Production of proteoglycans by human lung fibroblasts (IMR-90) maintained in a low concentration of serum

Kathryn G. VOGEL and Robert E. SAPIÉN

Department of Biology, The University of New Mexico, Albuquerque, NM 87131, U.S.A.

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Maintenance of fibroblasts in 0.5% serum results in viable but non-proliferative cells that may be analogous to fibroblasts *in vivo*. The synthesis of proteoglycans by human embryo lung fibroblasts in Eagle's minimal essential medium with 0.5% newborn-bovine serum or with 10% serum has been compared. A similar amount of [^{35}S]sulphate-labelled glycosaminoglycan per cell was secreted by fibroblasts in 10% or 0.5% serum. $^{35}\text{SO}_4^{2-}$ incorporation into sulphated glycosaminoglycans was enhanced in 0.5% serum when expressed per mg of cell protein, but [^3H]glucosamine incorporation was decreased. The charge density of these glycosaminoglycans was not changed as determined by ion-exchange chromatography. It was concluded that decreased protein/cell resulted in an apparent increase in ^{35}S -labelled glycosaminoglycan synthesis/mg of cell protein, whereas decreased uptake of [^3H]glucosamine resulted in a decrease in their glucosamine labelling. The proteoglycans secreted by fibroblasts in 0.5% serum were similar in glycosaminoglycan composition, chain length and buoyant density to the dermatan sulphate proteoglycan, which is the major secreted component of cells in 10% serum. Larger heparan sulphate and chondroitin sulphate proteoglycans, which comprise about 40% of the total secreted proteoglycans of cultures in 10% serum, were greatly diminished in the medium of cultures in 0.5% serum. The proteoglycan profile of medium from density-inhibited cultures in 10% serum resembles that of proliferating cultures, indicating that lack of proliferation was not responsible for the alteration. The dermatan sulphate proteoglycan, participating in extracellular matrix structure, may be the primary tissue product of lung fibroblasts *in vivo*.

Fibroblasts in normal tissue are not in direct contact with high levels of serum and they do not proliferate in the rapid and repeated manner of fibroblasts in culture medium containing 10–20% serum. Dell'Orco (1974) has reviewed the characteristics of fibroblasts maintained in medium with a low serum content (0.5%) and concludes that their lengthy viability with very low proliferation makes them a reasonable model of fibroblasts *in vivo*.

Proteoglycans are major constituents of connective tissue and are produced by fibroblasts in cell culture. The characteristics of proteoglycans produced by proliferating human lung fibroblasts in medium plus 10% serum have been reported (Vogel & Peterson, 1981). There are three distinct mole-

cular types in the culture medium. The two larger proteoglycans are composed either of chondroitin sulphate or heparan sulphate glycosaminoglycan chains and are also associated with the cell layer. The smallest, a dermatan sulphate proteoglycan, is the most abundant and found only in the culture medium. Although the cellular functions of proteoglycans are not understood, roles in adhesion (Culp *et al.*, 1979; Schubert & LaCorbiere, 1981) and aggregation (Underhill & Dorfman, 1978) have been suggested. In addition, certain glycosaminoglycans may play a role in regulating proliferation (Castellot *et al.*, 1981). The present study was undertaken because it was reasoned that useful clues about proteoglycan regulation and function might be gained by studying the composition and distribution of proteoglycans synthesized by cells having the altered characteristics induced by culture in a low concentration of serum.

Abbreviations used: MEM, Eagle's minimal essential medium; CPC, cetylpyridinium chloride; PBS, Dulbecco's phosphate-buffered saline; TCA, trichloroacetic acid; ^{35}S -GAG, [^{35}S]sulphate-labelled glycosaminoglycans.

Materials and methods

Cell culture

Human embryo lung fibroblasts (IMR-90) used in this study were obtained from the National Institutes on Aging Repository at the Institute for Medical Research, Camden, NJ, U.S.A. (Das & Murphy, 1981). Cells were maintained in Corning polystyrene flasks with MEM plus 10% newborn-bovine serum (GIBCO) as previously described (Vogel & Kendall, 1980). Only cells in the proliferative phase of their lifespan *in vitro* (population doubling levels 15–35) were used.

For comparative experiments cells were seeded into replicate flasks in MEM + 10% serum and allowed to attach and grow for 2 days. At this point half of the cultures were exchanged into MEM + 0.5% serum and maintained for 2 days. Labelling was initiated by addition of fresh medium containing $\text{Na}_2^{35}\text{SO}_4$ (5 $\mu\text{Ci/ml}$; sp. radioactivity 0.8–0.2 Ci/mmol) and/or D-[6(n)- ^3H]glucosamine (0.5 $\mu\text{Ci/ml}$, sp. radioactivity 19.5 Ci/mmol) or D-[1- ^{14}C]glucosamine (0.5 $\mu\text{Ci/ml}$, sp. radioactivity 49 Ci/mol). All isotopes were purchased from New England Nuclear. Uptake was continued for 48 h unless otherwise indicated.

