially degraded proteoglycans may have different elution properties on DEAE-cellulose by comparison with the individual glycosaminoglycan chains.

Fig. 2. DEAE-cellulose chromatography of glycosaminoglycans in Triton X-100 and guanidinium chloride extracts of fibroblasts grown on collagen gels

Confluent monolayers of cells grown on collagen gels were incubated with $\text{Na}_2^{35}\text{SO}_4$ and $[^3\text{H}]\text{glucosamine}$. After incubation, the medium and interstitial fluid were removed and the washed gels were extracted sequentially with 1% (w/v) Triton X-100 and 4.0 M-guanidinium chloride (see the Materials and methods section). Extracts were analysed by DEAE-cellulose chromatography. Peaks I, II and III are as identified in Fig. 1. (a) Triton X-100 extracts; (b) guanidinium chloride extract. —, ^3H radioactivity; ---, ^{35}S radioactivity. ^{35}S axes are different.

Triton X-100 and guanidinium chloride extracts.

The above findings suggest that the composition of surface-membrane-associated glycosaminoglycans, presumed to be represented by the trypsin extract, was modified by growth on collagen. To check this possibility further an alternative preparation procedure was used. Instead of first extracting extracellular matrix glycosaminoglycans with collagenase, followed by trypsin to prepare surface-membrane glycosaminoglycans as described above, cells on collagen were treated first with the non-ionic detergent Triton X-100, which dissolved the cells and released membrane-associated glycosaminoglycans. The cell-free collagen gel remaining after Triton X-100 extraction was then treated with either 4.0 M-guanidinium chloride or collagenase, both procedures leading to complete dissolution of the gel

Fig. 3. Binding to collagen gels of glycosaminoglycans released into the culture medium by cells grown on plastic

Cells cultured on plastic surfaces were incubated for 48 h with [^3H]glucosamine and $\text{Na}_2^{35}\text{SO}_4$. The culture medium was divided equally, half being fractionated directly on DEAE-cellulose, the other half transferred to collagen gels and incubated at 37°C for 24 h. The medium and interstitial fluid were separated from the collagen gel and the washed gel was then dissolved by using collagenase. Collagen-gel-bound and -non-bound glycosaminoglycans were then separated by DEAE-cellulose chromatography. For full experimental details see the text. (a) Medium from cells on plastic; (b) collagenase extract containing collagen-binding glycosaminoglycans in the medium shown in (a); (c) glycosaminoglycans in medium shown in (a) that remained in the medium after transfer to collagen gels. —, ^3H radioactivity; ---, ^{35}S radioactivity.

and release of the collagen-bound or extracellular-matrix glycosaminoglycans. When material in these extracts was fractionated by DEAE-cellulose chromatography, the composition of the Triton X-100 extract was very similar to that of the trypsin extract (cf. Fig. 2a and Fig. 1c, broken lines, for cells grown on collagen). Furthermore the subsequent guanidinium chloride or collagenase extracts (Fig. 2b) were virtually identical in composition (i.e. considerably enriched in dermatan sulphate) with the collagenase-extracted material prepared by addition of this enzyme before removal of surface-membrane-bound glycosaminoglycans with trypsin (Fig. 1b, broken line). The Triton X-100 extract would of course release intracellular as well as cell-surface components, but we have found, in accord with other workers (Malmström *et al.*, 1975; Gill *et al.*, 1981), that, after trypsin treatment to remove cell-surface glycosaminoglycans, the residual cell pellet contains less than 5% of the total ^{35}S -labelled glycosaminoglycans produced by human skin fibroblasts. Hence the contribution from the intracellular pool will be small, and the [^{35}S]sulphated species in the Triton X-100 extract should be derived mainly from the cell-surface membrane.

