Interferon γ differentially affects the synthesis of chondroitin/dermatan sulphate and heparan sulphate by human skin fibroblasts

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Interferon γ (IFN γ) is often considered to be an antifibrotic cytokine because it inhibits collagen synthesis in fibroblasts. Here we report the effects of recombinant human IFN γ on sulphated glycosaminoglycan chains produced by normal skin fibroblasts from adult donors. IFN γ (250 i.u./ml) induced an increase in incorporation of D-[1- 3 H]glucosamine into glycosaminoglycans, either secreted into the culture medium or associated with the cell layer. The structures of these molecules were analysed by using various cleavage agents (heparinases I and II, heparitinase/chondroitinases ABC and AC/periodate oxidation) followed by size-exclusion and anion-exchange HPLC.

No modification was detected in the structure of the heparan sulphate chains. In contrast, the cytokine induced changes in the microcomposition of chondroitin/dermatan sulphate chains. More precisely, we found a decrease in the iduronic acid content, associated with down-regulation of the 4-O-sulphation on the GalNAc residues. In contrast, the 6-O-sulphation on these GalNAc residues was potentiated by the cytokine. These results indicate that IFN γ is able to modulate not only collagen but also the structure of galactosaminoglycans synthesized by human skin fibroblasts.

INTRODUCTION

Chondroitin/dermatan sulphate and heparan sulphate proteoglycans constitute a heterogeneous family of macromolecules that contain a core protein covalently linked to polyanionic glycosaminoglycan chains [1]. The sequences of some core proteins have been determined and this has revealed a broad range of proteic modules, determining the localization and functions of these proteoglycans [2]. Some of them, such as the large chondroitin sulphate proteoglycan aggrecan in the cartilage, are embedded in the extracellular matrix as aggregates with hyaluronic acid [3]. Owing to the high density of their charge, they retain water and show resistance to compressive forces. Other proteoglycans, such as decorin, biglycan and fibromodulin [4], are also secreted into the extracellular matrix and bind to separate sites on fibrillar collagens [5], controlling collagen fibrillogenesis [6]. Proteoglycans also show some affinity for soluble cytokines, controlling their bioavailability, their stability and their activity [7]. In particular, detailed investigations have been performed on the interaction between transforming growth factor β 1 (TGF β 1) and decorin [8]. Decorin core protein was reported to neutralize the growth-promoting effect of TGF *in vitro* [9] and its fibrogenic action in experimental glomerulonephritis in rats [10]. Many studies have also described interactions between heparan sulphate chains and cytokines, for example basic fibroblast growth factor [11] or interferon γ (IFN γ) [12].

Chondroitin/dermatan sulphate galactosaminoglycans are first synthesized as repetitive sequences of alternating GalNAc and GlcA residues. Then, changes occur such as sulphations at the C-4 and/or C-6 positions of GalNAc and/or the C-2 position of the uronic acid. Some of the GlcA residues can also be epimerized at their C5 position into iduronic acid (IdoA), but this modification occurs only in chains identified as dermatan

sulphate [13,14]. Therefore the major difference between chondroitin sulphate and dermatan sulphate lies in the absence of IdoA residues in chondroitin sulphate as opposed to the presence of a variable percentage of IdoA in dermatan sulphate [15]. The ratio GlcA/IdoA in a given dermatan sulphate proteoglycan depends essentially on its cellular and tissue origins [16]. This ratio is also modulated in particular pathological contexts or by the presence of cytokines [17].

IFN γ is a cytokine that displays a broad range of activities [18]. Indeed, the molecule possesses antiviral and immunoregulatory properties. IFN γ is also antiproliferative [19] and acts as an inhibitor of collagen synthesis when added to fibroblasts in culture [20]. In fact this cytokine is suspected of suppressing collagen synthesis in these cells not only at transcriptional [21] or post-transcriptional levels [22], but also by blocking the maturation of collagen through the inhibition of prolyl 4-hydroxylase [23]. These results have suggested that IFN γ could be used as an antifibrotic agent in some pathological cases where there is an abnormal deposit of matrix overproduced by fibroblasts, as can be observed in hypertrophic scars [24]. However, the effects of IFN γ on the other components of the matrix (including glycoproteins and glycosaminoglycans) remain to be elucidated. Some investigations have characterized the effects of cytokine on glycosaminoglycans synthesized by fibroblasts. However, conflicting results have been published about the quantitative effect of cytokine on sulphated glycosaminoglycans [25–27].

In the present study we have analysed the effects of IFN γ on sulphated glycosaminoglycans produced by adult human skin fibroblasts. Heparan sulphate chains were not changed in a significant manner either quantitatively or qualitatively. However, we found an increase of 66% in the production of chondroitin/dermatan sulphate chains secreted into the culture medium, and an increase of 37% for those associated with the cell layer. The cleavage of the galactosaminoglycans from the cell

Abbreviations used: IdoA, iduronic acid; IFN γ , interferon γ ; UA, uronic acid; GlcNS, N-sulphated glucosamine.