Cell number was determined by visual counting of nuclei in six different fields by phase-contrast light microscopy at 400 $\times$ (magnification). Protein content was determined by the dye-binding method of Bradford (1976) with reagents from Bio-Rad Laboratories, with bovine serum albumin as standard. Thymidine labelling indices were determined as previously described (Vogel *et al.*, 1981a). For scanning electron microscopy, cells were seeded on to glass coverslips and fixed in 2.5% glutaraldehyde (in 0.067 M-cacodylate buffer, pH 7.4, for 1 h at room temperature) 3 days after exchange into MEM + 10% or 0.5% serum. Cells were post-fixed in 1% OsO_4 (0.067 M-cacodylate buffer for 2 h at room temperature) and sputter-coated with Au-Pd alloy before viewing with an ETEC Autoscan microscope.

Characterization of glycosaminoglycans

The amount of radioactive glycosaminoglycan produced in each culture compartment was quantified by CPC precipitation as previously described (Vogel & Kendall, 1980). The composition of glycosaminoglycans in each fraction was determined by cellulose t.l.c. separation of the disaccharides produced after digestion with chondroitin ABC lyase or chondroitin AC lyase (Miles Laboratories). These methods have been described previously (Vogel & Kendall, 1980). By this procedure the percentages of sulphate-labelled disaccharides corresponding to chondroitin 6-sulphate and chondroitin 4-sulphate with either glucuronic acid or iduronic acid are

determined directly. Dermatan sulphate is reported as the percentage of radioactivity migrating as the disaccharide of chondroitin 4-sulphate digestion after chondroitinase ABC minus the amount migrating as chondroitin 4-sulphate after digestion with chondroitinase AC. As noted by Yanagishita *et al.* (1979), this method may somewhat overestimate the amount of iduronic acid in a heteropolymer because chondroitinase AC is most active as an exo-enzyme. The term 'dermatan sulphate proteoglycan' refers to a component that contains chondroitin sulphate rich in iduronic acid. Heparan sulphate is determined in this assay as macromolecular sulphate-labelled material that is insensitive to chondroitinase ABC digestion and remains at the origin of the t.l.c. plate. We have found that the percentage of macromolecular [^{35}S]sulphate insensitive to chondroitinase ABC corresponds to the percentage that becomes retarded on Sephadex G-50 filtration after HNO_2 treatment.

For ion-exchange chromatography samples were digested with trypsin (0.1 mg/ml for 4 h at 37°C) and then dialysed for at least 24 h against several changes of water. Up to 8 ml of sample was applied to a column (1 cm $\times$ 8 cm) of DEAE-cellulose (Whatman DE-52) that had been prepared by successive washes in 1 M-NaOH and 3 M-HCl with final equilibrium in 0.05 M-PBS. The column was flushed with 80 ml of 0.05 M-PBS and then eluted with a gradient of NaCl (0.01 M–0.8 M; 100 ml of each).

Characterization of proteoglycans

Proteoglycans of the medium were analysed by adding solid chemicals to produce medium containing 4 M-guanidine hydrochloride (grade I; Sigma Chemical Co.), 0.05 M-sodium acetate, 0.1 M-disodium EDTA and 0.005 M-benzamidine hydrochloride. To obtain only heparan sulphate proteoglycans, medium was digested with chondroitin ABC lyase (0.1 unit/ml for 5 h at 37°C) before addition of guanidine hydrochloride and proteinase inhibitors. Samples were then passed through a column (1 cm $\times$ 40 cm) of Sephadex G-50 eluting with 4 M-guanidine hydrochloride, 0.05 M-sodium acetate, pH 5.8, to remove labelling isotope, and analysed by gel-filtration chromatography directly or after density-gradient centrifugation.

Proteoglycans of the cell layer were solubilized by the techniques of Kimura *et al.* (1981) as previously described (Vogel & Peterson, 1981). In this procedure the cell layer was solubilized by addition of 4% Zwittergent (Calbiochem–Behring Corp.) to the rinsed monolayer. After 20 min at room temperature the cells were scraped from the flask and an equal volume of 8 M-guanidine hydrochloride in 0.1 M-sodium acetate plus proteinase inhibitors was added. Alternatively, detergent and 4 M-guanidine hydrochloride + proteinase inhibitors were added simul-

