CHANGES IN THE SYNTHESIS, DISTRIBUTION AND SULPHATION OF GLYCOSAMINOGLYCANS OF CULTURED HUMAN SKIN FIBROBLASTS UPON ASCORBATE FEEDING

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SUMMARY
The effect of ascorbic acid on the synthesis, distribution and sulphation of glycosaminoglycans by human skin fibroblasts has been examined. Medium was supplemented with ascorbate over several days, and cultures incubated with [3H]glucosamine and Na2[35SO4] for 48 h, followed by analysis of the glycosaminoglycans in the medium, in collagenase and trypsin extracts, and in cell fractions. Ascorbate feeding resulted in a reduction in hyaluronate synthesis, which was the main [3H]-labelled component and was distributed mainly in the medium fractions. Sulphated glycosaminoglycans showed a reduction in incorporation of [3H] label, but increased sulphation following ascorbate feeding. In control cultures 53% of [3H]-labelled sulphated glycosaminoglycans and 63% of [35S]-labelled glycosaminoglycans were present in the medium fraction, while in ascorbate-fed cultures, 41% of [3H] label and 38% [35S] label were incorporated into medium-sulphated glycosaminoglycans. Ascorbate also caused an increase in cell density and in collagen production and deposition.

INTRODUCTION
The biochemical nature of the extracellular matrix is now known to play an important role in cell shape, migration, control of cell growth and differentiation. Three of the major components of the extracellular matrix, namely fibronectin, collagen and glycosaminoglycans, have been extensively studied. Fibronectins have been shown to play an important role in cell adhesion (Grinnell, 1978), spreading, morphology and migration (Ali & Hynes, 1978). Glycosaminoglycans are components of the pericellular envelope of virtually all mammalian cells (Dietrich, Sampaio & Toledo, 1976), the composition of which varies depending on the stage of development of the tissue (Dietrich, Sampaio, Toledo & Cassaro, 1977), during the passage and senescence of cultured cells (Vannucchi & Chiarugi, 1977), after transformation (Johnston, Keller & Keller, 1979), and during tissue regeneration (Edward, Long, Watson & Williamson, 1980), while Kraemer & Tobey (1972) showed a loss of heparan sulphate from the cell surface preceding cell division. Collagen substrates were found to enhance the expression of a differentiated phenotype in cultured cells (Hay, 1977), while Gallagher, Gasiunas & Schor (1980) demonstrated increased synthesis of glycosaminoglycans by human skin fibroblasts grown on collagen gels.

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compared to those cultured on plastic. Human skin fibroblasts grown on plastic substrata in the presence of ascorbic acid synthesize fibrous collagen, which is maximal after the cells reach confluence, when an extensive extracellular matrix is deposited (Russell, Russell & Trupin, 1981).

In view of these observations, it appears that multiple interactions of fibronectin, collagen and glycosaminoglycans may play an important role in the deposition of these substances into an insoluble extracellular matrix. To investigate the possible influence of endogenously synthesized collagen and ascorbate on the synthesis and deposition of glycosaminoglycans, we analysed the glycosaminoglycans of the medium, a collagenase extract, a trypsin extract and cell-associated fractions of human skin fibroblasts grown in the presence and absence of ascorbate.

MATERIALS AND METHODS

Materials

Eagle's minimal essential medium, heat-inactivated foetal calf serum, penicillin/streptomycin, L-glutamine and non-essential amino acids were obtained from Gibco-Biocult, Paisley, Scotland. Chondroitinase AC from Arthrobacter aurescens, chondroitinase ABC from Proteus vulgaris, Pronase E from Streptomyces griseus, bovine pancreatic ribonuclease A and deoxyribonuclease I, highly purified collagenase Sigma type VII from Clostridium histolyticum, and soybean trypsin inhibitor type 1-s were purchased from Sigma Chemical Company, Poole, Dorset, England. Hyaluronidase from Streptomyces hyalurolyticus was from Miles Laboratories Ltd, Slough, England, while twice-crystallized trypsin from bovine pancreas was obtained from Armour Pharmaceutical Company Ltd, Eastbourne, England. D-[6-3H]glucosamine hydrochloride (24.8 Ci/mmol) and Na2SO4 (780 mCi/mmol) were from Amersham International Ltd, Amersham, England, while cetylpyridinium chloride and ascorbic acid were purchased from British Drug Houses Ltd, Poole, England. Sephaphore III cellulose acetate strips were from Gelman Instrument Company, Ann Arbor, MI, U.S.A. All other reagents were of analytical grade.