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layer with different agents (periodate oxidation, chondroitinase AC), and the separation by anion-exchange HPLC of their constitutive disaccharides after chondroitinase ABC digestion, demonstrated that a concomitant change in the disaccharide composition also occurred after treatment with IFN γ . Indeed, we observed a decrease in IdoA and 4-O-sulphated GalNAc content concurrent with an increase in the number of 6-O-sulphated GalNAc residues. These epimerization and O-sulphation changes in chondroitin/dermatan sulphate chains after treatment with IFN γ could have many implications for chondroitin/dermatan sulphate functions such as the organization of extracellular matrix or the regulation of cellular proliferation.

EXPERIMENTAL

Materials

D-[1-³H]Glucosamine (specific radioactivity 138 mCi/mg) was purchased from Amersham International. Cell-culture media and foetal calf serum were supplied by Gibco. Dialysis membranes (Spectra por 6; cut-off 1000) came from Spectrum. Chondroitinase ABC (EC 4.2.2.4) from *Proteus vulgaris*, chondroitinase AC (EC 4.2.2.5), heparinase (EC 4.2.2.7), heparinase II (no EC number assigned) and heparitinase (EC 4.2.2.8) from *Flavobacterium heparinum* were supplied by Sigma. DEAE-Sephacel gel was obtained from Pharmacia. Bio-Gel P-6 (–400 mesh) and Bio-Gel P-10 fine came from Bio-Rad. HPLC was performed on a Waters 625 LC system with a Spherisorb SAX column (5 μ m particle size, 4.6 mm $\times$ 25 cm) from Supelco. The absorbance of the column eluate was monitored with a Waters 484 detector, and peak heights and areas were calculated with a HP 3396 series II integrator (Hewlett Packard). Unsaturated heparan sulphate disaccharides [IVA, IVS, IIA, IIS, IIIS, IS, which are UA-GlcNAc, UA-GlcNS, UA-GlcNAc(6S), UA-GlcNS(6S), UA(2S)-GlcNS and UA(2S)-GlcNS(6S) respectively; GlcNS is N-sulphated glucosamine and UA is uronic acid] and chondroitin/dermatan sulphate disaccharides [Δ Di-6S, Δ Di-4S, Δ Di-UA-2S, Δ Di-diS_B, Δ Di-diS_B, which are UA-GalNAc(6S), UA-GalNAc(4S), UA(2S)-GalNAc, UA-GalNAc(4S,6S) and UA(2S)-GalNAc(4S) respectively] were obtained from Sigma.

Cell culture and radiolabelling

Human skin fibroblasts either from children or adults were maintained at 37 °C (5% CO₂ in air) in Dulbecco's modified Eagle's medium, 25 mM Hepes (with sodium pyruvate and 1 g/l glucose) supplemented with 10% (v/v) heat-inactivated foetal calf serum, penicillin (100 i.u./ml) and streptomycin (100 μ g/ml). Cultures at different numbers of passages (P4, P5, P13, P15) were grown to confluence in eight 75 cm² flasks (Falcon). Half of them were washed, and fresh medium with 1% serum and 250 i.u./ml IFN γ was added for 36 h. Confluent cultures and low serum concentration were used to avoid growth-related events. The concentration of IFN γ (250 i.u./ml) chosen for the experiment was selected on the basis of previous studies performed in our laboratory. The other four flasks did not receive any IFN γ and acted as control cultures. Both the treated and untreated cultures were then allowed to incorporate D-[1-³H]glucosamine (6 μ Ci/ml) for 36 h with or without 250 i.u./ml IFN γ respectively.

Extraction of proteoglycans

The culture medium was removed and the cell layers were carefully washed twice with 20 mM sodium phosphate/0.15 M NaCl, pH 6.8. The medium and the first rinsing were pooled and

centrifuged (200 g for 5 min). The supernatant was kept for the separation of proteoglycans on a DEAE-Sephacel column.

Cell-associated proteoglycans were extracted by gentle shaking of the cell layers for 12 h at 4 °C in 4 M guanidinium chloride/0.05 M sodium acetate/2% (v/v) Triton X-100, pH 6.0. Solubilized macromolecules and a wash were combined and centrifuged at 200 g for 5 min. Supernatant was desalted on a Bio-Gel P-6 DG column (2.5 cm $\times$ 30 cm) eluted with 20 mM sodium phosphate/0.15 M NaCl/6 M urea/0.1% CHAPS, pH 6.8. The flow rate was 1.6 ml/min and the volume of each fraction collected was 2.4 ml.

Proteoglycans were subjected to initial purification by ion-exchange chromatography on a DEAE-Sephacel column (0.9 cm $\times$ 6 cm). For the proteoglycans secreted into the medium, the column was washed with 20 mM sodium phosphate/0.15 M NaCl, pH 6.8, to elute contaminating proteins. Then the proteoglycans were eluted with a linear gradient of 0.15–0.8 M NaCl in the same buffer at a flow rate of 0.2 ml/min. Fractions of 2.4 ml were collected. For the cell-associated proteoglycans, the same procedure was used except that the buffer was 20 mM sodium phosphate/0.15 M NaCl/6 M urea/0.1% CHAPS, pH 6.8. The nature of each peak was assessed by specific enzymes (hyaluronidase for hyaluronic acid, heparinase, heparinase II and heparitinase for heparan sulphate, and chondroitinase ABC for chondroitin/dermatan sulphate). Fractions corresponding to heparan sulphate proteoglycans and to chondroitin/dermatan sulphate proteoglycans were pooled, dialysed against distilled water and freeze-dried.