taneously to the cell layers, giving a similar result. Extraction in 2% Zwittergent and 4 M-guanidine hydrochloride was carried out for 24 h at 4°C and produced a clear solution. This material was then passed through Sephadex G-50 (eluted with 4 M-guanidine hydrochloride/0.05 M-sodium acetate, pH 5.8) to remove residual labelling isotope. Proteoglycans of the surface were obtained by treating the cell layer with trypsin (0.1 mg/ml in Earle's balanced salt solution for 15 min at 37°C). The trypsin-removable material was adjusted to 4 M-guanidine hydrochloride/0.05 M-sodium acetate/0.1 M-disodium EDTA/0.005 M-benzamidine hydrochloride and, in addition, soya-bean trypsin inhibitor (0.1 mg/ml; Sigma Chemical Co.) was added. Cellular material remained attached to the substratum after trypsin treatment and was then extracted as described above.

For density-gradient centrifugation of the medium, solid CsCl (grade I; Sigma Chemical Co.) was added to macromolecular material in 4 M-guanidine hydrochloride, giving an initial density of 1.47 g/ml. This material was centrifuged at 40000 rev./min for 48 h (10°C; type 65 rotor; Spinco model L ultracentrifuge). Each tube was divided into four equivalent sections and fractions D1 (bottom), D2, D3 and D4 (top) were slowly withdrawn from the top. Density was determined by using a 100 µl glass pipette as a pycnometer.

Hydrodynamic volume of various fractions was determined by gel-filtration chromatography on Sepharose CL-2B or CL-4B (Pharmacia Fine Chemicals; 1 cm × 100 cm column; elution with 4 M-guanidine hydrochloride in 0.1 M-sodium acetate, pH 5.8; flow rate 10 ml/h; 2 ml fractions). The proteoglycan nature of various radiolabelled components was determined by elution position from

Sepharose CL-6B before and after treatment with NaOH (0.2 M-NaOH and 1 M-NaBH₄, 45°C, 24 h, neutralized with acetic acid) to cleave intact glycosaminoglycans from the core proteins. The V_0 of 4B columns was marked by Blue Dextran (M_r 2×10^6) and the V_1 by vitamin B₁₂ (M_r 1350). Portions of each fraction were counted for radioactivity in 4 ml of Scint-A (Packard Instrument Co.) fluid with a Packard 300 CD liquid-scintillation system using Spectral Index of External Standard programming to calculate d.p.m.

Determination of TCA-soluble pool

Intracellular *N*-acetylhexosamine was determined as the TCA-soluble dialysable pool by the method described by Angello & Hauschka (1980). Radioactivity corresponding to *N*-acetylglucosamine and *N*-acetylgalactosamine was determined by subtracting the radioactivity in the TCA-soluble dialysis residue from total TCA-soluble radioactivity.

Results

Cellular characteristics

Although the cell number in 0.5% serum increased more slowly than in 10% serum, some proliferation was evident (Table 1). The 48 h [³H]thymidine labelling index of proliferating cultures in 10% serum was 63%. The labelling index for a 24 h period beginning 2 days after a shift to 0.5% serum was 2.2%. Confluent control cultures in 10% serum had only 4.1% of the nuclei labelled. By contrast, cultures that were in 0.5% serum for 5 days and then shifted back into 10% serum showed labelling of 83.1% of nuclei during the 24 h period beginning 1 day after re-addition of serum. The amount of total

Table 1. Incorporation of Na₂³⁵SO₄ into glycosaminoglycans secreted by fibroblasts over a 7 day period in MEM + 10% serum (high) or MEM + 0.5% serum (low)

Cultures were seeded in MEM + 10% serum and half exchanged into 0.5% serum on day 0. Labelling with Na₂³⁵SO₄ was initiated 48 h before the day indicated. At time of harvesting, medium was decanted to determine ³⁵S-GAG by CPC-filtration; the cell layer was rinsed and fixed in 0.2 M-HClO₄ for visual counting and then cell-layer protein content was determined. Details of the procedure are presented in the Materials and methods section. Results are means ± s.d. of duplicate flasks.

Day	Serum content	Cells/10 ⁶ flask	Protein/flask (mg)	Protein/10 ⁶ cells (mg)	³⁵ S-GAG (d.p.m.)	³⁵ S-GAG (d.p.m./mg of protein)	³⁵ S-GAG (d.p.m./10 ⁶ cells)
0	High	2.4 ± 0.1	0.62 ± 0.01	0.26	19900 ± 500	32 100	8300
1	High	3.6 ± 0.2	0.87 ± 0.03	0.24	25 100 ± 3500	28 900	7000
	Low	2.6 ± 0	0.34 ± 0.01	0.13	12 700 ± 1000	37 400	4900
3	High	5.0 ± 0.1	0.86 ± 0.06	0.17	34 700 ± 3500	40 300	7000
	Low	2.8 ± 0.2	0.40 ± 0.09	0.14	22 700 ± 2200	56 800	8100
5	High	5.2 ± 0.2	0.90 ± 0.02	0.17	45 700 ± 4200	50 800	8800
	Low	3.3 ± 0.1	0.48 ± 0.04	0.15	25 700 ± 700	53 500	7800
7	High	5.3 ± 0.1	1.53 ± 0.07	0.29	55 100 ± 1300	20 130	10 300
	Low	3.0 ± 0.2	0.58 ± 0.12	0.19	30 800 ± 2100	53 100	10 400

