Interferon-γ inhibits ^{35}S incorporation in heparan sulfate synthesized by human skin fibroblasts

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Abstract Glycosaminoglycans synthesized by human skin fibroblasts were simultaneously radiolabelled with D-[1-^{3}H]glucosamine and $Na_2{}^{35}SO_4$. Considering ^{3}H incorporation, we found that IFNγ increased the production of glycosaminoglycan synthesis, including hyaluronic acid, heparan and chondroitin/dermatan sulfate. In contrast, the production of heparan and chondroitin/dermatan sulfate was slightly decreased on the basis of the ^{35}S signal. Furthermore, when heparan sulfate was treated with nitrous acid, the release of free ^{35}S was greater in control than in treated cells, although the ^{3}H patterns of depolymerization with this agent were similar. These data demonstrate that IFNγ inhibits the incorporation of sulfate from extracellular medium into heparan sulfate.

Key words: Interferon-γ; Sulfate assimilation; Heparan sulfate; Glycosaminoglycans; Metabolic labelling

1. Introduction

Interferon-gamma (IFNγ) is a cytokine having a broad range of activities [1] including antiviral, immunoregulatory [2] and antiproliferative [3] properties. It also acts as an inhibitor of collagen synthesis when added to fibroblasts in culture [4]. However, the effects of IFNγ on the other components of the matrix (mainly glycoproteins and glycosaminoglycans) remain to be elucidated. Contradictory results have been published on the quantitative effect of IFNγ on glycosaminoglycans synthesized by human skin fibroblasts. Indeed, when D-[^{3}H]glucosamine was used for radiolabelling these molecules, an increase in the total production of hyaluronic acid and proteoglycans was found [5]. However, when ^{35}S incorporation was investigated, no increase was observed [6,7]. To clarify this discrepancy, we used in this study the two isotopic reagents simultaneously for the radiolabelling of sulfated glycosaminoglycans associated with the cell layer of human skin fibroblasts.

2. Materials and methods

2.1. Cell culture, IFNγ treatment and radiolabelling

Children's skin fibroblasts were cultured at 37°C (5% CO_2 in air) in DMEM 25 mM HEPES (Gibco), supplemented with 10% (v/v) fetal calf serum (heat inactivated), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were grown to confluence at the fifth passage in two 75-cm^2 flasks (Falcon, UK). One flask was washed and received fresh medium with 1% of serum and 250 U/ml of IFNγ (Roussel Uclaf, RU 42369) for 24 h. The other flask (control) received the same medium without IFNγ. Cells were then allowed to incorporate for 36 h D-[1-^{3}H]glucosamine (32 μCi/ml) and $Na_2{}^{35}SO_4$ (32 μCi/ml) purchased from Amersham International, in DMEM devoid of sulfate.

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2.2. Extraction of hyaluronic acid and proteoglycans from cell layer

Cell-associated proteoglycans were extracted by incubation in 4 M guanidine HCl, 0.05 M sodium acetate (pH 6), 2% (v/v) Triton X 100 for 12 h at 4°C. Solubilized macromolecules were buffer exchanged on a Bio-Gel P-6 DG (Bio-Rad) column (2.5 cm × 30 cm) eluted with 20 mM sodium phosphate buffer, 0.15 M NaCl, 6 M urea, 0.1% CHAPS (w/v), pH 6.8, at a flow rate of 1.6 ml/min (fractions of 2.4 ml) Proteoglycans were separated on a anion-exchange DEAE Sephacel (Pharmacia) column (0.9 cm×6 cm) eluted at a flow rate of 0.2 ml/min with a 200 ml linear gradient from 0.15 to 0.8 M NaCl in 20 mM sodium phosphate, 6 M urea and 0.1% CHAPS, pH 6.8. Fractions corresponding to heparan sulfate proteoglycans were pooled, dialyzed (Spectra por 6; cut-off 1000, from Spectrum) against distilled water, and freeze-dried [8].