Preparation of intact heparan sulphate chains

Traces of chondroitin/dermatan sulphate in fractions containing heparan sulphate proteoglycans were removed by treating them with chondroitinase ABC (1 unit/ml) overnight at 37 °C, in 100 mM Tris acetate/10 mM EDTA, pH 8. Core proteins were then digested with papain (1 mg/ml) for 24 h at 65 °C in 0.1 M sodium acetate/5 mM EDTA/5 mM cysteine, pH 7.0. A second digestion was performed with papain for 5 h under the same conditions. Ice-cold 100% (w/v) trichloroacetic acid was added to the extract at 4 °C to give a final concentration of 10% (v/v). After 30 min at 4 °C, the precipitate was centrifuged at 10000 g for 30 min at 4 °C. The supernatant was recovered. The pellet was resuspended in 10% trichloroacetic acid and allowed to stand for 30 min at 4 °C. The centrifugation was repeated in the same way. The two supernatants were pooled and dialysed against 0.15 M sodium phosphate buffer, pH 6.8. Heparan sulphate chains were then recovered by stepwise elution with 1 M NaCl from a 2 ml DEAE-Sephacel column after a wash with 0.3 M NaCl to elute chondroitin/dermatan sulphate oligosaccharides and peptide fragments from the core proteins. After dialysis against distilled water, the heparan sulphate chains were freeze-dried for further analysis.

Preparation of intact galactosaminoglycans

Traces of heparan sulphate were removed by HNO₂ deaminative cleavage performed with the low-pH method of Shively and Conrad [28]. Samples were dried by centrifugal evaporation, reconstituted in 2 ml of 0.5 M HNO₂ solution and incubated for 15 min at room temperature. The reaction was stopped by adding 1 ml of 1 M Na₂CO₃. After dialysis against distilled water, the core proteins were removed by papain and the chondroitin/dermatan sulphate chains were purified by the procedure used for the heparan sulphate chains.

Depolymerization of heparan sulphate chains

Heparitinase was used to cleave heparan sulphate chains into oligosaccharides, which were analysed by size-exclusion chromatography. Samples were incubated with the enzyme (10 units/ml; 1 unit of heparitinase/heparitinase forms 0.1 μ mol of product/h) in 50 mM Tris/HCl, pH 7.6, overnight at 35 °C. To obtain only disaccharides for the anion-exchange HPLC analysis, heparinase, heparitinase and heparinase II were added sequentially at 2 h intervals at a concentration of 10 units/ml in 50 mM Tris/HCl containing 100 mM NaCl, pH 7.2, and incubated overnight at 35 °C. All the digestions were stopped by heating them at 100 °C for 2 min.

Depolymerization of galactosaminoglycans

Chondroitin/dermatan sulphate chains were cleaved with chemical (periodate oxidation) or enzymic agents (chondroitinases).

Periodate oxidation of galactosaminoglycans was performed in accordance with Fransson's method [29]. Briefly, chondroitin/dermatan sulphate chains in 50 mM sodium citrate/200 mM sodium perchlorate/20 mM sodium periodate, pH 3.0, were incubated at 4 °C for 24 h in the dark. The reaction was terminated by adding 0.1 vol. of 10% (w/v) mannitol. The oxyglycan was subsequently cleaved by alkaline elimination [30]. For this purpose the solution of oxydermatan sulphate was adjusted to pH 12 with 1 M NaOH. After being left at room temperature for 30 min, the solution was neutralized with 1 M acetic acid.

Chondroitinases AC and ABC were used overnight at 37 °C at a concentration of 1 unit/ml, in 100 mM Tris acetate, pH 7.4, and 100 mM Tris acetate/10 mM EDTA, pH 8.0, respectively. Enzymes were deactivated by heating them at 100 °C for 2 min. In control digests with unlabelled glycosaminoglycans, the increase in A_{232} was measured to ensure that depolymerization was complete in each digestion.

Gel chromatography

Enzymic and chemical degradation products of glycosaminoglycans were resolved by gel chromatography on Bio-Gel P-6 and Bio-Gel P-10 columns (1 cm $\times$ 120 cm) in 0.5 M NH_4HCO_3 eluted at a flow rate of 4 ml/h. Fractions of 1 ml were collected and aliquots were taken for liquid-scintillation counting of radioactivity. The content of GlcA (y ; see below) was determined on heparitinase and chondroitinase AC profiles by the method of Sjöberg et al. [31].

