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Lack of acidic fibroblast growth factor activation by heparan sulfate species from diabetic rat skin

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The glucosaminoglycans isolated from the skin of control and streptozotocin-diabetic rats were fractionated on ion-exchange chromatography into a heparan sulfate (HS)-like and a heparin-like species. In addition, a low sulfated fraction was isolated from the diabetics. The HS and heparin-like fractions isolated from the diabetics (in contrast to the low sulfated fractions) retained high affinity for the acidic (FGF-1) and basic (FGF-2) fibroblast growth factors. In culture, the fractions purified from the control rats and the heparin-like material isolated from the diabetics mediated the biological activity of both FGFs in a dose-dependent manner. By contrast, the diabetic HS-like fractions promoted the biological activity of FGF-2 but not of FGF-1. The results support the idea that the structural motives in HS required for FGF-1 and FGF-2 mediated receptor signalling are different. They may be relevant to the impaired wound healing observed in the disease.

***Keywords*: diabetes, fibroblast growth factor, heparan sulfate**

Introduction

Heparan sulfate proteoglycans (HSPGs) not only play an important role in extracellular matrix organization [1] but have also been shown to modulate fibroblast growth factor activities [2, 3]. Heparin and heparan sulfate (HS) are copolymers of uronic acid (either glucuronic or iduronic) and glucosamine. The biosynthesis of the polysaccharide is a stepwise process which transforms alternating precursor sequences of *N*-acetyl D-glucosamine and D-glucuronic acid units into a glycosaminoglycan (GAG) sulfated in various positions. The extent of sulfation depends on the initial glucosaminyl *N*-deacetylation step which is a prerequisite for the other polymer modifications [4]. The final polysaccharide heparin contains essentially highly sulfated and iduronic acid rich regions while HS is organized by clusters of low and high sulfated regions [5].

Acidic and basic FGF (FGF-1 and FGF-2 respectively) are the prototypes of a family which comprises nine members. They are polypeptides that affect growth and differentiation of a variety of cell types and tissues [6]. They are potent mitogenic and chemotactic factors and have been implicated in the wound healing process [7]. They bind tightly to heparin, although with different affinities [8] and to cellular HS. The mitogenic activity of the FGFs is mediated by the activation of transmembrane tyrosine kinase receptors (FGFRs). The FGFR1 (Flg) and FGFR2 (Bek) share approximately 71% sequence homology and bind FGF-1 and FGF-2 with approximately equal affinities [9, 10].

Heparin or integral membrane HSPGs were shown to be necessary for binding of FGFs to the cell receptor FGFRs and therefore for cellular responses to the FGFs [11–14]. It has been suggested that HSPGs, usually considered as 'low affinity' receptors for the FGFs at the cell surface present the growth factor in an active form to the tyrosine kinase receptor following liberation of HS-FGF complexes [15–17], although degradative enzymes may not be needed [18]. More recently another concept has emerged. The polysaccharide was shown to bind both the FGF and the FGFR, the ternary complex HS-FGF-FGFR being the high affinity receptor complex [19, 20]. A heparin-binding sequence was identified in the second Ig-like loop of the FGFR1 [20]. Heparin and HS sequences of defined structure and size with afinity for FGF-2 were identified [21–24]. Different studies have aimed at identifying the structural requirements in heparin involved in the interaction with the tyrosine kinase receptor. They may differ depending on the FGFs [25–27].

Delayed wound healing and cutaneous extracellular matrix disorganization are common complications associated with diabetes [28] which may be accounted for abnormal biosynthesis of the glycosaminoglycans [29]. In

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Diabetes Mellitus, a defective function of the glucosaminyl *N*-deacetylase was described in the liver and in the kidney [30, 31]. It can be hypothesized that impaired synthesis of HS in diabetic tissue may result in decreased availability of the heparin binding growth factors at the site of injury and/or lack of formation of the active FGF-HS-FGFR complexes. In the present study, heparin/HS fractions were isolated from the skin of normal and diabetic Hairless rats, and their properties examined. The results show that the major HS-like fraction isolated from the diabetics, although still able to bind with high affinity to both FGFs, lack the ability to promote the FGF-1 cellular response.