2.3. Preparation of intact heparan sulfate chains

Traces of chondroitin sulfate were removed by treatment with chondroitinase ABC (EC 4.2.2.4) from *Proteus vulgaris* supplied by Sigma (1 U/ml), in 100 mM Tris-acetate, 100 mM EDTA, pH 8, for one night at 37°C. Core proteins were eliminated by two successive digestions with papain (1 mg/ml) for 24 h and 5 h at 65°C in 0.1 M sodium acetate, 5 mM EDTA, 5 mM cysteine, pH 7.0. Heparan sulfate was recovered in the supernatants of two successive precipitations with ice-cold trichloroacetic acid 100% (w/v) added to the extract at a final concentration of 10% (v/v) for 30 min at 4°C, followed by centrifugations at 10000×*g* for 30 min at 4°C. After a dialysis against 0.15 M sodium phosphate buffer, pH 6.8, samples were loaded on a 2 ml DEAE Sephacel column. After extensive washing with 0.3 M NaCl, heparan sulfate chains were obtained by elution with 1 M NaCl, dialyzed against distilled water and freeze-dried [8].

2.4. Depolymerization of heparan sulfate chains with nitrous acid and separation of the resulting oligosaccharides

Freeze-dried heparan sulfate was submitted to low-pH nitrous acid deaminative cleavage following the method of Shively and Conrad [9]. Oligosaccharides were resolved by gel filtration chromatography on a Bio-Gel P-6 (minus 400 mesh, from Bio-Rad) column (120 cm×1 cm) in 0.5 M NH_4HCO_3 eluted at a flow rate of 4 ml/h. Aliquots from 1 ml fractions were counted by liquid scintillation. Disaccharides were recovered and fractionated on an MonoQ HR5/5 anion exchange column (0.5 cm×5 cm) with an FPLC system from Pharmacia. A 15 ml linear gradient from 0 to 1 M NaCl in 10 mM HCl, pH 2.0, was used at a flow rate of 1 ml/min. 50 fractions of 0.5 ml were collected, mixed with 10 ml of PicoFluor 15 (Packard) and counted in a Tri-Carb 2100 TR liquid scintillation analyser (Packard). The degree of sulfation was identified by co-injection of 1 μg of different heparan sulfate disaccharide standards.

3. Results

In the total cell extract, we compared the glycosaminoglycan production by control and IFNγ-treated cells. We calculated the treated/untreated ratios for each isotope used for the radiolabelling and obtained a value of 3.07 for D-[1-^{3}H]glucosamine against only 0.88 for $Na_2{}^{35}SO_4$ (data not shown). It thus seems plausible that IFNγ increases in higher proportion hyaluronic acid (unsulfated glycosaminoglycan) in comparison with sulfated proteoglycans (with heparan and/or chondroitin/dermatan sulfate side chains). To evaluate the

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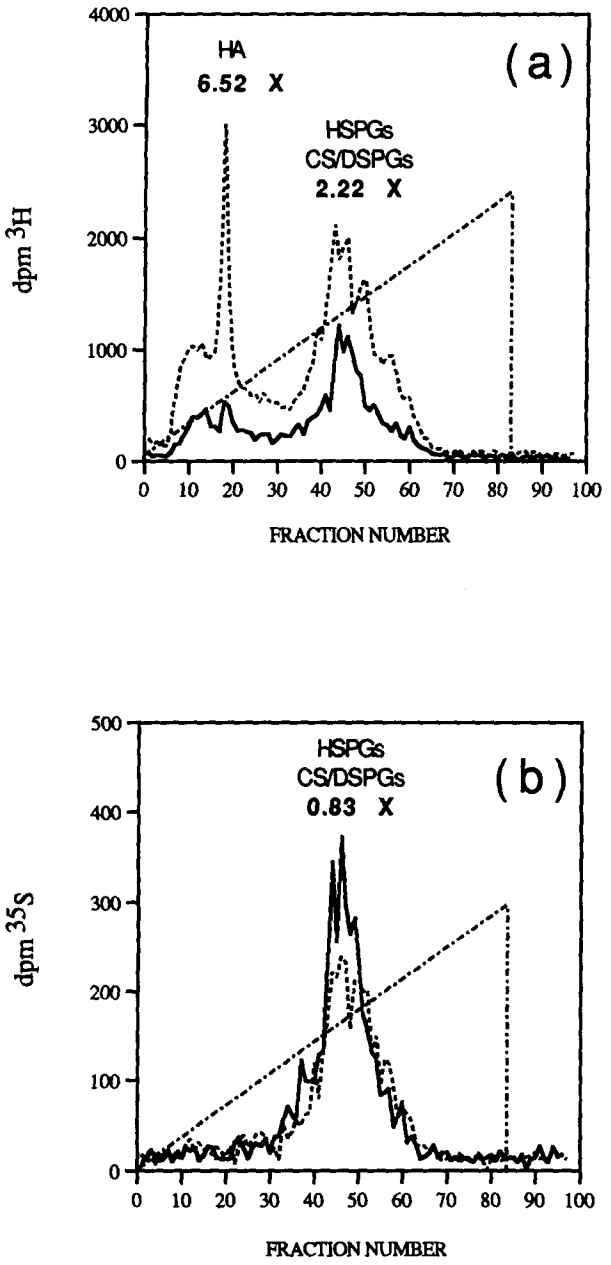