protein in a flask of cells decreased within 24 h of serum reduction. When combined, these measurements indicate that protein/cell decreased rapidly when cells were first placed in 0.5% serum. Subsequently the amount of protein/cell increased somewhat and remained at a level that was about 40% below cells of the confluent cultures in 10% serum.

By phase-contrast microscopy cultures in 0.5% serum looked quite similar to those in 10% serum. Cellular overlapping at high density was somewhat reduced and the cells in 0.5% serum had a greater tendency to cluster after gentle trypsin treatment. By scanning electron microscopy the cells in 0.5% serum appeared extremely flattened on the substratum (Fig. 1). Although cells were apparently in contact with the substratum over a greater proportion of their surface, they did not demonstrate greater resistance to trypsin release. Most experiments of this study were performed on cultures that had been in 0.5% serum for 4 days; the cultures remained healthy in MEM + 0.5% serum for several weeks, however.

Fig. 1. Scanning electron microscopy of IMR-90 fibroblasts growing in MEM containing (a) 10% serum or (b) 0.5% serum

Cells were first seeded into MEM + 10% serum and then some transferred to MEM + 0.5% serum for 3 days before fixation. Cells in low serum had few microvillus projections and were more flattened on to the substratum than those in 10% serum.

Incorporation of [^{35}S]sulphate and [^3H]glucosamine

Cultures of human embryo lung fibroblasts incubated in medium containing 0.5% serum incorporated $\text{Na}_2^{35}\text{SO}_4$ into ^{35}S -GAG, which were primarily released to the culture medium (Fig. 2). A small amount of the ^{35}S -GAG was retained at the cell surface and could be released by trypsin treatment; additional ^{35}S -GAG remained associated with the cell after trypsin treatment, presumably in an intracellular location. When expressed as incorporated radioactivity per mg of cellular protein, the production of medium ^{35}S -GAG by cultures in 0.5% serum was increased by 50–100% (Table 2). However, uptake of [^3H]glucosamine into these same ^{35}S -GAG was reduced by approx. 50%. Incorporation of $^{35}\text{SO}_4^{2-}$ into glycosaminoglycans of the surface and intracellular culture compartments was somewhat reduced in cultures in 0.5% serum, but incorporation of [^3H]glucosamine into the sulphated glycosaminoglycans and into the glycoprotein and hyaluronic acid fractions of all culture compartments was reduced to 50% or less than that of cultures in 10% serum. The amount of medium ^{35}S -GAG produced per cell was essentially unchanged, however (Table 1). This result was con-

Fig. 2. Incorporation of [^{35}S]sulphate into glycosaminoglycans by fibroblasts in MEM + 0.5% serum

Uptake was initiated after 48 h in MEM + 0.5% serum by addition of $\text{Na}_2^{35}\text{SO}_4$ ($10 \mu\text{Ci/ml}$). The amount of ^{35}S -GAG in each culture compartment was determined by CPC precipitation. Results shown are means of two 25 cm^2 flasks at each time point in one experiment.

Table 2. *Production of glycoproteins and glycosaminoglycans by fibroblasts in MEM + 10% (high) or MEM + 0.5% serum (low)*

Cultures of IMR-90 fibroblasts were seeded in MEM + 10% serum and exchanged into either high or low serum after 2 days. Cells were then labelled by addition of $\text{Na}_2^{35}\text{SO}_4$ ($5\mu\text{Ci/ml}$) and $[^3\text{H}]\text{glucosamine}$ ($0.5\mu\text{Ci/ml}$) for 48 h. Macromolecular components of the medium, surface and intracellular culture compartments were analysed for glycosaminoglycan and oligosaccharide content by differential solubilization of the CPC precipitate by the method of Saarni & Tammi (1977).