Materials and methods

Chemicals

The streptozotocin, and pronase (protease E) were purchased from Sigma (MO, USA). Heparin lyases (heparinase I and II, E.C. 4.2.2.7; heparinase III, E.C. 4.2.2.8), chondroitin ABC lyase (chondroitinase ABC, E.C. 4.2.2.4) and chondroitinase AC-IIlyase (chondroitinase AC-II, E.C. 4.2.2.5) were obtained from Sigma. The collagenase was a product from Worthington Diagnostic (NJ, USA). Heparan sulfate from bovine kidney, heparin from porcin intestinal mucosa (Hep-1) purchased from Sigma, and a highly sulfated heparin (Hep-2) which was a gift from J. Choay (Sanofi, France) were used as standards. The recombinant human FGF-1 [33] was prepared in the laboratory by High Trap heparin-Sepharose chromatography (Pharmacia Biotech SA, Sweden) of a bacterial lysate. The bovine bacterial recombinant FGF-2 was kindly provided by G. Mazue (Carlo Erba, Italy). [^{35}S]-sodium sulfate (carrier free), C6[^{3}H]-D glucosamine (40 Ci mmol^{-1}) and methyl [^{3}H]-thymidine (specific activity 35 Ci mmol^{-1}) were obtained from ICN Biochemicals (France). The [^{3}H]-acetylated *E.coli* K5 capsular polysaccharide ([^{3}H]Ac-K5 PS, specific radioactivity of 2.10^5 cpm per μg of polysaccharide) prepared as described [32] was a generous gift of Professor Ulf Lindahl (Uppsala University, Sweden).

Chromatographies

Ion-exchange chromatography was performed on DEAE-Trisacryl LS (IBF, France) and gel filtration on Sephadex G50 superfine (Phamacia). Affinity matrices were prepared according to the manufacturer's instructions. Briefly, 2 mg of FGF-1 or FGF-2 were incubated overnight at 4 °C with 1 ml of activated Sepharose-CH4B (Pharmacia) in the presence of acetylated heparin in sodium carbonate, pH 8. The remaining activated groups were blocked with 0.5 M Tris. The gel was washed and tested for its ability to bind heparin. At least 250 μg of standard heparin (Hep-1) bound to 0.5 ml of affinity matrix and eluted with 1 M NaCl from the FGF-1 and 1.5 M from the FGF-2 columns. The samples of rat skin glucosaminoglycans applied to the columns amounted up to ≈ 50 μg of polysaccharide. The elution was performed stepwise as indicated in the legend to the figure.

Streptozotocin induced diabetes

Hairless male rats, 9 weeks old, were obtained from IFFA CREDO (France). The rats were rendered diabetic by intravenous injection of a single dose (45 mg kg^{-1} body weight) of extemporaneously prepared streptozotocin in 0.05 M citrate, pH 4.5). The rats with a glycaemia over 17 mM (normal glycemia 5 mM) 2 days after the injection of the toxin were kept separately and the development of the diabetes was followed over a period of 30 days. While the normal rats gained on average 30% of their initial weight, the diabetics showed a significant loss (average 11.5%).

Labelling of the glycosaminoglycans

The GAGs were metabolically labelled with 1 mCi [^{35}S]-sulfate and 0.5 mCi [^{3}H]-glucosamine. The isotopes were administered in a total volume of 500 μl phosphate buffer saline by local subcutaneous injections (5 × 100 μl). An area of 30 cm^2 was previously delimited on the dorsal skin of each animal. A total of 10 animals were injected (four normal and six diabetic rats). The rats were killed 24 h later by pentothal intraperitoneal injection, the full skin corresponding to the defined area was excised and immediately frozen in liquid nitrogen.

Extraction and isolation of the glycosaminoglycans

The frozen skin was chopped with a sterilized cutter and reduced into powder by a magnetic grinder in liquid nitrogen. A volume of 50 ml of 0.02 M Hepes, 0.1 M NaCl, pH 8, was added and the tissue was further homogenized at 4 °C with an ultraturax. The extracts were centrifuged and the supernatants filtered to remove fat deposits. The filtrates were incubated overnight at 37 °C with 0.5 mg ml^{-1} of collagenase. The solutions were then adjusted to 2 mM $CaCl_2$, pH 7.8, pronase was added to a final concentration of 0.5 mg ml^{-1} and the extracts were further incubated for 6 h at 50 °C. The digests were cleared by centrifugation and each homogenate was mixed with 5 ml of DEAE-Trisacryl. After 30 min of incubation at 4 °C, they were centrifuged and the gel subsequently washed with 20 ml of 0.1 M NaCl, 0.05 M Tris-HCl, pH 8, and 20 ml of 0.1 M NaCl, 0.05 M sodium acetate, pH 4. The GAGs bound to the matrix were recovered by batch elution with 1.5 M NaCl in sodium acetate buffer. Alkaline β-elimination was then performed overnight at 4 °C with 0.5 M NaOH. Sodium borohydride was added to a final concentration of 15 mM to prevent polymer modification. After neutralization with acetic acid, the extracts were dialysed against 0.05 M Tris-HCl, 0.1 M NaCl, pH 8, and again subjected to ion-exchange chromatography (3 ml of DEAE-Trisacryl). The free polysaccharide chains were recovered in 10 ml of 1.5 M NaCl,