HPLC procedure

Heparan sulphate and chondroitin/dermatan sulphate disaccharides from gel-filtration chromatography were submitted to anion-exchange HPLC and fractionated on a SAX Spherisorb column with a linear NaCl gradient. Before injection, the column was pre-equilibrated with distilled water adjusted to pH 3.5 with HCl. Freeze-dried samples resuspended in 550 μ l of this solution were injected on to the analytical column. The elution was performed with the use of a linear gradient of NaCl at pH 3.5 (0–0.5 M over 60 min) with a flow rate of 1 ml/min. The elution profile was monitored by measuring A_{232} . Simultaneously, 0.50 ml fractions were collected for liquid-scintillation counting. Peak identification was performed by co-injection of 1 μ g of each unsaturated heparan sulphate or chondroitin/dermatan sulphate disaccharide standard. We were not able to separate Δ Di-4S from Δ Di-UA-2S chondroitin/dermatan sulphate standards because these two monosulphated disaccharides always co-eluted regardless of the type of NaCl gradient or the flow rate used.

Liquid-scintillation counting

Samples were mixed with PicoFluor®15 (Packard) and the level of radioactivity determined in a Tri-Carb 2100 TR (Packard) liquid-scintillation analyser.

RESULTS

IFN γ increases ^3H incorporation into glycosaminoglycans

Human skin fibroblasts from an adult (28 years old) at passage 15 were cultured in the presence or absence of IFN γ (250 i.u./ml) with the concomitant radiolabelling of their glycosaminoglycans with D-[1- ^3H]glucosamine. After the extraction of the proteoglycans from the cell layer, we calculated a ratio of 2.7 between the incorporation of ^3H into the IFN γ -treated cells and into the untreated ones. To analyse the effect of the cytokine on each proteoglycan, the labelled material was fractionated on a DEAE-Sephacel column. As shown in Figure 1, the concentrations of the hyaluronic acid and the sulphated proteoglycans (heparan sulphate and chondroitin/dermatan sulphate proteoglycans) produced by the fibroblasts were all increased by the treatment, both in the medium (Figure 1a) and in the cell layer (Figure 1b). After pooling the fractions corresponding to these molecules, we calculated the ratio 'treated/untreated' for ^3H incorporation into hyaluronic acid, heparan sulphate proteoglycans and

Figure 1 Fractionation of radiolabelled proteoglycans by chromatography on DEAE-Sephacel

Radioactive materials from medium (a) and from the cell layer (b) of adult human skin fibroblasts were applied to a DEAE-Sephacel column (0.9 cm $\times$ 6 cm) in PBS at pH 6.8. After washing with 25 ml of the starting buffer, a linear gradient of NaCl was developed from 0.15 to 0.8 M in a total volume of 200 ml. Fractions of 2.4 ml were collected for liquid-scintillation counting. The order of elution was hyaluronic acid (HA), heparan sulphate proteoglycans (HSPGs) and chondroitin/dermatan sulphate proteoglycans (CS/DSPGs). The ratios between IFN γ -treated (broken line) and control cells (solid line) are indicated in bold above each peak. The molarity in the NaCl gradient is also indicated in italics for the maximum value of each peak.

Table 1 Ratios between ^3H incorporation in IFN γ -treated cells and control cells, either in the medium or in the cell layer

This table summarizes the ratios between the IFN γ -treated and the untreated values of ^3H incorporation in hyaluronic acid (HA), heparan sulphate proteoglycans (HSPGs) and chondroitin/dermatan sulphate proteoglycans (CS/DSPGs) synthesized by different cell lines from children and adults. Asterisk values indicates that a combined ratio was calculated for HSPGs and CS/DSPGs in the cell layer of these fibroblasts. The ratios for the cell line used in this work are indicated in bold.

Donor	Number of passages	Medium			Cell layer	
		HA	HSPGs	CS/DSPGs	HA	HSPGs + CS/DSPGs
Child 1	P4	5.68	9.38	14.89	5.02	2.28*
Child 2	P5	3.14	2.51	1.96	2.54	2.20*
Adult 1	P13	1.71	1.75	1.62	3.42	1.73*
Adult 2	P15	1.36	1.66	1.60	4.25	1.51 + 1.33

chondroitin/dermatan sulphate proteoglycans to be 1.36, 1.66 and 1.60 in the medium, and 4.25, 1.51 and 1.33 in the cell layer respectively.

To ensure that this effect was not restricted to one fibroblastic cell line, dermal fibroblasts at different numbers of passages and from donors of different ages were tested under the same conditions. We found important interindividual variation (Table 1). However, IFN γ treatment led to an increase in the production of hyaluronic acid and sulphated proteoglycans in every case. We continued our analysis with adult fibroblasts in passage 15 (shown in bold in Table 1).

The ratios 'treated/untreated' for the incorporation of ^3H in chondroitin/dermatan sulphate proteoglycans (1.60 in the medium and 1.33 in the cell layer) were not modified by the purification of the galactosaminoglycan chains (1.66 in the medium and 1.37 in the cell layer). This result suggested that the increase in the incorporation of ^3H after IFN γ treatment was due to the galactosaminoglycan chains and not to other glycosylations on the core protein. Because galactosaminoglycan chains from the cell layer have a similar molecular mass in control (27 kDa) and treated cells (24 kDa) as evaluated by molecular sieving on a 1 cm $\times$ 120 cm CL-6B column (results not shown), we concluded that the increase in ^3H incorporation observed after IFN γ treatment was not due to a lengthening of these chains.