Cell culture

Human skin fibroblasts (obtained at abortion from 26-week gestation foetuses passage 4-8) were grown in Eagle's minimal essential medium supplemented with 10% heat-inactivated foetal calf serum, 1 mm-sodium pyruvate, non-essential amino acids, 2 mm-L-glutamine, penicillin (100 units/ml) and streptomycin (100 µg/ml) at 37°C in an atmosphere of 5% CO2 in air. For radio-labeling studies, cells were seeded in 175 cm2 tissue-culture flasks (NUNC) at an initial density of 1 X 10^6 cells/flask. The medium was changed every 3 days and one flask received ascorbic acid (50 µg/ml) daily (Furcht, Wendelschafer-Crabb, Mosher & Foidart, 1980), 24 h after seeding. Following 7 days incubation, the confluent cultures were incubated with D-[3H]glucosamine (5 µCi/ml) and Na2SO4 (20 µCi/ml) for 48 h.

Collagenase and trypsin extracts of cultures

After 48 h incubation with radiolabels, the medium was removed and the cell layer was washed twice with 10 ml phosphate-buffered saline (PBS; Dulbecco A) at 37°C, and the washes were added to the medium. The cell layer was then incubated for 2 h at 37°C with 5 ml collagenase (70 units/ml) in PBS, the collagenase extract was removed, and the cell layer was gently washed twice with 10 ml of PBS, which was then added to the enzyme extract. The cells were then treated with 5 ml of 0.05% trypsin in PBS for 10 min at 37°C, before the addition of trypsin inhibitor (1 ml of a 0.2% solution in PBS) and the cell suspension was centrifuged at 500 g for 5 min, the supernatant removed, and the cell pellet was washed once with 10 ml PBS, the wash being added to the trypsin extract. The cells were counted and viability, as judged by Trypan Blue exclusion, was found to be in excess of 90%.
Isolation of radiolabelled glycosaminoglycans

Medium, collagenase, trypsin and cell fractions, which were resuspended in 20 ml 0.05 M-Tris-HCl (pH 7.6), were boiled for 10 min. The medium and enzyme extracts were dialysed against several changes of distilled water for 24 h, then against 0.05 M-Tris-HCl (pH 7.6) for a further 24 h. All fractions were then incubated with Pronase (5 mg/ml) for 24 h at 30°C, additional Pronase (2 mg/ml) being added after 12 h, followed by boiling for 5 min. The cell fraction was then made 12 mM with respect to MgSO₄ and incubated for a further 24 h at 37°C with deoxyribonuclease 1 (1938 units) and ribonuclease A (152 units) before heat inactivation. Samples were then combined with carrier glycosaminoglycans (chondroitin sulphate A, hyaluronic acid, dermatan sulphate and heparan sulphate; 100 ng each). All fractions were then dialysed for 24 h against several changes of distilled water, concentrated on a rotary evaporator and split into two equal portions. Glycosaminoglycans were identified and estimated by their susceptibility to hydrolysis by hyaluronidase, chondroitinase AC, chondroitinase ABC, and HNO₂. For susceptibility to hyaluronidase digestion, samples were dissolved in acetate buffer (0.9 ml, 0.05 M, pH 6.0) containing NaCl (0.1 M) to which was added 0.1 ml hyaluronidase in acetate buffer (5 TRU units), and digests were incubated for 18 h at 37°C followed by heat inactivation. Control samples were treated in parallel, but received buffer instead of enzyme solution. Glycosaminoglycans were then precipitated by the addition of 1/5 vol. of 10% cetylpyridinium chloride in 0.03 M-NaCl. After 1 h at 40°C the precipitates were collected by centrifugation, washed once in 0.1% cetylpyridinium chloride in 0.03 M-NaCl, and dissolved in 2 M-NaCl. The glycosaminoglycans were then reprecipitated by the addition of 3 vol. 95% ethanol, dissolved in Tris/acetate buffer (1.0 ml, 0.05 M, pH 8.0) containing NaCl (0.15 M), and bovine serum albumin (0.1%, w/v) and triplicate samples (33.3 μl) were taken for scintillation counting in 5 ml Triton/toluene scintillant. Chondroitinase AC (100 μl, 5 units/ml) was then added, and the mixtures were incubated at 37°C for 18 h, followed by boiling for 3 min. The digests were precipitated as before, redissolved in Tris/acetate buffer (1.0 ml, 0.05 M, pH 8.0) and triplicate samples were taken for scintillation counting. The digests were then incubated for a further 18 h at 37°C with chondroitinase ABC (100 μl, 5 units/ml), heat-inactivated, and precipitated as before. The precipitates were dissolved in water, triplicate samples were taken for counting, and the remaining glycosaminoglycan solutions were dried prior to treatment with HNO₂. Deamination of glycosaminoglycans with HNO₂ was by treatment with 1.8 M-acetic acid containing 0.24 M-sodium nitrite for 2 h at room temperature (Lindahl, Backström, Jansson & Hallen, 1973), followed by cetylpyridinium chloride precipitation and sampling as before.