0.05 M sodium acetate, pH 4. The eluates were extensively dialysed against water and concentrated by evaporation.

The glucosaminoglycans (*ie* heparin and HS) were isolated after chondroitinase ABC digestion overnight at 37 °C (1 U ml^{-1} in 0.05 M Tris-HCl, 30 mM sodium acetate, pH 8) of part of the pool containing the total GAGs.The digest was loaded on a column of Sephadex G50 (1 × 50 cm) eluted at 10 ml h^{-1} with 0.2 M ammonium bicarbonate. The undigested material, recovered in the void volume of the column, was desalted, lyophilyzed and dissolved in a small volume of water for further analysis. The galactosaminoglycans (*ie* chondroitin and dermatan sulfate) were isolated according to a similar procedure, except that the enzymatic digestion was performed with heparin lyases (6 mIU ml^{-1} in 0.05 M Tris-acetate, 3 mM $CaCl_2$, pH 6.8). In order to estimate the relative amounts of dermatan sulfate and chondroitin sulfate, samples of galactosaminoglycans were incubated overnight at 37 °C with chondroitinase AC-II (0.5 U ml^{-1} in 0.05 M Tris-HCl, 30 μM sodium acetate, pH 8). Gel filtration on G50 was then performed as described above. Only ≈ 10% of the radioactive material applied to the columns were degraded to disaccharides. The dermatan sulfate (*ie* chondroitinase AC resistant material) was recovered in the void volume of the columns, desalted and concentrated by evaporation.

Anionic properties of the glucosaminoglycans

Samples of the total glucosaminoglycans recovered on G50 after chondroitinase ABC digestion were subjected to analytical ion-exchange chromatography. The glucosaminoglycans isolated from each rat were analysed independently. The samples were applied to a 3 ml column of DEAE-Trisacryl, in 0.05 M Tris-HCl, 0.05 M NaCl, pH 7.5. The columns were washed with 15 ml of the same buffer and then 15 ml of 0.05 M sodium acetate, 0.05 M LiCl, pH 4. A linear salt gradient (total volume 100 ml) ranging from 0.05 M to 1.5 M LiCl in sodium acetate, pH 4, was applied at a flow rate of 12 ml h^{-1} with a Pharmacia FPLC pump. Fractions of 1 ml were collected and the radioactivity content determined.

Specific activity

The amounts of radioisotopes incorporated was measured by counting an aliquot of the various fractions mixed with 3 ml of QuickSafe 400 scintillation cocktail (Wallac, France) and a RackBeta 1209 counter (Wallac). The uronic acid content was determined by the carbazole method of Bitter and Muir [34] with glucuronolactone as a standard. The specific radioactivity of the GAGs was expressed in dpm per μg uronic acid.

Tissue dry weight determination

Two biopsies of 6 mm diameter were taken on each rat at close vicinity of the experimental excision. The degree of hydration of the tissues was calculated from the difference in the weights of the wet and the lyophilyzed biopsies.

Glucosaminyl *N*-deacetylase assay

On day 30, when the rats were killed, skin biopsies of 2 cm^2 and the median lobe of the liver were taken, immediately frozen in liquid nitrogen and kept at − 80 °C until use. The frozen skin was reduced to powder as described above. The tissue were homogenized at 4 °C with an ultraurax in 10 volumes of 0.05 M Tris-HCl, pH 7.5, 1% Triton X 100, 2 mM EDTA. The extracts were allowed to stand for 1 h in an ice bath and centrifuged at 12 000 × g at 4 °C for 10 min. The supernatants were further concentrated by centrifugation on a centricon-10 (Amicon, MA, USA). The liver was washed quickly with cold phosphate buffer to remove excess blood. The tissue was then homogenized and the microsomal fraction prepared as for the skin. The enzymatic assay was performed as described by Navia *et al.* [35]. A sample containing 10 000 cpm of [^{3}H]Ac-K5 PS was incubated with an aliquot of each enzyme extract (corresponding to 0.2 mg protein) in 150 μl of 50 mM MES, 10 mM $MnCl_2$, 1% Triton X 100, pH 6.3. After 1 or 2 h incubation at 37 °C (for the liver and the skin enzyme respectively) the reaction was stopped by adding 150 μl of 1 M chloroacetic acid, 0.5 M NaOH, 2 M NaCl. The radioactivity corresponding to the tritiated acetyl groups released by the enzyme was measured in a two phase system with 4 ml of Optiscint (Pharmacia) scintillation cocktail containing 10% isoamylalcohol. Zero-time values were determined by adding the stopping solution prior to the substrate and subtracted from the experimental values.