To see whether this quantitative effect was associated with changes in the disaccharide composition of glycosaminoglycan chains, we used chemical and enzymic degradations followed by size-exclusion and anion-exchange HPLC.

IFN γ does not change the structure of heparan sulphate chains

Heparitinase requires a GlcA residue to cleave heparan sulphate chains, and only recognizes the linkages GlcNR α 1-4GlcA (R = Ac or SO $_3^-$). The cleavage thus releases Δ -oligosaccharides with the general structure $\Delta\text{UA}[\text{GlcNSO}_3^- \text{IdoA}]_{n-1} \text{GlcNR}$, where UA denotes either GlcA or 2-O-sulphated IdoA residues. The profiles obtained for the heparan sulphate chains secreted into the medium (Figure 2a) or associated with the cell layer (Figure 2b) were almost identical for control and treated cells. We calculated the content of GlcA residues (y) with the formula:

$$y = \sum_{n=1}^7 A_n/n \quad (1)$$

where A_n denotes the percentage of total ^3H radioactivity in the peak corresponding to the Δ -oligosaccharide with n disaccharides

Figure 2 Bio-Gel P-6 gel filtration of oligosaccharides produced by heparitinase on heparan sulphate chains

Heparan sulphate chains from medium (a) and from the cell layer (b) of adult human skin fibroblasts, treated with IFN γ (broken line) and untreated (solid line), were degraded with heparitinase as described in the Experimental section. The fragments obtained have the general structure $\Delta\text{UA}[\text{GlcNSO}_3^- \text{IdoA}]_{n-1} \text{GlcNR}$ (R = Ac or SO $_3^-$). These oligosaccharides were fractionated on a Bio-Gel P-6 gel-filtration column (1 cm $\times$ 120 cm) eluted with 0.5 M NH $_4$ HCO $_3$ at a flow rate of 4 ml/h. Aliquots were counted by liquid scintillation. Under the degree of polymerization (2, disaccharides; 4, tetrasaccharides; and so on) is indicated the proportion of each peak in the total collected radioactivity. Control values precede those for IFN γ -treated samples.

in its formula [31]. Heparan sulphate chains in medium derived from untreated cells contained 71.4% of GlcA as opposed to 75.3% in medium derived from treated cells. Heparan sulphate chains associated with the untreated cell layer contained 71.6% of GlcA as opposed to 74.7% for the treated cell layer. Thus IFN γ has no significant effect on the epimerization of GlcA into IdoA residues in fibroblastic heparan sulphate chains.

To determine whether the pattern of O-sulphations in heparan sulphate chains was changed by IFN γ , we analysed the disaccharide composition of the chains by anion-exchange HPLC.

Disaccharides obtained by exhaustive digestion of heparan sulphate chains by heparitinase were recovered after size-exclusion chromatography on Bio-Gel P-6 for separation by anion-exchange HPLC. Each peak was identified by the co-injection of unlabelled standards (Figure 3). The percentage of total ^3H radioactivity found in each peak was determined for the control and treated cells, both in the medium (Figure 3a) and in the cell layer (Figure 3b). We concluded from this experiment that the

Figure 3 HPLC identification of disaccharides generated from heparan sulphate chains by heparinase, heparinase II and heparitinase digestion

Disaccharides produced by heparinase, heparinase II and heparitinase digestion on heparan sulphate chains from medium (a) and from the cell layer (b) of adult human skin fibroblasts, treated with IFN γ (broken line) and untreated (solid line), were prepared by Bio-Gel P-6 chromatography, freeze-dried and analysed by strong-anion-exchange chromatography. The column (SAX Spherisorb, 4.6 mm $\times$ 25 cm) was equilibrated in mobile phase (distilled water adjusted to pH 3.5 with HCl). Elution was performed with a linear gradient of NaCl (0–0.5 M over 60 min) in the same mobile phase at a flow rate of 1 ml/min with a Waters 625 LC system. Samples of 550 μ l were injected and 0.50 ml fractions collected for liquid-scintillation counting. The nature of each peak was identified by the co-injection of disaccharide standards: IVA, Δ UAGlcNAc; IVS, Δ UAGlcNSO $_3^-$; IIA, Δ UAGlcNAc(6S); A, Δ UA(2S)GlcNAc; IIS, Δ UAGlcNSO $_3^-$ (6S); IIS, Δ UA(2S)GlcNSO $_3^-$; IS, Δ UA(2S)GlcNSO $_3^-$ (6S). The percentages correspond to the proportion of each disaccharide in the total collected radioactivity. Control values precede those for IFN γ -treated samples.

pattern of O-sulphations in heparan sulphate chains was not significantly modified by IFN γ .