Fig. 1. Fractionation of radiolabelled hyaluronic acid and sulfated proteoglycans by chromatography on DEAE Sephacel. The order of elution in the 200 ml linear gradient from 0.15 to 0.8 M NaCl (- · - · -) was hyaluronic acid (HA), heparan sulfate proteoglycans (HSPGs) and chondroitin/dermatan sulfate proteoglycans (CS/DSPGs). Superposition of the ^{3}H (a) and ^{35}S signals (b) for control (——) and IFNγ-treated cells (- - -). The ratio between IFNγ-treated and control cells is indicated in bold letters above each peak.

IFNγ effect on individual glycosaminoglycans, the cell extract was fractionated by anion exchange chromatography (Fig. 1). IFNγ induced a 6.52- and 2.22-fold increase in the ^{3}H signal found in hyaluronic acid and sulfated glycosaminoglycans (heparan sulfate and chondroitin/dermatan sulfate) peaks, respectively (Fig. 1a). In contrast, the extent of ^{35}S incorporation was slightly reduced in the sulfated proteoglycans after IFNγ treatment (Fig. 1b). This led to a decrease in the $^{35}S/^{3}H$ ratio for sulfated proteoglycans from 0.24 for control cells to 0.09 for treated cells. This shift in the $^{35}S/^{3}H$ ratio was almost identical for purified heparan sulfate chains (0.23 and 0.10, respectively) and therefore was not related to changes in the degree of glycosylation of the core proteins.

At this stage of our study, the data were consistent with the hypothesis that IFNγ decreases the number of *N*- and/or *O*-sulfations on heparan sulfate chains. In order to characterize the *N*-sulfation on these chains, we analyzed the nitrous acid pattern of depolymerization of heparan sulfate isolated either from control or IFNγ-treated cells (Fig. 2). This agent cleaves each *N*-sulfated glucosamine with the concomitant release of the *N*-sulfate associated group [9]. Considering the ^{3}H signal, identical patterns of cleavage were obtained (Fig. 2a), demonstrating that IFNγ did not induce changes in the number and