Culture and biological assays

Chinese hamster lung fibroblasts (CCL39) were obtained from the American Type Culture Collection. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, France) supplemented with 10% fetal calf serum (FCS, D.A.P., France) in 7% CO_2 atmosphere at 37 °C. The cells were seeded at a cell density of 35 000 cells per cm^2 and used before passage 25. The plastic culture (75 cm^2 flasks and 48 well plates) was from Costar (MA, USA). The ability of heparin and HS fractions to mediate the biological activity of either FGF-1 (5 ng ml^{-1}) or FGF-2 (2.5 ng ml^{-1}) was evaluated by measuring the incorporation of [^{3}H]-thymidine in CCL39 fibroblasts. The FGFs were used at doses corresponding to the ED_{50} (5 ng ml^{-1} or 2.5 ng ml^{-1} respectively [36]). The stimulation unit (ED_{50}) was defined as the concentration of growth factors required to obtain half the maximum [^{3}H]thymidine incorporation. The cells were seeded in 48 well plates at a density of 35 000 cells per cm^2, in DMEM supplemented with 10% FCS and incubated at 37 °C in 7% CO_2. Forty-eight h later, the culture medium was replaced by serum free medium which was maintained for 24 h. The cultures were rinsed and

incubated in the presence of heparinase II in DMEM (1 U ml^{-1}) for 3 h as previously described [37]. Fresh serum free DMEM was added with 5 mM chlorate and DNA synthesis was reinitiated by adding the FGFs. The methyl [^{3}H]-thymidine (1 μCi ml^{-1}) was added 20 h later and the cells were further incubated for 4 h. When tested, the HS samples were added at the same time as the FGFs. The radioactivity incorporated by the cells was measured in a 1450 microbeta counter (Wallac) with Hisafe scintillation cocktail.

Results

Purification of the glucosaminoglycans

The GAGs, labelled with [^{35}S]-sulfate and [^{3}H]-glucosamine, were recovered from each tissue homogenate, after collagenase and pronase digestion, by ion-exchange chromatography. The presence of uronic acid containing material was detected only in the salt eluate. Rat skin heparin was previously shown to be linked to a [ser-gly]-rich protein core which resists proteolytic digestion, thus the eluate was subjected to alkaline β-elimination [38]. After neutralization and dialysis an additional ion-exchange chromatography was performed. The isolated GAGs chains again bound to the matrix. The GAG contents of the control and diabetic rat skins are shown in Table 1. While the values found for the untreated rats were all of the same order (≈ 1 mg g^{-1} tissue) some variation was observed among the diabetics. The galactosaminoglycans were removed by gel filtration on Sephadex-G50 following chondroitinase ABC digestion. Heparin and HS-like materials were recovered in the void volume of the column (not shown). The amounts of glucosaminoglycans and the ratio between [^{35}S]-sulfate and the [^{3}H]-glucosamine incorporated were lower for the diabetic than for the control rats (Table 1). When analysed by the *t*-test, the average values for the diabetic and the normal rats were found significantly different ($p < 0.05$).

Different species of heparan-like material were isolated on ion-exchange chromatography

A salt gradient elution was performed for each isolated glucosaminoglycan sample. The results shown in Figure 1 are representative of the different elution profiles observed. The glucosaminoglycans from the untreated rat skin (Figure 1a) separated into two peaks, a HS-like and a heparin-like fractions, denoted N-HSP2 and N-HSP3 respectively. The fraction N-HSP2 emerged at 0.77 M LiCl, while N-HSP3 was eluted with 1.35 mM LiCl. On the basis of [^{3}H]-glucosamine incorporation, the N-HSP2 and the N-HSP3 fractions represented 72.5% and 27.5% respectively of the GAGs recovered. By contrast, a third peak of low sulfate content (D-HSP1) was observed with the GAGs isolated from the diabetics. However, the amounts of material eluted with $\leqslant 0.5$ M NaCl differed from one diabetic rat to another. These differences raised the possibility that alteration in the structure of HS-like and heparin-like materials could exist which were not revealed by ion-exchange chromatography. Thus, according to the elution profiles obtained, the diabetic rats fell into two groups. In group I (Figure 1b), the material of low sulfate content emerging as DI-HSP1 represented $\leqslant 10\%$ of the glucosaminoglycans while in group II (Figure 1c) this species (DII-HSP1) amounted up to 25–30% of the material recovered. This fraction was digested to oligosaccharides by heparitinase (not shown). The presence of this material is likely to account for the decrease in the ^{35}S/^{3}H ratio of the total glucosaminoglycans observed in the diabetics (Table 1). The chromatographic properties of the dermatan sulfate samples isolated from the skin of the different animals were also compared. A single peak eluted at 0.87 M LiCl. With the exception of one animal which presented a minor fraction at 0.45 M LiCl, no significant difference was observed between the control and the diabetic rats (not shown).