IFN γ changes the pattern of galactosaminoglycan depolymerization with periodate oxidation/alkaline elimination

Periodate oxidation followed by alkaline elimination cleaves only at the non-sulphated IdoA residues in dermatan sulphate. 2-O-Sulphated IdoA and GlcA residues are both resistant to cleavage [30]. Thus this chemical reagent releases oligosaccharides with the general structure GalNAc[UAGalNAc] $_n$ R, where UA denotes either GlcA or 2-O-sulphated IdoA residues, R is a C-4 fragment of an oxidized IdoA residue and n represents the number of UA residues in the oligosaccharide fragments [31]. Fractionation of these oligosaccharides obtained by this method was performed on a Bio-Gel P-10 column. The profiles of

Figure 4 Bio-Gel P-6 gel filtration of oligosaccharides produced by periodate oxidation/alkaline elimination on galactosaminoglycans

Galactosaminoglycans from medium (a) and from the cell layer (b) of adult human skin fibroblasts, treated with IFN γ (broken line) and untreated (solid line), were degraded with periodate oxidation/alkaline elimination as described in the Experimental section. The resulting oligosaccharide mixture was fractionated on a Bio-Gel P-6 gel filtration column (1 cm $\times$ 120 cm) eluted with 0.5 M NH $_4$ HCO $_3$ at a flow rate of 4 ml/h. Fractions of 1 ml were collected for scintillation counting. The fragments obtained have the general backbone GalNAc[UAGalNAc] $_n$ R, where UA denotes either GlcA or 2-O-sulphated IdoA residues, and R is a C-4 fragment of an oxidized IdoA residue; n represents the number of UA residues in the oligosaccharide fragments.

separation are shown in Figure 4. Galactosaminoglycans secreted into the control cell medium yielded a cleavage pattern similar to that of the medium derived from IFN γ -treated cells (Figure 4a). By contrast, the galactosaminoglycans from the control cell layer were degraded to a greater extent than those from the treated cell layer (Figure 4b). We concluded from this experiment that the chondroitin/dermatan sulphate chains from the control and treated media contained similar quantities of unsulphated IdoA residues. The content of unsulphated IdoA, in contrast, was lower in chondroitin/dermatan sulphate chains from the cell layer of treated cells compared with the control cells. Because of the presence of overlapping peaks in the patterns of cleavage with periodate oxidation, we were unable to quantify the effect of IFN γ on galactosaminoglycans associated with the cell layer. This is why we decided to use chondroitinase AC as an alternative agent of cleavage.

IFN γ induces a change in the respective proportions of GlcA and IdoA residues in galactosaminoglycans

Chondroitinase AC degrades the chondroitin sulphate portions of galactosaminoglycan chains and releases resistant dermatan sulphate fragments (IdoA-containing oligosaccharides) with the general structure UAGalNAc[IdoAGalNAc] $_{n-1}$ [31], where $n-1$

Figure 5 Bio-Gel P-10 gel filtration of oligosaccharides produced by chondroitinase AC digestion of galactosaminoglycans

Galactosaminoglycans from medium (a) and from the cell layer (b) of adult human skin fibroblasts, treated with IFN γ (broken line) and untreated (solid line), were degraded with chondroitinase AC as described in the Experimental section. The fragments obtained after this cleavage present the general carbohydrate sequence $\Delta\text{UAGalNAc}[\text{IdoAGalNAc}]_{n-1}$, with n varying from 1 to 3. These oligosaccharides were fractionated on a Bio-Gel P-10 gel-filtration column (1 cm $\times$ 120 cm) eluted with 0.5 M NH_4HCO_3 at a flow rate of 4 ml/h. Fractions of 1 ml were collected for liquid-scintillation counting. Under the degree of polymerization (defined as in Figure 2) is indicated the proportion of each peak in the total collected radioactivity. Control values precede those for IFN γ -treated samples.

represents the number of IdoA residues in the oligosaccharide fragments. The cleavage profiles obtained for the medium (Figure 5a) and for the cell layer (Figure 5b) revealed three types of oligosaccharide length (disaccharides, tetrasaccharides and hexasaccharides) in both the control and treated cells. The fact that only short sequences were resistant to chondroitinase AC indicated that dermatan sulphate chains produced by fibroblasts did not contain more than three contiguous (IdoAGalNAc) disaccharides. In the medium, IFN γ did not induce significant changes in the pattern of cleavage with chondroitinase AC (Figure 5a). In contrast, we observed in the cell layer (Figure 5b) that IFN γ strongly diminished the proportion of the hexasaccharides ($n = 3$) to one-third and reduced the proportion of the tetrasaccharides ($n = 2$) to one-half. At the same time the proportion of disaccharides ($n = 1$) was doubled after cytokine treatment. This result was consistent with the changes observed in the cleavage profile after periodate oxidation and alkaline degradation (Figure 4b). We calculated the content of GlcA residues (y) from eqn. (1). The percentage of GlcA for galactosaminoglycans secreted into the medium was not significantly modified by IFN γ , with a value of 61.3% for control cells and 58.5% for treated ones. In contrast, the quantity of GlcA residues in galactosaminoglycans associated with the cell layer

Figure 6 HPLC identification of disaccharides generated from galactosaminoglycans by chondroitinase ABC

