Co-polymeric glycosaminoglycans in transformed cells

Transformation-dependent changes in the co-polymeric structure of heparan sulphate

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1. Heparan sulphates from normal 3T3 fibroblasts are association-prone as indicated by their affinity for agarose gels substituted with cognate heparan sulphate species. Heparan sulphates from SV40-transformed or polyoma-virus-transformed cells have no affinity for the same gels. 2. Heparan sulphates from the medium, the pericellular and intracellular pools of normal, SV40-transformed and polyoma-transformed 3T3 cells were separated into four subfractions (HS1–HS4) by ion-exchange chromatography. In general, HS1–HS3 were found in cell-derived heparan sulphates, whereas HS3–HS4 were present in the medium. The heparan sulphates from transformed cells were more heterogeneous and of lower charge density than those from the normal counterpart. 3. Degradations via periodate oxidation/alkaline elimination yielded the oligomers glucosamine-(hexuronate-glucosamine)_n-R with $n = 1-5$ and a large proportion of *N*-sulphate groups. There was a large contribution of fragments $n = 4-5$ from heparan sulphates of normal cells. These fragments were less common in low-sulphated heparan sulphates of transformed cells. In the case of medium-derived heparan sulphates all species had a low content of fragments $n = 4-5$. 4. The size distribution of (glucuronate-*N*-acetylglucosamine)_n regions was assessed after deaminative cleavage. It was broad and ranged from $n = 1-10$ for all heparan sulphate species. In the case of medium-derived heparan sulphates there were distinct differences between normal and transformed cells. In the latter chains the *N*-acetyl-rich segments were both shorter and longer than in the normal case. The shape of the disaccharide peak was consistent with a lower content of *O*-sulphate in the heparan sulphates from transformed cells. 5. It was concluded that heparan sulphates from medium or transformed cells exhibit the greatest structural deviation from the normal case. The finding of lower proportions of extended, iduronate/glucuronate-bearing, *N*-sulphate-rich segments in heparan sulphates of transformed cells was particularly interesting in view of the fact that these elements have been associated with ability to self-interact.

Heparan sulphate, which is a ubiquitous cell surface component, suffers quantitative and qualitative changes as a result of viral transformation of the cell (for references see Fransson *et al.*, 1981a). This is expressed in a lowering of the *O*-sulphate

Abbreviations used: GlcN, 2-amino-2-deoxy-D-glucose; GlcNAc, 2-acetamido-2-deoxy-D-glucose; GlcNSO₃, 2-sulphamido-2-deoxy-D-glucose; HexA, hexuronic acid; IdoA, L-iduronic acid; GlcA, D-glucuronic acid; -OSO₃, ester sulphate; R, remnant of an oxidized and degraded GlcA residue; aMan, D-anhydromannose.

content of heparan sulphate and an altered metabolism of this macromolecule. We have recently explored the self-associating properties of various heparan sulphates both from beef lung tissue (Fransson *et al.*, 1980a,b) and human lung fibroblasts (Fransson *et al.*, 1981a) and arrived at the conclusion that there are specific and possibly complementary interactions between these chains (Fransson *et al.*, 1981b). Using an affinity chromatography assay and examining binding of free chains onto heparan sulphate-agaroses (Fransson *et al.*, 1981b), we have observed that heparan

sulphates from SV40-transformed or polyoma-transformed 3T3 cells have no affinity for their corresponding heparan sulphate gels (Fransson *et al.*, 1981a). As the self-association property is correlated with the occurrence of alternating or mixed IdoA- and GlcA-containing repeating disaccharides (Fransson *et al.*, 1980a,b) it is conceivable that transformation-dependent loss of aggregatability may be caused by subtle changes in the co-polymeric structure. This has prompted us to examine the structural features of heparan sulphates from normal and transformed 3T3 cells.

Experimental

Materials

Heparan sulphate was purified from heparin byproducts as described extensively elsewhere (Fransson *et al.*, 1980a,b,c). The total pool was subfractionated according to charge density by stepwise precipitation with cetylpyridinium chloride in the presence of decreasing concentrations of NaCl. The following fractions were obtained: HS1 (0.2–0.4 M-NaCl), HS2 (0.4–0.6 M), HS3 (0.6–0.8 M), HS4 (0.8–1.0 M), HS5 (1.0–1.2 M) and HS6 (complexes not soluble in 1.2 M-NaCl). The subfractions HS2–HS4 were separated into more or less association-prone variants by gel chromatography as described in a preceding report (Fransson *et al.*, 1981b), where also the analytical data for these materials were given. The fractions containing aggregatable species were designated HS2-A, HS3-A and HS4-A, respectively.