Table 1. Glycosaminoglycans in normal and diabetic rat skin[a].

Rats	*GAGs* (mg g^{-1})	*DS and CS*[b] (mg g^{-1})	*HS-Hep*[c] (mg g^{-1})	*HS-Hep*[c] (^{35}S/^{3}H)
Controls				
Mean ± SD	1.023 ± 0.049	0.628 ± 0.057	0.394 ± 0.050	1.440 ± 0.529
Diabetics				
Mean ± SD	0.848 ± 0.295	0.532 ± 0.330	0.286 ± 0.047	0.837 ± 0.244
t-test	NS[d]	NS	$p < 0.01$	$p < 0.05$

[a]The glycosaminoglycans (GAGs) were isolated after subcutaneous injection of [^{35}S]-sulfate and [^{3}H]-glucosamine as described in Materials and methods. The overall sulfation of the glucosaminoglycans was estimated from the relative amounts of radioisotopes (^{35}S/^{3}H) incorporated per μg uronic acid.
[b]DS and CS: dermatan sulfate and chondroitin sulfate;
[c]HS-Hep: heparan sulfate and heparin-like material;
[d]NS: non-significant. Four normal and six diabetic rats were used in the above experiments.

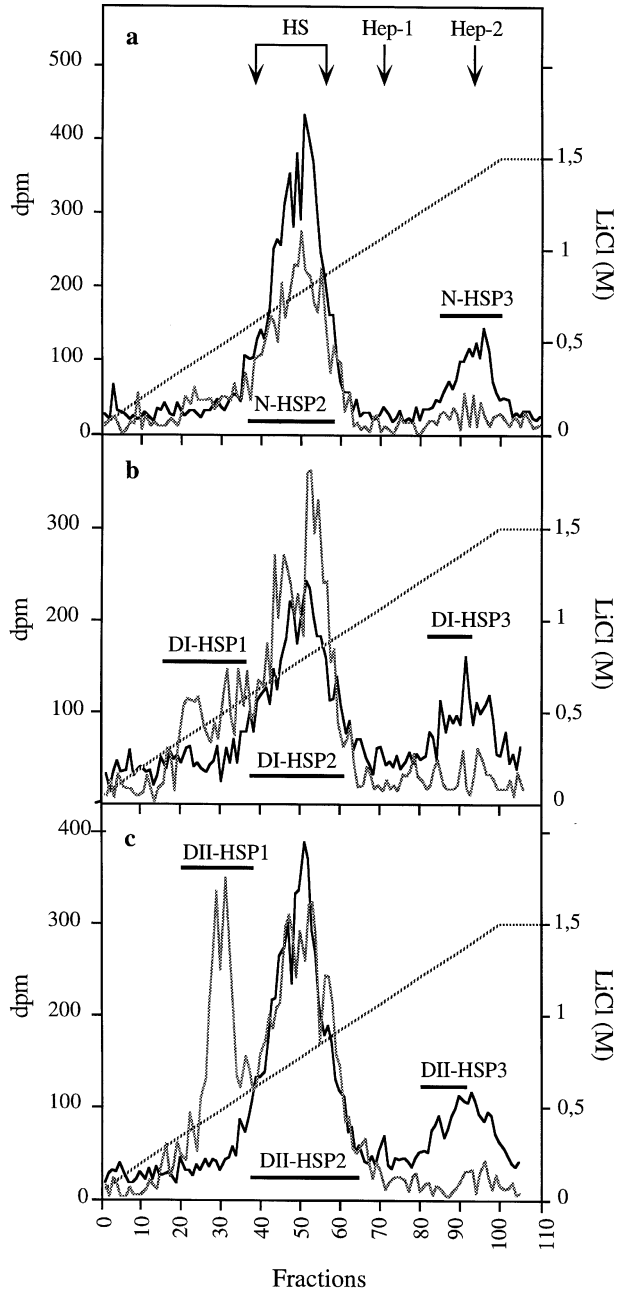

Figure 1. The rat skin glucosaminoglycans separate into subspecies on ion-exchange chromatography. Samples of purified glucosaminoglycans from the normal (a) and the diabetic (b and c) rats were loaded on 3 ml columns of DEAE-Trisacryl, in 0.05 M Tris-HCl, 0.05 M NaCl, pH 7.5. The column was washed with 15 ml of the same buffer and then 15 ml of 0.05 M sodium acetate, 0.05 M LiCl, pH 4. A linear salt gradient (total volume 100 ml) ranging from 0.05 M to 1.5 M LiCl in sodium acetate, pH 4 was applied at a flow rate of 12 ml h^{-1}. Fractions of 1 ml were collected and analysed for their content in [^{3}H]-glucosamine (^{3}H-dpm ----) and [^{35}S]-sulfate (^{35}S-dpm ——). They were pooled as indicated by the bars. The elution positions of heparan sulfate (HS) and heparin standards (Hep-1 and Hep-2) are indicated on top of the graph.