Collagen-binding glycosaminoglycans from cells grown on plastic surfaces

In evaluating the effects of the culture substratum on glycosaminoglycan distribution, it was desirable to test whether cells grown on plastic surfaces would produce collagen-binding glycosaminoglycans. Unfractionated spent medium from confluent fibroblast monolayers, incubated for 48 h with [^3H]glucosamine and $\text{Na}_2^{35}\text{SO}_4$, was transferred to collagen gels and incubated at 37°C for 24 h. The medium plus interstitial fluid was separated from the collagen gel, and the gel was then dissolved with collagenase. Analysis by DEAE-cellulose chromatography showed that of the two sulphated glycosaminoglycans the separated medium contained mainly heparan sulphate (Fig. 3c), whereas the collagenase extract was predominantly dermatan sulphate (Fig. 3b). The overall composition was therefore similar to collagenase extracts of collagen gels used as substrata for the cultured fibroblasts (Figs. 1b and 2b). Since the culture medium that was used in the medium transfer experiment was slightly enriched in heparan sulphate (Fig. 3a), the results provide an impressive demonstration of the selective binding of a major fraction of fibroblast-derived dermatan sulphate to collagen. The findings also indicate that the presence of the preformed collagen gel is sufficient to account for the high content of dermatan sulphate in the extracellular-matrix compartment of cells grown on collagen (Figs. 1 and 2).

When the ^{35}S -labelled glycosaminoglycans that failed to bind to collagen were transferred to a

Fig. 4. Net synthesis and distribution of glycosaminoglycans produced by skin fibroblasts on plastic and collagen-gel substrates

Confluent cultures were incubated for 48 h with [3 H]glucosamine and $\text{Na}_2^{35}\text{SO}_4$. Glycosaminoglycans were then determined in the culture medium (▨), the collagenase extract (▤) and the trypsin extract (□). For full experimental details see the text. For simplicity only the ^{35}S -radioactivity scale is shown.

second set of collagen gels, very little material (<2%) was found in the collagenase extracts. Hyaluronic acid remained principally with the medium fraction in the medium-transfer experiment (Fig. 3).

Stimulation of sulphated glycosaminoglycan synthesis

When the human foreskin fibroblasts were grown on collagen, a 3-fold increase in net synthesis of sulphated glycosaminoglycans occurred, and this stimulation was due mostly to a selective effect on the production of dermatan sulphate (Fig. 4). Dermatan sulphate is the main sulphated glycosaminoglycan in mammalian skin (Toledo & Dietrich, 1977; Damle *et al.*, 1982). By primarily stimulating the formation of dermatan sulphate in cultured skin fibroblasts, collagen gels may be promoting the expression of patterns of glycosaminoglycan biosynthesis that occur *in vivo*.

Co-polymeric structure of dermatan sulphate

The co-polymeric structure of dermatan sulphate produced by skin fibroblasts and prepared by DEAE-cellulose chromatography was examined by subjecting samples to degradation by chondroitin

AC lyase and examining the products by exclusion gel chromatography on Bio-Gel P-4. Di-, tetra- and hexa-saccharide fragments of glycosaminoglycans were clearly resolved by this technique. Fig. 5 shows a comparison of the degree and patterns of degradation of collagen-binding dermatan sulphate (material found in the collagenase extract) with that of corresponding material that did not bind to collagen (components isolated in the medium) when cells were grown on collagen gels (see Fig. 1). It is clear that the medium-derived material was more extensively degraded than was that found in the collagenase extract: the dermatan sulphate species in the medium yielded a disaccharide peak as the major degradation product, in contrast with the relatively small proportion of disaccharides in the dermatan sulphate that bound to collagen (cf. Figs. 5a and 5b). In both chromatograms radioactive material in the void volume was almost entirely degraded to disaccharides by chondroitin ABC lyase. Similar degradation patterns were seen when collagen-binding and collagen-non-binding dermatan sulphate from the medium-transfer experiment (Fig. 3) were treated with chondroitin AC lyase (see also Table 1).

Chondroitin AC lyase is an endo-hexosaminidase that will cleave dermatan sulphate only between *N*-acetylgalactosamine (GalNAc) and glucuronic acid (GlcA) residues: *N*-acetylgalactosamine residues linked to iduronic acid (IdoA) residues are resistant to the enzyme. The glycan fragments formed have the general formula:

where n is the number of disaccharide units in the fragment that contains iduronic acid.