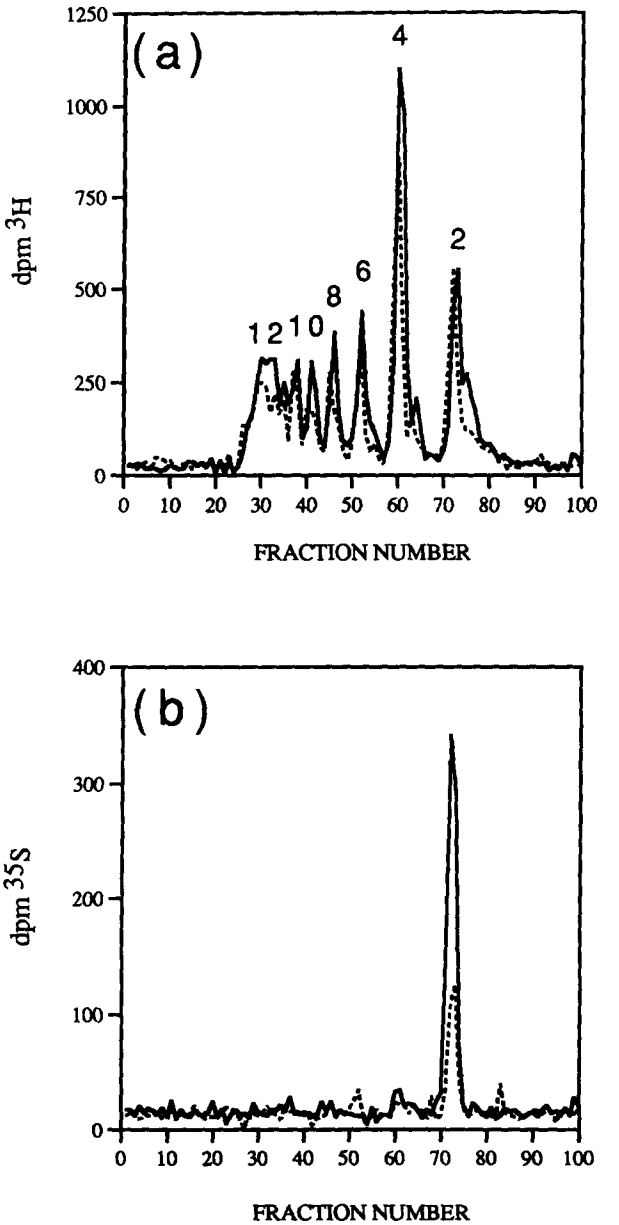

Fig. 2. Bio-Gel P-6 gel filtration of oligosaccharides produced by low-pH nitrous acid deaminative cleavage on heparan sulfate chains. (a) Superposition of the ^{3}H signal for control (——) and IFNγ-treated cells (- - -). (b) Superposition of the ^{35}S signal for control (——) and IFNγ treated cells (- - -). The degree of polymerization (2, disaccharides; 4, tetrasaccharides,...) is indicated above each peak.