Affinity chromatography on FGFs

The glucosaminoglycans species separated on ion-exchange chromatography were pooled as indicated in Figure 1. The affinity of each fraction for immobilized FGF-1 and FGF-2 was examined. As expected, the HSP2 and HSP3 fractions isolated from the untreated rats bound strongly to either FGFs matrices (Figure 2). The N-HSP2 fraction required a higher salt concentration to be eluted from the FGF-2 than from the FGF-1 column (1–1.2 M and 0.8–1 M NaCl respectively). The highly sulfated fraction N-HSP3 showed a even stronger interaction with both FGFs than the HS-like fraction. Similarly, the fractions isolated from the diabetic rats (*ie* D-HSP2 and D-HSP3) showed high affinity for both FGFs (Figure 3b, e and 3c, f) although they eluted slightly earlier from the FGF-1 (Figure 3b, c) column than the control species (Figure 2). The DII-HSP1 fraction did not show affinity for the FGFs (Figure 3a, d).

Glucosaminyl *N*-deacetylase assay

The glucosaminyl *N*-deacetylase, a key enzyme in the biosynthesis of heparin/HS, was assayed in the microsomal fractions prepared from rat skin and liver. The assay was performed on each sample, then the results obtained in each category were grouped to allow a *t*-test analysis (Figure 4). The activity of the enzyme in the liver microsomal fractions of the diabetics was significantly lower than in the control rats ($p < 0.01$). However such result was not observed in the skin preparations. A greater variation in the values was found among the diabetics. Again, no significant difference was found when the results obtained for the diabetic rats of group DI and group DII were analysed separately (not shown).

The biological activity of FGF-1 is not promoted by the major diabetic heparan sulfate species

In order to elicit full biological activity, FGF-1 and FGF-2 require heparin or HS [11]. Therefore the fractions HSP2 and HSP3, which exhibited affinity for the immobilized growth factors, were studied for their ability to promote the biological activities of the growth factors on heparinase treated CCL39 fibroblasts. Heparinase/heparitinase treatment was previously reported to decrease the binding of FGF-2 to the tyrosine kinase receptors to 17% of that of normal [37]. The assays were performed with doses of either 2.5 ng ml^{-1} of FGF-2 or 5 mg ml^{-1} of FGF-1 which corresponded to the ED_{50} determined on these cells [36]. The dose response curve obtained with increasing amounts of standard heparin (either Hep-1 or Hep-2) and HS reached a maximum for 40 µg ml^{-1}. Higher concentration led to decreased potentiation. Thus rat skin HS/heparin-like species which showed affinity for the FGFs were assayed under these conditions. Although all the fractions tested were able to promote the biological activity of FGF-2, the effect of the diabetic HS-like fractions on the acitivity of FGF-1 was