		Radioactivity (d.p.m./mg of cell protein)			
		Glycoproteins	Hyaluronic acid	Sulphated glycosaminoglycans	
Serum content				³ H	³⁵ S
Medium	High	617 200	135 939	132 953	49 923
	Low	473 270	47 713	74 775	74 767
Surface	High	100 788	7665	6448	1439
	Low	56 715	4160	2629	1050
Intracellular	High	312 527	19 368	23 871	6966
	Low	138 659	6575	10 891	5334

Table 3. *Composition of ^{35}S -labelled glycosaminoglycans in various compartments of fibroblast cultures in the presence of high (10%) or low (0.5%) serum*

At 2 days after seeding in MEM + 10% serum, cultures were exchanged into MEM + 0.5% serum or MEM + 10% serum for 2 days and then labelled with $\text{Na}_2^{35}\text{SO}_4$ for 48 h. Glycosaminoglycan composition was determined for each culture compartment by t.l.c. separation of disaccharide products resulting from digestion with chondroitinase ABC and AC. Details of sample preparation and calculation are presented in the Materials and methods section. Abbreviations: HS, heparan sulphate; C6-S, chondroitin 6-sulphate; C4-S, chondroitin 4-sulphate; DS, dermatan sulphate. Data are expressed as means $\pm$ s.d. of percentage of total ^{35}S -labelled glycosaminoglycan in each compartment. For cultures in 0.5% serum, $n = 3$; for cultures in 10% serum, $n = 2$.

Culture compartment	Glycosaminoglycan	Low serum (0.5%)	High serum (10%)
Medium	HS	21 ± 2	30 ± 2
	C6-S	28 ± 4	28 ± 4
	C4-S	4 ± 1	6 ± 2
	DS	47 ± 1	34 ± 3
Cell surface	HS	66 ± 2	57 ± 3
	C6-S	26 ± 4	25 ± 4
	C4-S	4 ± 3	14 ± 2
	DS	4 ± 2	4 ± 1
Intracellular	HS	45 ± 6	73 ± 1
	C6-S	26 ± 5	12 ± 1
	C4-S	5 ± 2	10 ± 5
	DS	24 ± 1	6 ± 2

sistently seen in both sparse and crowded cultures and for all time points after 24 h in 0.5% serum.

The composition of ^{35}S -GAG released to the medium of cultures in 0.5% serum was somewhat altered in that the medium contained less heparan sulphate and a greater percentage of dermatan sulphate (Table 3). The intracellular compartment also contained a greater percentage of dermatan sulphate. Hyaluronic acid was a lesser percentage of total glycosaminoglycans in medium of cultures in 0.5% serum (35%) than of cultures in 10% serum (50%), as determined both by CPC precipitation and by comparison of DEAE-cellulose chromato-

graphic profiles after incorporation of $[^3\text{H}]\text{glucosamine}$.

Proteoglycan characterization

Gel-filtration chromatography of proteoglycans released to the medium by cultures in 10% serum demonstrated components of two size classes. By contrast, proteoglycans in the medium of similar cultures in 0.5% serum were almost exclusively composed of the smaller-sized component (Fig. 3). By CsCl -density-gradient centrifugation the major proteoglycan component of medium from cultures in 0.5% serum was of only one size and had a density

Fig. 3. *Sepharose 4B* profile of ^{35}S -labelled proteoglycans from the medium of cells in 10% and 0.5% serum. Cultures were labelled for 48 h with $\text{Na}_2^{35}\text{SO}_4$ ($20\ \mu\text{Ci/ml}$). Medium was made to 4 M-guanidine hydrochloride and passed through Sephadex G-50 to obtain macromolecular material before *Sepharose* CL-4B filtration in 4 M-guanidine HCl/0.1 M-sodium acetate, pH 5.8; a portion (0.4 ml) of each 2 ml fraction was counted for radioactivity. (a) Cells exchanged to MEM + 0.5% serum for 2 days before labelling; (b) cells in MEM + 10% serum.

$\leq 1.42\text{ g/ml}$ (Fig. 4). The glycosaminoglycan composition of material in fraction D3 was 40% chondroitin 4-sulphate + dermatan sulphate, 43% chondroitin 6-sulphate and 17% heparan sulphate; for the D4 material, proportions were 56%, 33% and 11% respectively. Elution position on *Sepharose* CL-6B shifted from $K_{av} = 0.32$ to $K_{av} = 0.47$ after alkaline treatment (Fig. 5a). The proteoglycan was digested to free glycosaminoglycan chains ($K_{av} = 0.45$), by treatment with trypsin + Pronase (Fig. 5b) and more than 80% of this radioactivity was sensitive to chondroitinase ABC (Fig. 5b). Some cultures were maintained in 10% serum for 15 days until maximum density-dependent inhibition of growth was obtained. These extremely dense cultures produced a profile of ^{35}S -labelled proteoglycans that resembled that of cultures proliferating in 10% serum, that is, the large proteoglycans were present (results not shown).