Other sources of materials were as follows: glycosaminoglycan standards from the Chicago collection; Instagel, Packard AB, Bandhagen, Sweden; Sephadex gels, Pharmacia; microgranular DEAE-cellulose (DE-52), Whatman. Other Chemicals were of analytical grade.

Preparation of radiolabelled heparan sulphates

³H- and ³⁵S-labelled heparan sulphate was from a Balb-3T3 cell line and SV40-transformed or polyoma-transformed lines as described previously (Vannucchi & Chiarugi, 1977; Chiarugi & Dietrich, 1979). After incorporation of radioactivity from [³H]glucosamine and ³⁵SO₄, respectively, for 48 h, medium and cells were collected separately. The cells were digested briefly with trypsin and material released by this treatment (pericellular material) as well as the remaining material (intracellular) was recovered. The various fractions were processed as described elsewhere (Malmström *et al.*, 1975; Sjöberg & Fransson, 1977, 1980). Thus, labelled glycosaminoglycans were released as free chains by papain digestion and the total pool of sulphated glycosaminoglycans was separated from hyaluronate and glycopeptides by ion-exchange chromatography.

The yields of sulphated glycosaminoglycans [as 10⁻⁴ × ³H radioactivity (d.p.m.)/plate (≈10⁷ cells)] were as follows (for the intra-, peri- and extracellular sources, respectively): normal cells, 5.95, 3.19 and 7.05; for SV40-transformed cells, 17.83, 11.14 and 15.15; and for polyoma-transformed cells, 31.60, 43.00 and 96.01. After depolymerization of the galactosaminoglycan components by chondroitinase-ABC digestion, the heparan sulphates were isolated by gel chromatography on Sephadex G-50 (void volume fraction). The proportions of heparan sulphate (% of total) were as follows (for the intra-, peri- and extracellular sources, respectively): normal cells, 74, 89 and 72%; SV40-transformed cells, 52, 88 and 62%; and for polyoma-transformed cells, 57, 78 and 67%.

Degradation methods

Periodate oxidations were conducted with 0.02 M-sodium metaperiodate/0.05 M-sodium formate (pH 3.0) at 4°C for 24 h in the dark (Fransson, 1978; Fransson *et al.*, 1980d). Reactions were terminated by the addition of 0.1 vol. of 10% (w/v) D-mannitol. After dialysis against distilled water, oxidized products were cleaved by alkaline elimination at pH 12.0 for 30 min at room temperature.

Deaminative cleavage of bonds between GlcNSO₃ and hexuronic acid was performed with the pH 1.5/HNO₂ method (Shively & Conrad, 1976). Excess HNO₂ was evaporated with methanol. An outline of the result of the two degradation methods is depicted in Scheme 1.

Chromatographic methods

Radiolabelled heparan sulphates were subjected to ion-exchange chromatography on columns (6 mm × 140 mm) of DE-52 DEAE-cellulose that were equilibrated with 0.1 M-sodium acetate, pH 5.0. Elution was performed with a linear gradient of 0.1–2.5 M-sodium acetate, pH 5.0 (total volume, 100 ml) at a rate of 3 ml/h. The shape of the gradient was checked by conductivity measurements. The effluent was analysed for radioactivity in a Packard 2650 liquid-scintillation counter with automatic quench correction using Instagel (0.5 ml of sample and 5 ml of liquid) as scintillator. Gel chromatography of degradation products was performed on columns (8 mm × 1700 mm) of Sephadex G-50, superfine grade, that were eluted with 0.2 M-pyridine acetate (pH 5.0) at a rate of 6 ml/h. The effluents were analysed as described above.

Results and discussion

The ion-exchange chromatography profiles of the ³H- and ³⁵S-labelled heparan sulphates derived from the three pools of normal, SV40-transformed and polyoma-transformed 3T3 cells are shown in Fig. 1.

Scheme 1. Degradation of a heparan sulphate chain (a) by periodate oxidation/alkaline elimination (b) or deaminative cleavage (c)

In general, the heparan sulphates from transformed cells were more heterogeneous and of lower charge density than those from the normal counterpart (see in particular Figs. 1g and 1h). In both normal and transformed cells the heparan sulphates from the medium (Figs. 1c, 1f and 1i) were more retarded on the column than were the corresponding cell-associated materials. Heparan sulphate components that eluted at different ionic strengths were isolated from the various pools of normal, SV40-transformed and polyoma-transformed cells. The results, which were reproducible between different cell cultures (see also Vannucchi & Chiarugi, 1977; Chiarugi & Dietrich, 1979), are in keeping with the general notion that there is a lowering of the *O*-sulphate content in heparan sulphates from transformed cells.