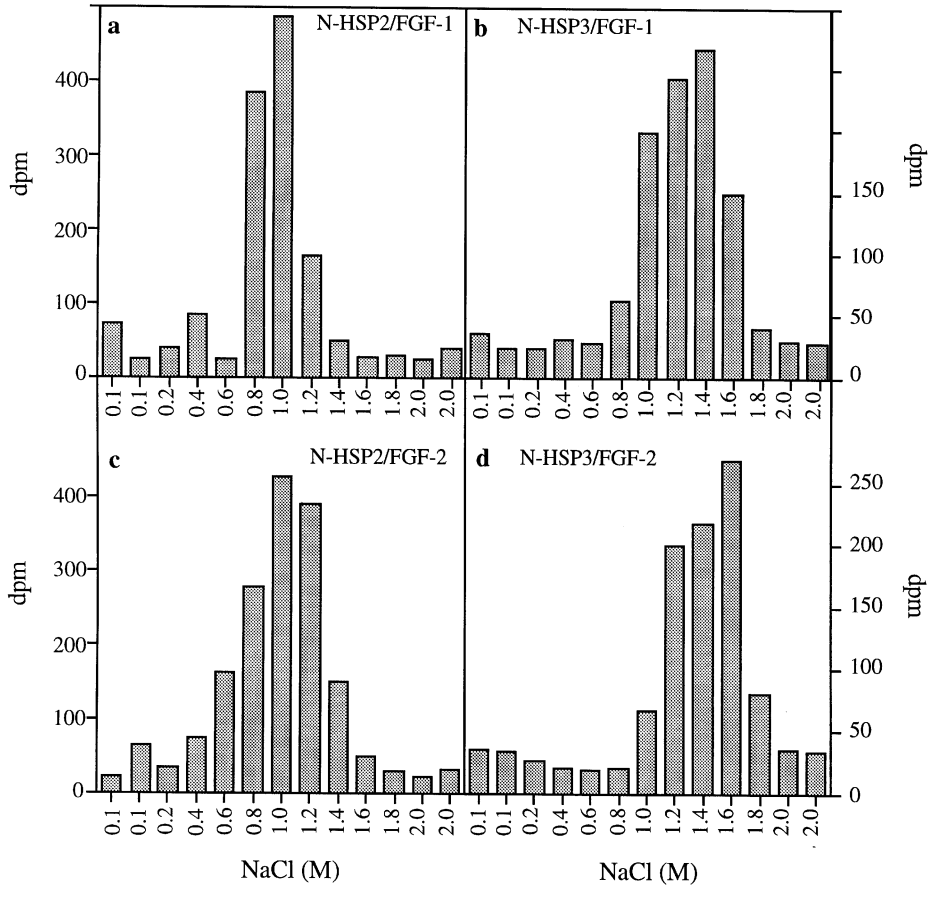

Figure 2. The affinity chromatography on FGF-1 and FGF-2 of the heparan sulfate and heparin-like species from control rat skin. Aliquots of the HS-like (N-HSP2) and heparin-like (N-HSP3) pools were applied in 0.5 ml of 0.1 M NaCl, 0.05 M Tris pH 7.4 to 0.5 ml of FGF-Sepharose (1 mg of either FGF-1 or FGF-2). The columns were washed once with 1.5 ml of the same buffer and the elution performed stepwise with 1.5 ml of 0.05 M Tris -HCl pH 7.4 containing increasing amounts of NaCl (0.2 M increments). The amounts of radioactivity (expressed in ^{35}S-dpm) eluted in each NaCl step was determined by counting 0.5 ml of each fraction.

quite poor. Dose-response experiments were then performed with doses ranging from 0.01 to 100 $\mu g\,ml^{-1}$ of polysaccharide (Figure 5). The heparin-like fractions from the normal rats (N-HSP3) and the diabetics (D-HSP3) were able to promote the biological activity of both the FGF-2 and the FGF-1. No significant differences were noted when the HS-like fractions N-HSP2, DI-HSP2 and DII-HSP2 were tested with FGF-2 as a mitogen (Figure 5a and 5b). The control N-HSP2 fraction also induced the cellular response to FGF-1 (Figure 5c). On the contrary, the HS-like fractions derived from the diabetic rats (*ie* DI-HSP2 and DII-HSP2) failed to mediate the biological activity of FGF-1 (Figure 5d).

Detection of the FGF-1 and FGF-2 in the skin extracts

Taking in account the lower content in total glucosaminoglycans in the diabetics, compared to the controls, and the presence of the low sulfated HSP1 fraction in the diabetic rat skin, devoid of affinity for the FGFs and since heparin/HS represent binding sites for the FGFs, it could be hypothesized that the amounts of FGFs retained within the tissue would be reduced. To test this hypothesis, immunodetection by Western dot blot of FGF-1 and FGF-2 was performed on tissue extract of all the rats used in this study. The growth factors were detected in all cases for nearly equal amounts of protein extract. Moreover, they bound to heparin-Sepharose (not shown).

Discussion

The purpose of this study was to investigate the biological properties of skin glucosaminoglycans in diabetic Hairless rat, a strain commonly used for skin repair studies. The HS-like and the heparin-like fractions from the control rats showed high affinity for the immobilized FGFs and eluted earlier from the FGF-1 than from the FGF-2 column, a result expected since FGF-1 has a lower affinity for heparin than FGF-2 [8]. The N-HSP3 fraction bound with