Disaccharides produced by chondroitinase ABC digestion of chondroitin/dermatan sulphate chains from the cell layer of adult human skin fibroblasts, treated with IFN γ (broken line) and untreated (solid line), were prepared by Bio-Gel P-6 chromatography, freeze-dried and analysed by strong-anion-exchange chromatography on a SAX Spherisorb column. Chromatographic conditions were identical with those indicated for Figure 3. The nature of each peak was identified by co-injection of disaccharide standards: $\Delta\text{Di-0S}$, $\Delta\text{UAGalNAc}$; $\Delta\text{Di-6S}$, $\Delta\text{U-GalNAc(6S)}$; $\Delta\text{Di-4S}$, $\Delta\text{UAGalNAc(4S)}$; $\Delta\text{Di-UA-2S}$, $\Delta\text{UA(2S)GalNAc}$; $\Delta\text{Di-diS}_D$, $\Delta\text{UA(2S)GalNAc(6S)}$; $\Delta\text{Di-diS}_E$, $\Delta\text{UAGalNAc(4S,6S)}$; $\Delta\text{Di-diS}_B$, $\Delta\text{UA(2S)GalNAc(4S)}$. The percentages correspond to the proportion of each disaccharide in the total collected radioactivity. Control values precede those for IFN γ -treated samples.

was 60.3% for the control cells and reached 84.9% for the treated cells. Thus IFN γ induced a decrease in the epimerization of GlcA into IdoA residues in the total galactosaminoglycan population associated with the fibroblastic cell layer.

To determine whether the pattern of O-sulphations in galactosaminoglycans from cell layers was also regulated by IFN γ , we analysed the disaccharide composition of chondroitin/dermatan sulphate chains by anion-exchange HPLC.

IFN γ modifies the pattern of O-sulphations in galactosaminoglycans

Disaccharides obtained by exhaustive chondroitinase ABC digestion on chondroitin/dermatan sulphate chains from cell layers were recovered from size-exclusion chromatography on Bio-Gel P-6 for separation by anion-exchange HPLC. Each peak was identified by co-injection of unlabelled standards (Figure 6). We were unable to separate $\Delta\text{Di-4S}$ from $\Delta\text{Di-UA-2S}$ standards. However, material being eluted at this position is likely to be $\Delta\text{Di-4S}$ -type because this disaccharide is predominant in the structure of dermatan sulphate (the main glycosaminoglycan synthesized by fibroblasts [32]). In the galactosaminoglycans

secreted into the medium, no significant variation was observed between control and treated cells (results not shown). The situation was different in the cell layer (Figure 6). Indeed, IFN γ treatment was found to promote the occurrence of Δ Di-6S disaccharides, reducing the amount of Δ Di-4S disaccharides by the same proportion. The other types of disaccharide, either poorly represented (Δ Di-0S, Δ Di-diS_D, Δ Di-diS_E, Δ Di-diS_B) or absent [Δ Di-TriS = UA(2S)GalNAc(4S,6S)], were not changed by the cytokine in a relevant fashion.

Therefore IFN γ changed the pattern of sulphations of GalNAc residues in galactosaminoglycans from the cell layer with a decrease in their 4-O-sulphation balanced by an increase in their 6-O-sulphation. The experiment was repeated three times with the same results.

DISCUSSION

In this study we found that IFN γ increases the incorporation of D-[1-³H]glucosamine into hyaluronic acid and into the two populations of sulphated proteoglycans (heparan sulphate and chondroitin/dermatan sulphate proteoglycans) synthesized by adult human skin fibroblasts. The fact that the same increase in incorporation of D-[1-³H]glucosamine was found after removal of core proteins from sulphated proteoglycans suggests that IFN γ affects the glycosaminoglycan part rather than the glycosylated part of the core proteins. We also observed that IFN γ does not lengthen glycosaminoglycan chains. Therefore IFN γ either potentiates the total production of chondroitin/dermatan sulphate and heparan sulphate proteoglycans or the use of a greater number of acceptor sites on the core protein by xylosyltransferase during the biosynthesis of sulphated glycosaminoglycans. The IFN γ effect was found in cells from different origins and did not depend on the age of the donors or the number of passages of cells in culture. However, contradictory results have been published on the quantitative effect of IFN γ . Indeed, when D-[1-³H]glucosamine was used for radiolabelling, the total production of hyaluronic acid and proteoglycans was found to increase [26]. Nevertheless, when ³⁵S incorporation was used to radiolabel proteoglycans, no increase was observed [25,27]. We tried to use this isotopic reagent and we observed that cytokine interfered with the incorporation of radioactive sulphate in glycosaminoglycans [33]. For this reason, experiments involving ³⁵S incorporation after IFN γ treatment should be considered with caution.