Periodate oxidation of GlcA in $(\text{GlcA-GlcNAc})_n$ -block regions of heparan sulphate chains (see Scheme 1) followed by scission in alkali produces a fragmentation of the chains whereby the *N*-sulphate-rich segments may be isolated and their size distribution assessed. This analysis was performed with heparan sulphates from the intracellular pools of normal, SV40-transformed and polyoma-transformed cells (Fig. 2). The oligosaccharides obtained from the total cellular heparan sulphate pool of normal cells (Fig. 2a) ranged from $n = 1$ to $n = 5$ in the general formula $\text{GlcN}(\text{HexA-GlcN})_n\text{-R}$. There was a large contribution from oligomers with $n = 4-5$. Moreover, the size distribution obtained with the fragments from normal 3T3 cell heparan sulphate was very similar to the one seen in the case of lung fibroblast heparan sulphate (Sjöberg & Fransson, 1980). The profiles obtained with the oligosaccharides derived from SV40-transformed cell heparan sulphate (Fig. 2b) and the two heparan

sulphate subfractions (HS1a and HS2) from polyoma-transformed cells (Figs. 2c and 2d, respectively) were all markedly different from that of the normal counterpart. The common feature was a decrease in the amount of oligosaccharides with $n = 4-5$.

When heparan sulphates derived from the pericellular pool of the various cell lines were subjected to the same degradation procedure the results shown in Fig. 3 were obtained. The two heparan sulphate subfractions HS2 and HS3 of normal cells (Figs. 3a and 3b) had similar degradation profiles and, like the intracellular material, oligomers with $n = 4-5$ were present in significant amounts. However, the subfractions HS2 and HS1a from transformed cells (Figs. 3c and 3e) were more extensively degraded and thus were similar to their intracellular counterparts (see Figs. 2b-2d). In contrast, heparan sulphate subfractions HS3 of SV40-transformed cells (Fig. 3d) and HS2 of polyoma-transformed cells (Fig. 3f) gave similar oligosaccharide patterns as compared with normal HS3 (Fig. 3b). Thus, the heparan sulphates of higher charge density had similar co-polymeric structure in the case of the pericellular pool of normal and transformed cells, whereas the chains of lower charge density had shorter *N*-sulphate-rich segments in the case of the transformed cells. The oligomers with $n = 4-5$ that were obtained in the above cases were susceptible to re-oxidation with periodate, indicating that they comprised some GlcA-GlcNSO_3 repeats (Fransson *et al.*, 1980b,c).

From pools of the medium of the transformed and non-transformed cells two heparan sulphate subfractions, HS3 and HS4, were isolated. In the case of the transformed cells HS3 was larger than HS4

Fig. 1. Ion-exchange chromatography of [^3H , ^{35}S]heparan sulphates from the intracellular (a, d, g), pericellular (b, e, h) and medium (c, f, i) pools of normal (a–c), SV40-transformed (d–f) and polyoma-transformed (g–i) cell lines

The heparan sulphates were prepared and chromatographed as described in the Experimental section. HS2, 3 and 4 indicate the elution position of the heparan sulphate subfractions from bovine lung. Fractions were pooled as indicated by vertical lines.

whereas in the normal case the reverse situation prevailed (see Figs. 1c, 1f and 1i). These subfractions were also degraded by periodate oxidation/alkaline elimination and chromatographed on Sephadex G-50. In this case all profiles from transformed as well as non-transformed cells looked strikingly similar to one another and showed the same general size distribution as did the fractions HS1a and HS2 from transformed cells (Figs. 2 and 3).

In another set of experiments the various heparan sulphates were degraded with HNO_2 . In this procedure (see Scheme 1) the *N*-sulphate-rich regions of the chains are extensively degraded and

the size distribution of the $(\text{GlcA-GlcNAc})_n$ block segments may be determined by gel chromatography on Sephadex G-25 or G-50. When such analyses were performed with the heparan sulphate fractions from the intracellular and pericellular pools of normal and transformed cells, the size distribution of the above segments was very broad and ranged between $n=1$ and $n=10$ in HexA- $(\text{GlcNAc-GlcA})_n$ -aMan with a maximum around 1–3. However, there were no significant differences between the heparan sulphates of normal and transformed cells. When compared with heparan sulphate from human lung fibroblasts (Sjöberg & Fransson, 1980), all the 3T3 cell heparan sulphates

Fig. 2. Gel chromatography on Sephadex G-50 of oxidation-degradation products of [^3H , ^{35}S]heparan sulphates from the intracellular pools of (a) normal, (b) SV40-transformed and (c and d) polyoma-transformed 3T3 cells