Electrophoresis

Electrophoretic mobilities at pH 1, which should reflect the glycosaminoglycan sulphate content, were determined by cellulose acetate electrophoresis in 0.1 M-HCl (Wessler, 1971). Samples (1 μl) were applied to the cellulose acetate strips, and electrophoresis was carried out for 1 h at 2 mA/cm. The acetate strips were then cut into 2 mm sections and radioactivity was determined in 5 ml Triton/toluene scintillant.

Electron microscopy and histology

Human skin fibroblasts were seeded into 35 mm Petri dishes and to one half ascorbic acid (50 μg/ml) was added daily. Following 10 days incubation, the control and ascorbate-treated cells were rinsed twice with PBS. For electron microscopy, cells were fixed for 1 h in 4% glutaraldehyde in 0.1 M-cacodylate buffer (pH 7.4), rinsed for 30 min in cacodylate, followed by 1% osmium tetroxide in 0.1 M-phosphate buffer (pH 7.2) for 1 h. Cell layers were then dehydrated, and embedded in Araldite. After polymerization, the Araldite layer was separated from the plastic, cut into 2 mm squares and mounted. Thin sections were taken and counterstained with uranyl acetate and lead citrate, and examined in an AEI 801 electron microscope.

For histological examination, cells were fixed in 10% formol/saline for 20 min, rinsed in 70% ethanol and dried. Collagen fibres were demonstrated by the silver impregnation method of Berman et al. (1978).
RESULTS

Supplementation of the culture medium with ascorbate resulted in an increase in cell growth, the deposition of an extensive extracellular matrix, a higher maximum cell density and altered glycosaminoglycan synthesis. Staining by silver impregnation revealed an extensive collagenous matrix aligned in parallel arrays in the ascorbate-fed cultures, while controls showed no such material. Electron microscopic examination of the cultures confirmed the presence of collagen in the ascorbate-fed cultures, the typical striated fibres being observed (Fig. 1). The incorporation of D-[3H]glucosamine and Na235SO4 into the glycosaminoglycans synthesized by ascorbate-fed and control cultures is shown in Table 1. The addition of ascorbate resulted in a large reduction in the amount of hyaluronic acid synthesized, which was found predominantly in the medium. Ascorbate feeding also resulted in a reduced incorporation of D-[3H]glucosamine into the sulphated glycosaminoglycans, while the Na235SO4 incorporation increased. In addition to altered radiolabelled precursor incorporation, ascorbate resulted in a redistribution of the glycosaminoglycans. While treatment of control cultures with highly purified collagenase released very little radiolabel, the ascorbate-fed cultures released substantial amounts, particularly of chondroitin sulphate. In control cultures, 54% of the 3H-labelled, and 63% of the 35S-labelled sulphated glycosaminoglycans were found in the medium, while in
Ascorbate and glycosaminoglycan synthesis

Table 1. Synthesis and distribution of glycosaminoglycans by human skin fibroblasts in the presence and absence of ascorbate

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Ascorbate</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
<td>Collagenase</td>
</tr>
<tr>
<td>Hyaluronate</td>
<td>3H</td>
<td>3H</td>
</tr>
<tr>
<td>Chondroitin SO₄</td>
<td>54.9</td>
<td>51.3</td>
</tr>
<tr>
<td>Dermatan SO₄</td>
<td>11.9</td>
<td>5.8</td>
</tr>
<tr>
<td>Heparan SO₄</td>
<td>2.3</td>
<td>3.4</td>
</tr>
<tr>
<td>Total</td>
<td>427.1</td>
<td>150.3</td>
</tr>
</tbody>
</table>

Data shown are the average results obtained from duplicate extractions of two different cell lines.