We then determined what biochemical changes in the composition of galactosaminoglycans were induced by IFN γ . What emerges from the patterns of cleavage with periodate oxidation/alkaline elimination or chondroitinase AC is a decrease in the number of IdoA residues in the galactosaminoglycans associated with the cell layer after cytokine treatment. A relationship has been suggested between uronic acid epimerization and one of the O-sulphations occurring on GalNAc residues (at the C-4 or C-6 positions) [15]. The discovery of the proteoglycan PG-100 reinforces this idea [34]. Indeed, the absence of GlcA epimerization in this chondroitin/dermatan sulphate proteoglycan is accompanied by a decrease in the 4-O-sulphation on GalNAc residues. Furthermore these two anabolic steps are disrupted by monensin (a drug that interferes with the traffic through the medial Golgi) whereas 6-O-sulphation is not affected [35]. Therefore there is a link between the enzymic actions of the uronosyl C-5 epimerase and the 4-O-sulphotransferase [36]. Our results relating to the chondroitin/dermatan sulphate associated with the cell layer support these findings. Indeed, the decrease in IdoA we found was concomitant with a decrease in GalNAc(4S) residues and also an increase in the proportion of GalNAc(6S)

residues. The latter probably derive from GalNAc unmodified by 4-O-sulphotransferase and thus available for 6-O-sulphotransferase action. Therefore there seems to be an equilibrium between the actions of the two O-sulphotransferases. Our results did not allow us to exclude the possibility that IFN γ induced a shift from chondroitin sulphate A (4-O-sulphated) to chondroitin sulphate C (6-O-sulphated) regions in galactosaminoglycan chains. The change in the ratio GlcA/IdoA induced by IFN γ in galactosaminoglycans associated with the cell layer could have many implications for the organization of the extracellular matrix and for the distribution of its components. Indeed, it has been demonstrated that the self-association of scleral proteodermatan sulphate occurs through contact zones composed of alternating or mixed IdoA-GalNAc(4S) and GlcA-GalNAc(4S) on the glycosaminoglycan chain [37]. This self-association of dermatan sulphate chains is considered to be important for the proper assembly of collagen fibrils [38]. Furthermore IdoA is a flexible residue with different conformers [39]. The interconversion of these conformers provides an extra degree of freedom in dermatan sulphate, enabling this galactosaminoglycan to interact with other components of the matrix [40], especially collagens. For example, when human skin fibroblasts are cultured in three-dimensional collagen gel, there is a specific association between the IdoA-rich dermatan sulphate synthesized by these cells and the collagen substratum [41]. IFN γ , by favouring the synthesis of IdoA-poor galactosaminoglycans, yields a less stable matrix, consistent with its known activity in matrix remodelling. The fact that changes have been observed for the cell-associated layer galactosaminoglycans but not for the secreted galactosaminoglycans suggests that IFN γ might affect both matrix organization and cell-matrix interactions, but not other glycosaminoglycan functions [1]. Through its inhibitory action on collagen synthesis when added to fibroblasts in culture [20], IFN γ also diminishes the potential binding sites for IdoA-rich dermatan sulphate chains in the cell layer. Therefore our results could have been explained by a change in the distribution of IdoA-rich dermatan sulphate chains, bound more weakly to the cell layer and consequently secreted in greater quantity into the medium. However, no increase in the quantity of IdoA-rich galactosaminoglycans was detected in the medium after treatment with IFN γ . Our results are thus consistent with the existence of a biosynthetic compartmentalization, with different subsites within the Golgi stack specialized in the epimerization of galactosaminoglycans either secreted or associated with the cell layer. This agrees with other studies indicating dual distribution of chondroitin sulphate chains in the medial and *trans*-Golgi regions. Furthermore it is known that Golgi-membrane-disrupting agents have differential effects on the synthesis of different proteochondroitins (reviewed in [42]). Similarly, the present study suggests that IFN γ might selectively affect one of these biosynthetic compartments.

The ratio GlcA/IdoA in glycosaminoglycan chains is also suspected of influencing subcellular localization. This is the case for heparan sulphate, which contains, like dermatan sulphate, the two epimers GlcA and IdoA in its structure. In hepatocytes, heparan sulphate with an unusually high content of GlcA(2S)GlcNSO₃⁻(6S) has been found in the nucleus [43]. This suggests a potential capacity of heparan sulphate for regulating nuclear activities. As regards galactosaminoglycans, the link between the structure and the subcellular localization has not yet been established. However, chondroitin sulphate has no effect on the growth of human lung fibroblasts whereas dermatan sulphate shows an antiproliferative activity that increases in proportion to its IdoA content [44]. Given that IFN γ -treated galactosaminoglycans are poorer in IdoA residues than are control molecules,

it remains to be determined whether this biochemical modification could have any effect on the proliferation of fibroblasts.

In contrast with chondroitin/dermatan sulphate, the proportion of GlcA and IdoA residues in heparan sulphate was not significantly modified by IFN γ , as shown by the identical profiles of depolymerization by heparitinase (Figure 2). This result indicates that the uronosyl C-5 epimerase responsible for uronic acid inversion during heparan sulphate synthesis was not affected by IFN γ . This agrees with the existence of two uronosyl C-5 epimerases during glycosaminoglycan biosynthesis: one for heparan sulphate/heparin [45] and one for chondroitin/dermatan sulphate [46]. Both these enzymes, which are known to differ in optimum pH, sensitivity to ionic strength and affinity for substrates, also display different degrees of responsiveness to IFN γ .

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