Disaccharides as degradation products of chondroitin AC lyase treatment of dermatan sulphate are therefore derived from regions of the polymer chain that consist of sequences of two or more glucuronic acid-containing repeat units. Tetra-, hexa- and higher oligo-saccharide fragments represent regions of increasing length of repeats of iduronic acid-containing units flanked by glucuronic acid residues.

When fragments from chondroitin AC lyase degradation of dermatan sulphate are separated by gel chromatography as indicated in Fig. 5, the percentages of glucuronic acid and iduronic acid residues in the original polymer chain may be calculated as described by Malmström *et al.* (1975). Results for a variety of dermatan sulphate extracts are given in Table 1. The data demonstrate the preponderance of iduronic acid residues in collagen-binding dermatan sulphate, in contrast with the high proportion of glucuronic acid residues in the species that do not bind to collagen (i.e. the

Fig. 5. Bio-Gel P-4 chromatography of chondroitin AC lyase-treated dermatan sulphate

Dermatan sulphate isolated from skin fibroblast cultures was treated with chondroitin AC lyase (—) or chondroitin ABC lyase (---), and the products were fractionated on a Bio-Gel P-4 column. For full experimental details see the text. The column was calibrated with saccharide fragments formed by treatment of chondroitin sulphate and hyaluronic acid with chondroitin ABC lyase and testicular hyaluronidase. Low-molecular-mass peaks I, II and III represent di-, tetra- and hexa-saccharide fragments of dermatan sulphate. Plots show the degradation patterns of dermatan sulphate from three compartments: the medium of

dermatan sulphate fraction distributed to the medium in the presence of collagen gels).

Discussion

Glycosaminoglycans were found in the culture medium, the extracellular-matrix extracts and the cell-surface extracts of cultured human skin fibroblasts, but their distribution into these compartments varied according to the nature of the culture substratum (Figs. 1, 2 and 4). Most of the dermatan sulphate was found in the extracellular-matrix compartment (i.e. collagenase or guanidinium chloride extracts; Figs. 1 and 2) when cells were grown on collagen gels, and the binding sites for this extracellular material were most probably provided by the native collagen fibres that form the framework of the gel. Some dermatan sulphate could, of course, be bound to collagen produced by the cultured cells, and our methods would not identify this fraction. It was significant, however, that most of the dermatan sulphate released into the medium by cells grown on plastic would bind to collagen gels (Fig. 3), and

Table 1. Percentages of iduronic acid and glucuronic acid residues in dermatan sulphate

Dermatan sulphate was treated with chondroitin AC lyase, and the products were fractionated on Bio-Gel P-4 to determine the percentages of iduronic acid and glucuronic acid residues (see Fig. 5).

Fraction	Iduronic acid residues (%)	Glucuronic acid residues (%)
Medium		
Cells grown on plastic	59	41
Cells grown on collagen	27	73
Collagenase extract of cells grown on collagen	69	31
Medium transfer*		
Medium	31	69
Collagenase	67	33
Guanidinium chloride extract† (Fig. 2)	70	30

* Data are for dermatan sulphate remaining in the medium after transfer to collagen gels of culture medium containing radioactive dermatan sulphate produced by cells grown on plastic: data for the corresponding collagen-gel-bound dermatan sulphate (the collagenase extract) are also given (see Fig. 3).

† The guanidinium chloride extract of the collagen matrix was prepared from collagen gels after extraction of the cell monolayer with Triton X-100 (Fig. 2).

cells grown on plastic (a), the medium (b) and the collagenase extract (c) of cells grown on collagen gels. The proportions of iduronic acid and glucuronic acid residues derived from these plots are given in Table 1.

collagenase treatment of such cells released very little sulphated glycosaminoglycan (Fig. 1), indicative of little collagen-fibre deposition.