The heparan sulphates were obtained as described in the Experimental section and in the legend to Fig. 1. The material from polyoma-transformed cells was separated into fractions 1a and 2 (Fig. 1g) each of which was subjected to analysis (c and d, respectively). The degradation products have the general carbohydrate sequence $\text{GlcN}-(\text{HexA}-\text{GlcN})_n-\text{R}$. The column was calibrated with a series of oligomers, $n=0-5$, that were obtained from beef lung heparan sulphate HS4 (Fransson *et al.*, 1980c). The vertical line separates oligomers with $n > 4$ from those with $n \leq 3$. The proportion of ^3H radioactivity obtained as oligomers $n \geq 4$ was for (a) 38%, (b) 10%, (c) 13% and (d) 16% of total radioactivity. SO_4 , the elution position of [^{35}S]sulphate.

afforded much more heterogeneous profiles; in the former case the $\text{GlcA}-\text{GlcNAc}$ disaccharides were much more narrowly clustered at approx. 3–4 such consecutive repeats.

Deaminative cleavage was also conducted with heparan sulphates from the medium pools of transformed and non-transformed cells. As shown in Figs. 4(a) and 4(d) HS3 and HS4 from normal cells differed in two respects. Firstly, the bulk of the degradation products from HS3 consisted of $\text{HexA}-\text{GlcNAc}-\text{GlcA}-\text{aMan}$ and $\text{HexA}-\text{aMan}$ (Fig. 4a) whereas HS4 also had a distinct $\text{HexA}-(\text{GlcNAc}-$

$\text{GlcA})_n-\text{aMan}$ component in the higher region. Secondly, the shape of the disaccharide peak suggests that the disulphated disaccharide $\text{IdoA}-(\text{-OSO}_3)-\text{aMan}-(\text{OSO}_3)$ was more common among the degradation products of HS4. [It has been shown (L.-Å. Fransson & B. Havsmark, unpublished work) that there is a separation between the variously sulphated disaccharides.] It should also be noted that the HS4 component was not found in the cell-associated fractions where HS2 and HS3 dominate. The variation in charge density and structure seen among the heparan sulphates of normal 3T3 cells is unlike the situation with heparan sulphates from human lung fibroblasts (Sjöberg & Fransson, 1980). In this case the chains had the same structural features irrespective of the source (extra-, inter-, peri- or intracellular).

When comparing heparan sulphates HS3 and HS4 from the medium of transformed cells (Figs. 4b, 4c, 4e and 4f) with those from the medium of non-transformed cells (Figs. 4a and 4d) it is clear that changes have taken place. Firstly, in the HS3 components the size distribution of $\text{HexA}-(\text{GlcNAc}-\text{GlcA})_n-\text{aMan}$ fragments is changed and a larger proportion of longer species is obtained in the two cases of transformation (Figs. 4b and 4c). The HS4 components were also changed but in the opposite direction, i.e. the GlcNAc -rich segments have become shorter (Figs. 4e and 4f). Secondly, there were alterations in the shape of the disaccharide peaks. The HS3 components from transformed cells yielded largely $\text{IdoA}-(\text{OSO}_3)-\text{aMan}-\text{OSO}_3$ (Figs. 4b and 4c) whereas the HS4 components from these cells (Figs. 4e and 4f) had a larger proportion of non- or monosulphated disaccharides than did the normal counterpart (Fig. 4d). These alterations seem complex, as they are in opposite directions, i.e. an increased proportion and length of the $(\text{GlcA}-\text{GlcNAc})_n$ segments was coupled with an increased O -sulphation within the N -sulphate-rich regions, while diminishing amounts of the former segments were coupled with lower sulphation in the latter regions. Therefore, these changes may cancel each other in terms of overall charge density and may explain why distinct HS3 and HS4 components are obtained in these cases despite marked variations in disaccharide composition and distribution.