Medium from cultures in 0.5% and 10% serum was digested with chondroitinase ABC before elution on *Sepharose* CL-4B in 4 M-guanidine hydrochloride (Fig. 6). The resistant material of cultures in 10% serum eluted primarily at $K_{av} = 0.2$, but there was a small amount of material that was retarded to a greater extent. In the cultures in 0.5% serum also, the chondroitinase ABC-insensitive material,

Fig. 4. *Sepharose 2B* profiles of ^{35}S -labelled proteoglycans from cells in MEM + 0.5% serum after dissociative CsCl -density-gradient centrifugation. Cells were exchanged into MEM + 0.5% serum 2 days before labelling with $\text{Na}_2^{35}\text{SO}_4$ ($20\ \mu\text{Ci/ml}$) for 48 h. Medium in 4 M-guanidine hydrochloride was passed through a *Sephadex* G-50 column and macromolecular material brought to density of 1.47 g/ml by addition of solid CsCl . After centrifugation, tube contents were divided into four fractions, D1 (most dense)–D4, and chromatographed on *Sepharose* CL-2B in 4 M-guanidine hydrochloride and 0.1 M-sodium acetate, pH 5.8; a portion (0.4 ml) of each 2 ml fraction was counted for radioactivity.

although very low in quantity, was of both size classes.

Proteoglycans released from the cell layer by detergent/guanidine hydrochloride were separated by *Sepharose 4B* chromatography into two distinct peaks (termed I and II; Fig. 7a) with $K_{av} = 0.2$ and 0.6. Treatment of labelled cell layers with trypsin (0.1 mg/ml , 15 min) released a fraction corresponding to peak I (Fig. 7b); extraction of the cell

Fig. 5. *Sepharose 6B* profiles of proteoglycan fractions from cells in MEM + 0.5% serum before and after alkaline treatment or enzyme digestion

Proteoglycans of the medium were treated with (a) alkaline borohydride or (b) proteinase enzymes followed by chondroitinase ABC digestion. Also shown is alkaline treatment of the trypsin-removable fraction (c) (as in Fig. 7b) and intracellular fraction (d) (as in Fig. 7c). Radioactivity of original (—) and treated (---) samples is presented on a relative scale to facilitate illustration.

layer after trypsin treatment showed the two peaks of the original cell layer but peak I was greatly diminished. The proteoglycan component released from cells by gentle trypsin treatment eluted from Sepharose 6B at $K_{av} = 0.1$ and shifted to $K_{av} = 0.4$ after alkaline treatment (Fig. 5c). [^{35}S]Sulphate-labelled material extracted from the cell layer after removing the trypsin-releasable fraction was not made smaller by alkaline treatment and thus was not in proteoglycan form (Fig. 5d).

Charge density of glycosaminoglycans

Glycosaminoglycans from the medium of cultures in 0.5% and 10% serum labelled with $\text{Na}_2^{35}\text{SO}_4$ were compared on replicate ion-exchange columns, and no shift in position of the peaks was found. The positions of all peaks were also similar in co-chromatography of medium from cultures labelled with [^3H]glucosamine (0.5% serum) and [^{14}C]glucosamine (10% serum) (results not shown).

Uptake of [^3H]glucosamine

Uptake of [^3H]glucosamine into an intracellular TCA-soluble dialysable pool was found to be more than 4-fold higher in cultures in 10% serum than in cultures in 0.5% serum (Fig. 8). Diminished uptake in 0.5% serum was found regardless of whether the results were expressed per mg of cell protein or per cell, and also in both growing and confluent cultures.

Discussion

The amount of [^{35}S]sulphate incorporated into sulphated glycosaminoglycans secreted to the medium of fibroblast cultures in 10% serum or in 0.5% serum was similar, when expressed per cell. When expressed per mg of cellular protein, however, incorporation of $^{35}\text{SO}_4^{2-}$ by cultures in 0.5% serum increased, whereas incorporation of [^3H]glucosamine decreased. This observation could have several explanations, such as the synthesis of a

Fig. 6. Sepharose 4B profiles of ^{35}S -labelled proteoglycans from medium before and after digestion with chondroitinase ABC

Cells in MEM containing (a) 0.5% serum or (b) 10% serum were labelled with $\text{Na}_2^{35}\text{SO}_4$ for 48 h. The medium was then chromatographed on Sepharose CL-4B in 4 M-guanidine hydrochloride, 0.1 M-sodium acetate, pH 5.8, untreated (—) or after digestion with chondroitinase ABC (----). Details of sample preparation are given in the Materials and methods section. Radioactivity at the V_t represents unincorporated labelling isotope and digestion products that were not completely removed by Sephadex G-50 filtration before Sepharose chromatography.

highly sulphated product, or alteration in the specific activity of the precursor pools such that the sulphate pool was enhanced and/or the glucosamine pool was diminished.