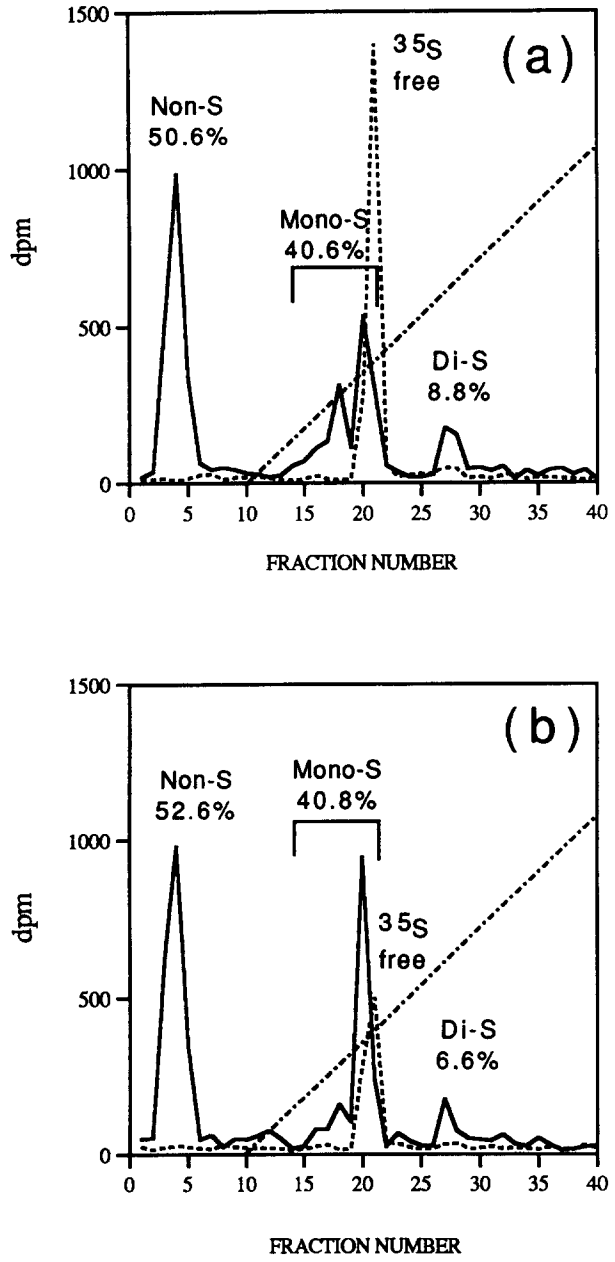

Fig. 3. FPLC fractionation on a MonoQ column of disaccharides generated by low-pH nitrous acid deaminative cleavage on heparan sulfate chains. *N*-desulfated disaccharides (peak 2 of Fig. 2a) were separated through a 15 ml linear gradient (- · · · -) from 0 to 1 M NaCl as a function of their degree of *O*-sulfation (Non-S, non-sulfated; Mono-S, monosulfated; Di-S, disulfated). (a) Superposition of ^{3}H (——) and ^{35}S (- - -) signals for control cell layer. (b) Superposition of ^{3}H (——) and ^{35}S (- - -) signals for treated cell layer. The indicated percentages correspond to the contribution of each disaccharide to the total collected radioactivity.

distribution of *N*-sulfated glucosamines. However, IFNγ led to a strong decrease in the ^{35}S signal (Fig. 2b). The ^{35}S signal was essentially detected in the disaccharide fractions, which was consistent with the fact that sulfated disaccharides are mainly present in the nitrous acid sensitive sequences in heparan sulfate chains [10]. In order to determine whether this ^{35}S signal arose from cleavage of *N*-sulfate groups by nitrous acid, or was incorporated in *O*-sulfate groups in disaccharides, this material was further analyzed on a MonoQ anion exchange column (Fig. 3). IFNγ did not lead to any significant change in the proportion of the different disaccharides separated as a function of their degree of *O*-sulfation (non-, mono- or di-sulfated which represent 51, 41 and 8%, respectively, of the total collected radioactivity). Therefore, the shift in ^{35}S signal induced by IFNγ could not be attributed to a change in *O*-sulfation. The ^{35}S signal did not coelute with any disaccharide standard (Fig. 3a,b) and corresponded to free sulfate liberated by the action of nitrous acid [11]. Therefore, IFNγ does not change the overall sulfation in heparan sulfate, but inhibits the incorporation of sulfate from the extracellular medium into this glycosaminoglycan.

4. Discussion

The present data demonstrate that IFNγ interferes with the incorporation of radioactive sulfate from the extracellular medium in heparan sulfate synthesized by human skin fibroblasts. Nevertheless, the cytokine does not affect the *O*- and *N*-sulfation of this glycosaminoglycan. This suggests that cells use endogenous sulfate for the synthesis of their heparan sulfate chains in the presence of IFNγ. In mammals, the sources of sulfate include diet, breakdown of proteins followed by oxidation of the resulting sulfur amino acids, and turn-over of glycosaminoglycans. Exogenous sulfate is transported into cells by proton/sulfate or sodium/sulfate symporters [12]. Then, this inorganic sulfate is activated into adenosine 3′-phosphate 5′-phosphosulfate (PAPS) by concerted action of the two enzymes ATP-sulfurylase and APS kinase [13,14]. The cytosolic product PAPS is then translocated in the lumen of the Golgi apparatus through a PAPS/3′-AMP antiport protein [15], where it acts as the sulfate donor in numerous sulfotransferase reactions [16]. Since IFNγ does not lead to an undersulfation of heparan sulfate chains, it should not affect any of the intracellular molecules involved in sulfate metabolism, but rather the plasma membrane transport systems (proton/sulfate or sodium/sulfate symporters). More work will be required to investigate a possible regulation of this transport systems by IFNγ.

An alternative hypothesis could also explain the effect of the cytokine on sulfate incorporation. Indeed, IFNγ increases hyaluronic acid in high proportions, and it has been demonstrated that this molecule inhibits sulfate incorporation in sulfate glycosaminoglycans sythesized by isolated adult chondrocytes [17].

Taken together, the present data provide a rational explanation for the discrepant results published about the effects of IFNγ on glycosaminoglycans. Whatever the stage affected by IFNγ in sulfate metabolism, this study highlights the fact that experiments realized with ^{35}S incorporation following IFNγ treatment should be considered with caution.

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