The collagen-binding dermatan sulphate was a co-polymeric macromolecule in which iduronic acid constituted about 70% of the hexuronic acid residues (Fig. 5 and Table 1). These findings are significant in relation to earlier biophysical data on the isolated macromolecules, in which collagen binding was associated with dermatan sulphate chains, defined in the traditional way as containing only iduronic acid residues, whereas the glucuronic acid-containing chondroitin sulphate showed little collagen affinity (Öbrink, 1973; for review see Lindahl & Höök, 1978). Our findings show clearly that glucuronic acid residues are present in collagen-binding dermatan sulphate, but the residue frequency is low (Table 1), and chondroitin AC lyase treatment showed that the individual glycosaminoglycan chains contained few repeat sequences of glucuronic acid-containing disaccharides (Fig. 5). These structural features clearly distinguish the collagen-binding species from the collagen-non-binding dermatan sulphate, which was enriched in glucuronic acid residues (Table 1) and contained a correspondingly high frequency of glucuronic acid-containing repeats (Fig. 5). The production of collagen-binding dermatan sulphate by skin fibroblasts in culture and the expression of this important biological property when cells are grown on collagen gels provide a suitable system for studying factors that influence the synthesis and degradation of glycosaminoglycans in the extracellular matrix.

The respective roles of the core protein and the glycosaminoglycan in determining the collagen affinity of dermatan sulphate proteoglycans have not yet been fully investigated, but we have established that after Pronase treatment the derived glycan chains will still bind to collagen gels (J. T. Gallagher, N. Gasiunas & S. Schor, unpublished work). However, high-affinity binding and the precise disposition of dermatan sulphate along the collagen fibres (Scott *et al.*, 1981) may require the native proteoglycan structure. In this connection it is notable that human embryonic skin fibroblasts produce iduronic acid-rich and glucuronic acid-rich dermatan sulphate chains that are bound to different protein cores (Carlstedt *et al.*, 1981). Therefore the protein constituent may determine the fine structure of the glycosaminoglycan chain, with the latter providing the necessary biological specificity.

Several reports have demonstrated that heparan sulphate is the major sulphated glycosaminoglycan of cell surfaces of cultured human skin fibroblasts (Malmström *et al.*, 1975; Kleinman *et al.*, 1975; Vogel & Kelley, 1977; Sjöberg *et al.*, 1979), and our findings when human foreskin fibroblasts were

grown on a plastic substrate (Fig. 1) were consistent with these results. On a collagenous substrate, however, heparan sulphate was no longer the most abundant sulphated species at the fibroblast surface, and, as determined radiochemically, dermatan sulphate was found in equal concentration to heparan sulphate in the plasma-membrane extracts (Figs. 1 and 2). Although both enzymic and chemical methods gave similar results, it must be borne in mind that there may be no discrete interface between the cell surface and the extracellular matrix, and in defining the glycosaminoglycan composition of these domains we are essentially making an empirical demarcation based on procedures designed for preferential extraction from one compartment rather than the other. Nevertheless, from the findings with two different experimental approaches, it seems reasonable to conclude that a change occurs in the composition of glycosaminoglycans bound to the surface membrane when cells are grown on collagen, and the increase in concentration of dermatan sulphate may more closely approximate to the plasma-membrane composition of skin fibroblasts in the collagenous environment of the dermis.

Although the synthesis of iduronic acid-rich and glucuronic acid-rich dermatan sulphate proteoglycans may be independently regulated (Vogel & Sapien, 1982), the stimulation of dermatan sulphate synthesis when fibroblasts were grown on collagen gels was not specific for the iduronic acid-rich collagen-binding species. This conclusion is evident from inspection of the chromatograms in Figs. 1 and 4. When cells were cultured on collagen, the ratio of collagen-binding to non-binding dermatan sulphate [i.e. collagenase-extractable (Fig. 1b) to medium-derived material (Fig. 1a)] was approximately 2:1. This proportion was in close agreement with the amount of collagen-binding dermatan sulphate found in the spent culture medium from cells grown on plastic surfaces (Fig. 3: about 65% of the dermatan sulphate bound to collagen). Thus the relative amounts of the two dermatan sulphate species produced were similar on both substrata. David & Bernfield (1979) suggested that the net increase in sulphated glycosaminoglycan synthesis of breast epithelial cells maintained on collagen gels was due to a decrease in the rate of degradation that followed from the binding of glycosaminoglycans to collagen. This conclusion is clearly inconsistent with our findings, and it may be that collagen substrates mediate changes in dermatan sulphate metabolism at the biosynthetic level.

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