Table 2. The percentage distribution of individual glycosaminoglycans in ascorbate-fed and control cultures

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Ascorbate</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
<td>Collagenase</td>
</tr>
<tr>
<td>Hyaluronate</td>
<td>3H</td>
<td>3H</td>
</tr>
<tr>
<td>Chondroitin SO₄</td>
<td>49.8</td>
<td>22.2</td>
</tr>
<tr>
<td>Dermatan SO₄</td>
<td>49.8</td>
<td>22.2</td>
</tr>
<tr>
<td>Heparan SO₄</td>
<td>2.3</td>
<td>3.4</td>
</tr>
<tr>
<td>Total</td>
<td>1499.3</td>
<td>347.1</td>
</tr>
</tbody>
</table>

Data shown are calculated from Table 1.

ascorbate-fed cultures, the medium contained 42% of ³H-labelled and 38% of ³⁵S-labelled sulphated glycosaminoglycans. Chondroitin sulphate A/C was the major sulphated glycosaminoglycan in both ascorbate and control cultures, while heparan sulphate was present in small amounts only. The percentage distribution of the individual glycosaminoglycans in the various fractions is shown in Table 2. Hyaluronic acid was found predominantly in the medium fraction, while 50% of the
Table 3. Glycosaminoglycan sulphation expressed as $^3$H: $^{35}$S ratios

<table>
<thead>
<tr>
<th>Fraction</th>
<th>+ Ascorbate</th>
<th></th>
<th>- Ascorbate</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CS A/C</td>
<td>DS</td>
<td>HS</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>1.12</td>
<td>2.45</td>
<td>4.26</td>
<td></td>
</tr>
<tr>
<td>Collagenase</td>
<td>1.07</td>
<td>1.50</td>
<td>3.77</td>
<td></td>
</tr>
<tr>
<td>Trypsinate</td>
<td>0.95</td>
<td>1.18</td>
<td>1.71</td>
<td></td>
</tr>
<tr>
<td>Cell-assoc.</td>
<td>1.38</td>
<td>0.69</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1.08</td>
<td>1.55</td>
<td>1.69</td>
<td></td>
</tr>
</tbody>
</table>

$^3$H: $^{35}$S ratios of chondroitin sulphate A or C (CS A/C), dermatan sulphate (DS) and heparan sulphate (HS) were calculated from data in Table 1.

Fig. 2. Cellulose acetate electrophoresis in 0.1 M-HCl of cell-associated chondroitin sulphate/dermatan sulphate from control (a) and ascorbate-fed (b) cultures. Samples were treated with hyaluronidase and HNO$_2$, precipitated with cetylpyridinium chloride and dissolved in distilled water before 1 μl samples were applied to the acetate strips.
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heparan sulphate was found in the trypsin extract of both ascorbate and control cultures. The major difference between the two cultures was the large amount of chondroitin sulphate A/C and dermatan sulphate extractable with highly purified collagenase.

The $^{3}$H: $^{35}$S ratios of the two cultures are shown in Table 3. The ratios suggest substantial differences in the degree of sulphation of the glycosaminoglycans from the two cultures. Glycosaminoglycans from ascorbate-fed cultures appeared to be highly sulphated, while in contrast control cultures showed a low degree of polymer sulphation. These differences in sulphation were demonstrated by cellulose acetate electrophoresis in 0-1 m-HCl (Fig. 2). Since carboxyl groups of uronic acids are undissociated in 0-1 m-HCl, the charge density of glycosaminoglycans in this medium should reflect their sulphate content. The mobilities of the sulphated glycosaminoglycans from ascorbate-fed cultures were greater than those of control cultures, confirming the higher degree of polymer sulphation.

DISCUSSION

The addition of ascorbate to the culture medium stimulates the production and deposition of collagen (Fig. 1), which may be partly responsible for the increased incorporation of sulphated glycosaminoglycans into the insoluble extracellular matrix. The effect of ascorbate is, however, not specific to collagen, but also causes a small increase in the apparent rate of synthesis of non-collagenous protein (Russell et al. 1981). The ascorbate-fed cultures grow to higher maximum cell density, and appear multi-layered, which may be facilitated by the extensive extracellular matrix synthesized. Cells grown in the presence of ascorbate synthesize only 28% of the hyaluronic acid produced by cells grown on plastic. However, comparison between the two cultures requires considerable caution, since cell density influences hyaluronic acid synthesis (Hopwood & Dorfman, 1977). The failure of ascorbate to stimulate increased incorporation of hyaluronic acid into the insoluble extracellular matrix presumably reflects the low affinity of hyaluronic acid for collagen, in contrast to the relatively high affinity demonstrated for sulphated glycosaminoglycans (Greenwald, Schwartz & Cantor, 1975).