Because glycosaminoglycans produced by cultures in 10% and 0.5% serum co-eluted from DEAE-cellulose, indicating similar charge densities, disparate sulphation was eliminated as an explanation for the increased ratio of $^{35}\text{S}/^3\text{H}$ found in sulphated glycosaminoglycans from low-serum cultures. Likewise, differential glucose metabolism is not an explanation for the increased ratio of $^{35}\text{S}/^3\text{H}$ because increased glucose metabolism would be

Fig. 7. Sepharose 4B profiles of ^{35}S -labelled material associated with cells in medium + 0.5% serum

Cells in MEM + 0.5% serum for 2 days were labelled for 48 h with $\text{Na}_2^{35}\text{SO}_4$ (20 $\mu\text{Ci}/\text{ml}$). The cell layer was rinsed with Earle's balanced salt solution (BSS) and then treated with BSS + trypsin (0.1 mg/ml, 15 min). Profile of untreated cells (a) is compared with trypsin-removable material (b) and components of the cell layer after trypsin treatment (c) prepared as described in the Materials and methods section. Fractions were passed through a Sephadex G-50 column to obtain macromolecular material before Sepharose CL-4B filtration in 4 M-guanidine hydrochloride and 0.1 M-sodium acetate, pH 5.8; a portion (0.4 ml) of each 2 ml fraction was counted for radioactivity.

required to dilute the *N*-acetylhexosamine precursor pool and thus lower incorporation of radioactivity into the sugars of glycosaminoglycans (Kim & Conrad, 1976). Glucose utilization in serum-arrested populations of foreskin fibroblasts was diminished by 25%, however (Dell'Orco, 1974).

Accumulation of intracellular *N*-acetylhexosamine by cells in 0.5% serum was diminished to approximately one-third of the level found for cultures in 10% serum. This reduction corresponds to the diminished amount of [^3H]glucosamine-labelled sulphated glycosaminoglycans produced by cells in 0.5% serum, suggesting that the apparent decrease in glucosamine incorporation may be

Fig. 8. Intracellular accumulation of *N*-acetylhexosamine by cells in medium containing 10% or 0.5% serum. Small flasks (25 cm²) of cells were labelled with [³H]glucosamine (0.5 µCi/ml) for 0.5, 2 or 6 h and intracellular TCA-soluble, dialysable radioactivity per mg of cell protein was determined.

caused by diminished cellular uptake or accumulation of the radioactive precursor, rather than by diminished synthesis. Cellular protein content was diminished by 20–40% during the first few days of culture in low-serum medium. A similar decrease in protein content has been reported for human foreskin fibroblasts (Dell'Orco *et al.*, 1973). Considering all of these factors we conclude that cells maintained in a low concentration of serum secrete a quantity of sulphated glycosaminoglycans to the medium that is similar to that produced by cells in 10% serum. The apparent increase in ³⁵S-GAG per mg of cell protein results from diminished protein content of cells in low serum; the increased ratio of [³⁵S]/[³H]glucosamine in sulphated glycosaminoglycans results from lowered uptake of radioactive glucosamine. This conclusion is distinctly different from results with the Swarm rat chondrosarcoma, where actual synthesis of sulphated proteoglycans was enhanced 200–800% by culture in 15% foetal-bovine serum (Stevens & Hascall, 1981).

The proteoglycan profile of medium from cultures in 0.5% serum indicated unchanged or enhanced production of the smallest proteoglycan component, but absence or greatly reduced production of the large heparan sulphate and chondroitin sulphate proteoglycans. Identity between the major proteoglycan constituents produced by cells in 10% serum and the single constituent of cells in 0.5% serum was confirmed by: (1) similar elution position from Sepharose CL-4B under dissociating conditions (K_{av} = 0.45); (2) similar shift in elution position from Sepharose CL-6B after alkaline treatment (from K_{av} = 0.32 to 0.47); (3) similar buoyant

density in CsCl ($d \leq 1.45$); and (4) similar glycosaminoglycan compositions (primarily chondroitin sulphate with high levels of iduronic acid and about 15% heparan sulphate). This observation suggests that synthesis of specific fibroblast proteoglycans is regulated in response to external signals. There is a precedent for this suggestion in a report by Yanagishita *et al.* (1981). They found that several hormones stimulated synthesis of proteoglycans by rat granulosa cells in culture, and the stimulation could be accounted for by increased synthesis of only one of the two major proteoglycans. It is of interest also that the ratio of type III/type I collagen was increased when IMR-90 fibroblasts were maintained in the absence of serum (Kelley *et al.*, 1981).