Conclusions

We have previously advocated (L.-Å. Fransson & B. Havsmark, unpublished work; Fransson *et al.*, 1981b) that specifically self-associating heparan sulphates may be distinguished by, at least, three structural criteria, i.e. (a) the size distribution and location of the $(\text{GlcA}-\text{GlcNAc})_n$ regions, (b) the alternating or mixed segments such as $-(\text{GlcNSO}_3-\text{IdoA})_m-\text{GlcNAc}-(\text{GlcA}-\text{GlcNSO}_3)_n-$ and (c) the

Fig. 3. Gel chromatography on Sephadex G-50 of the oxidation-degradation products of ^3H -labelled heparan sulphate fractions 2 and 3 from the pericellular pools of (a, b) normal cells and (c, d) SV40-transformed cells and (e, f) heparan sulphate fractions 1a and 2 from the same pool of polyoma-transformed 3T3 cells

The heparan sulphates were obtained as described in the Experimental section and in the legend to Fig. 1. The chains from normal and SV40-transformed cells (Figs. 1b and 1e) were separated into subfractions 2 and 3 and degraded; the results are shown in (a and c) and (b and d), respectively. Heparan sulphate from polyoma-transformed cells was separated into fractions 1a and 2 (Fig. 1h) that were degraded and chromatographed (e and f). For n see the legend to Fig. 2. The proportion of ^3H radioactivity obtained as oligomers $n \geq 4$ was for (a) 38%, (b) 35%, (c) 21%, (d) 32%, (e) 20% and (f) 28%.

variation of IdoA and GlcA in $(\text{HexA-GlcNSO}_3)_n$ segments. In the case of beef lung heparan sulphates we have shown (Fransson *et al.*, 1981b) that the extent of binding of free chains to immobilized chains is greatest when heparan sulphates of similar chemical features are combined. Thus, heparan sulphate from human lung fibroblasts, which has a characteristic sequential arrangement of its building blocks, also binds most efficiently to beef lung heparan sulphate HS4, which has the corresponding chemical characteristics (Fransson *et al.*, 1981a). In the present study these chemical characteristics were assessed by HNO_2 degradation [assaying criteria (a) and (b) above] and by periodate oxidation/alkaline degradation [assaying the presence of extended $(\text{HexA-GlcNSO}_3)_n$ segments]. The heparan sulphates from the medium of transformed cells (HS3 as well as HS4 types) exhibited the greatest structural deviation as compared with normal cell-associated heparan sulphate. The former showed both variations in the size distribution of the $(\text{GlcA-GlcNAc})_n$ segments and a low content of extended $(\text{HexA-GlcNSO}_3)_n$ segments. The heparan sulphates of the

intracellular and pericellular compartments of transformed cells displayed no alterations in the size distribution of $(\text{GlcA-GlcNAc})_n$ segments but in low-sulphated forms, the characteristic extended (HexA-GlcNSO_3) segments were present in lower proportions than in the normal counterpart (Figs. 2 and 3). The normal 3T3 cells, which are an established cell line, produced heparan sulphates which carried (HexA-GlcNSO_3) segments of the same size distribution as the heparan sulphates produced by human lung fibroblasts, a primary cell culture. However, the size distribution of $(\text{GlcA-GlcNAc})_n$ segments was more polydisperse in heparan sulphates from established cells than in those from the primary cell culture. Thus, the heparan sulphate from the primary cell culture exhibited the most limited structural heterogeneity. There was a progressively increasing 'disorder' when going from heparan sulphates of established cells to those of transformed cells, in particular in the medium-derived chains. The increased structural heterogeneity is also accompanied by changes in the self-associating properties (Fransson *et al.*, 1981a).

Fig. 4. Gel chromatography on Sephadex G-50 of the deaminative cleavage products of heparan sulphate fractions 3 (a–c) and 4 (d–f) from the medium of normal (a, d), SV40-transformed (b, e) and polyoma-transformed (c, f) 3T3 cells. The heparan sulphates were obtained as described in the Experimental section and in the legend to Fig. 1. The chains from the medium were separated into subfractions 3 and 4 (Figs. 1c, 1f and 1i), each of which were degraded by HNO_2 and chromatographed. Di, the elution position of the disaccharides uronosyl-anhydromannose (non-, mono- or di-sulphated) obtained from heparin by this procedure. As shown elsewhere (L.-Å. Fransson & B. Havsmark, unpublished work) there is a partial separation between these species.

The heparan sulphate chains from lung fibroblasts were bound to their cognate gel with a rather uniform affinity, whereas the heparan sulphate from 3T3 cells showed a broad range of affinities. Heparan sulphates from transformed cells were not bound at all.

There is no evidence for a template-regulated biosynthesis of heparan sulphate and the mechanisms that control the extent of the chemical heterogeneity are largely unknown. Nevertheless, current knowledge concerning the biosynthesis of heparin-related glycans (Riesenfeld, 1980) indicate that the first polymer-modifying enzyme, the *N*-deacetylase, may play a critical role as it controls the scope of variability inherent in the ensuing modification steps (epimerization of GlcA to IdoA and ester sulphation). More information about the nature of this enzyme in various cell types and its topological arrangement in the membranes of the endoplasmic reticulum are necessary before the observations reported in this paper can be adequately explained.

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