Treatment of the cell layer with highly purified collagenase releases substantial amounts of sulphated glycosaminoglycans in ascorbate-fed cells, but greatly reduced amounts in control cultures. This suggests that the synthesis and deposition of a collagenous matrix may be responsible for the increased incorporation of sulphate glycosaminoglycans. Ascorbate does not appear to stimulate the net synthesis of sulphated glycosaminoglycans, but reduces the incorporation of $^{3}$H-glucosamine while increasing $^{35}$SO$_{4}$ incorporation. Gallagher et al. (1980) showed an increase in dermatan sulphate synthesized by human skin fibroblasts cultured on collagen gels compared to those grown on plastic, and suggest a possible positive feedback mechanism activated by the accumulation of dermatan sulphate in the pericellular domain. However, the increased incorporation of sulphated glycosaminoglycans into the insoluble extracellular matrix in ascorbate-fed cultures is probably due to depletion of the
medium pool rather than stimulated synthesis. Treatment of the cell layers with trypsin following collagenase extraction releases significant amounts of sulphated glycosaminoglycans in both ascorbate and control cultures, and produces single cell suspensions, although ascorbate cultures require gentle pipetting. Substantial amounts of sulphated glycosaminoglycans are found in association with the cell pellet, in contrast to preliminary studies (Edward & Oliver, 1983), in which only negligible radioactivity was detected in the cell pellet. This may be due to the highly purified enzymes used in this study compared to the crude combined collagenase/trypsin extraction carried out in the preliminary study.

While heparan sulphate is found predominantly in the trypsin extract of both ascorbate and control cultures, chondroitin sulphate (59% \(^3\)H label; 60-5% \(^3\)S label) and derman sulphate (50-2% \(^3\)H label; 68-4% \(^3\)S label) predominate in the insoluble fractions of ascorbate-fed cultures, while chondroitin sulphate (52-4% \(^3\)H label; 60-9% \(^3\)S label) and derman sulphate (67-2% \(^3\)H label; 77-1% \(^3\)S label) predominate in the medium of control cultures. The presence of the bulk of the heparan sulphate in the trypsin extract of both ascorbate and control cultures is not surprising, as it is synthesized by many different mammalian cells and found associated with the cell surface (Roblin, Albert, Gelb & Black, 1975), mainly being released into the medium prior to mitosis (Kraemer & Tobey, 1972). Chondroitin sulphate and derman sulphate were estimated by their susceptibility to degradation by chondroitinase AC and chondroitinase ABC; however, it has been shown that human skin fibroblasts synthesize a chondroitin sulphate–derman sulphate copolymer (Malmström, Carlstedt, Aberg & Fransson, 1975; Sjöberg et al. 1979). Chondroitin sulphate is composed exclusively of glucuronic-acid-containing repeating units, while derman sulphate always contains iduronic acid in addition to glucuronic acid repeating units. Treatment of the sulphated copolymer with chondroitinase AC may therefore release blocks of glucuronic acid/\(\text{N-acetylgalactosamine} \) sulphate repeating units. Changes in the chondroitinase-AC-digestible material may therefore reflect alterations in the structure of the copolymer, as during biosynthesis of derman sulphate chondroitin-like chains are subject to inversion of configuration at \(\text{C}_5\) of glucuronic acid residues to form iduronic acid followed by sulphation on \(\text{N-acetylgalactosamine} \) moieties (Malmström, Fransson, Höök & Lindahl, 1975).

There appears to be some variation in the structure of the copolymer synthesized by human skin fibroblasts, as Gallagher et al. (1980) found no chondroitinase-AC-susceptible material, but they suggest that variation may be related to the age of the donor.

In addition to the incorporation of sulphated glycosaminoglycans into the collagenase-extractable fraction, ascorbate feeding results in increased glycosaminoglycan sulphation as suggested by the large differences in \(^3\)H: \(^3\)S ratios. Cellulose acetate electrophoresis at pH 1, confirmed that glycosaminoglycans from ascorbate-fed cultures are more highly sulphated. The electrophoretic mobilities in HCl do not depend on the structure of the polysaccharide backbone, as different glycosaminoglycans with similar sulphate contents have similar mobilities (Wessler, 1971). The reason for this altered sulphation is not clear, but may be partly due to possible
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changes in $^{35}$S uptake rates, or a decreased rate of desulphation, as the increased sulphation was apparent in all sulphated glycosaminoglycans.

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REFERENCES


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