A question concerning the size distribution of heparan sulphate is raised by these results. After CsCl-density-gradient centrifugation, the lighter fractions contained about 15% chondroitinase ABC-insensitive material. A similar result was found during analysis of the D3 and D4 components from medium with 10% serum [cf. Table II in Vogel & Peterson (1981)], but at that time it was assumed to be contamination from the other fractions that had substantial heparan sulphate content. Because the major heparan sulphate proteoglycan of the medium was greatly reduced in cultures in 0.5% serum, it was surprising to still find this 'contamination'. Its presence suggests that there could be two heparan sulphate fractions produced during culture in 10% serum, one as a dense proteoglycan and one in a smaller less dense form. Two heparan sulphates have also been identified as products of human embryo skin fibroblasts (Carlstedt *et al.*, 1981), a larger one associated with the cell layer and a smaller one found in the medium. Many lines of evidence suggest that skin fibroblasts retain greater amounts of proteoglycan in a pericellular matrix than do the IMR-90 lung fibroblasts. Thus the large heparan sulphate proteoglycan in medium of IMR-90 cultures in 10% serum could represent material shed directly from the cell layer without binding in a pericellular location. The results of this study are consistent with a conclusion that fibroblasts in both 10% serum and 0.5% serum produce heparan sulphate proteoglycans of two distinct size classes. It is the larger component, however, which is greatly reduced during maintenance in a low concentration of serum.

An important question is whether the alteration in proteoglycan type results from absence of certain stimulatory factors of serum, from the absence of proliferation itself, or from a particular morphology. Because the profile of proteoglycans produced by density-arrested cultures resembled that of growing cultures, we favour a serum-induced mechanism that is separate from the act of mitosis.

Several studies with fibroblasts have demon-

strated co-ordinate stimulation of glycosaminoglycan synthesis and proliferation by serum addition (Lembach, 1976; Moscatelli & Rubin, 1977). Addition of whole serum to arterial smooth-muscle cells made quiescent by maintenance in plasma-derived serum resulted in a 40–60% enhancement of proteoglycan synthesis (Wight *et al.*, 1980). Others have noted reduced glycosaminoglycan synthesis and secretion with increasing cell density. In one case this was apparently due to reduced sugar precursor transport (Cohn *et al.*, 1976) but in another, actual synthesis was reduced (Hronowski & Anastassiades, 1980). Although the relationship between proliferation and glycosaminoglycan synthesis is not clear, we believe that the important point of this investigation is that sulphated glycosaminoglycans continue to be secreted in essentially similar amounts by proliferating and non-proliferating cultures of human lung fibroblasts. Similarly, Breul *et al.* (1980) demonstrated that replicating human diploid lung fibroblasts continued to produce collagen at the same level as cells that were not replicating, indicating a very rigid control of collagen production by cells maintained in a constant extracellular milieu.

The striking morphological accompaniment to culture in low serum was a flattened shape suggesting close association with the substratum over a greater cellular area. If this condition represents a period of stable membrane–substratum association, then requirement for replacement of the surface-associated heparan sulphate and chondroitin sulphate proteoglycans might be lowered. Operation of such a regulatory mechanism is not supported, however, by recent work indicating that cells synthesize a constant amount of surface-associated sulphated glycosaminoglycan regardless of prior stripping by heparinase enzyme (Gill *et al.*, 1981).

If the low-serum culture condition is analogous to a state *in vivo*, we could postulate that the dermatan sulphate proteoglycan produced by arrested cells is the proteoglycan species that should be most typical of lung matrix. There is little experimental evidence on which to confirm or deny this suggestion. A dermatan sulphate proteoglycan of this size was not found in studies of bovine (Radhakrishnamurthy *et al.*, 1980a) and porcine (Sahu & Lynn, 1979) lung, and the major glycosaminoglycans from newborn-rabbit lung were hyaluronic acid and chondroitin 6-sulphate (Radhakrishnamurthy *et al.*, 1980b). Thus, this proteoglycan is not similar to the major components identified so far. On the other hand, microheterogeneity in glycosaminoglycan type and sulphation is to be expected in an organ as complex as the lung and although it is presumed to be alveolar (Bradley *et al.*, 1980), the specific source of IMR-90 lung fibroblasts is not known.

The small dermatan sulphate proteoglycan sec-

reted by IMR-90 fibroblasts is specifically adsorbed into a rat-tail tendon collagen gel (Vogel *et al.*, 1981b). Previous studies have proposed that dermatan sulphate participates in the formation of collagen fibrils (Toole & Lowther, 1968). This proteoglycan could, therefore, be present in lung as part of a highly insoluble complex with collagen. Secretion of dermatan sulphate proteoglycans may be the usual function of these fibroblasts *in vivo*. Sudden cellular contact with serum components, as a result of wounding, could stimulate synthesis of large heparan sulphate and chondroitin sulphate proteoglycans as a response to facilitate cellular motility, with adhesion and matrix orientation as primary goals.

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