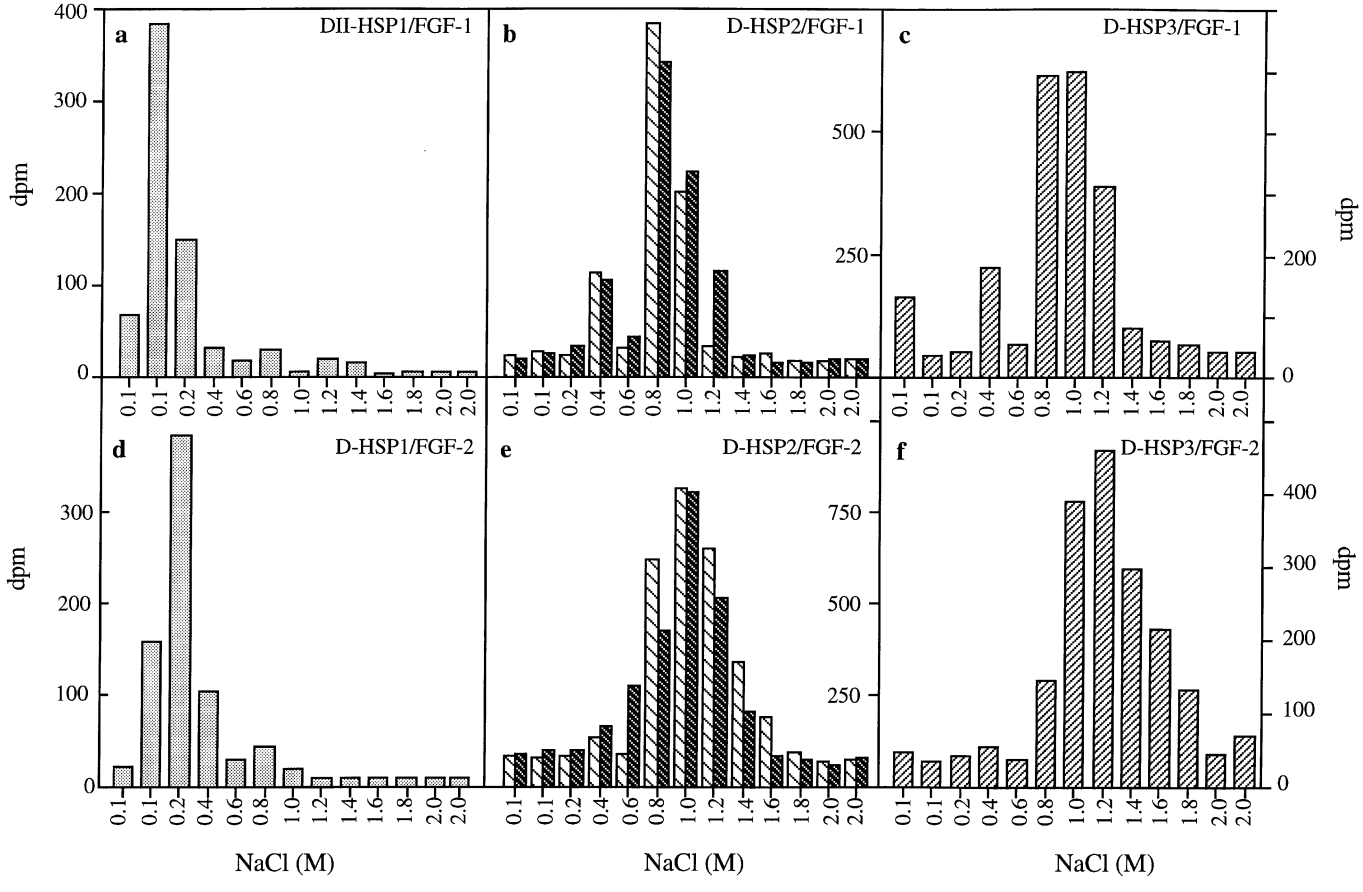

Figure 3. Affinity chromatography on FGF-1 and FGF-2 of the various heparan sulfate and heparin-like species from diabetic rat skin. The experiment was performed as in Figure 2. The pools are designated according to the ion-exchange chromatography profile (see Figure 1). The results are expressed in ^{3}H-dpm for the pool D-HSP1 and in ^{35}S-dpm for the HS-like (D-HSP2) and heparin-like (D-HSP3) pools. The results obtained for the different pools D-HSP2 corresponding to either the group I (▨) or the group II (▩) are shown separately.

a higher affinity to both FGFs than the N-HSP2 species, in agreement with the heterogeneity in charge density of the two populations of heparin and HS respectively. More surprising was the finding that, apart from the low sulfated fraction which was devoid of affinity for both FGFs, the skin HS-like and heparin-like species from the diabetic rat bound with similar affinity to the immobilized growth factors. Moreover, the HS-like species from the diabetics promoted the biological activity of FGF-2 but not of FGF-1.

The failure of the *N*-deacetylase/*N*-sulfotransferase enzyme to promote polymer modification along with the polysaccharide biosynthesis [30] in diabetic tissues should lead to a polysaccharide presenting with scattered iduronosyl-2-O-sulfate and N and/or 6-O-sulfated glucosaminyl residues. Such residues were implicated in the oligosaccharide sequences which interact with the FGFs and the FGFR1 and R2 [21–23, 25, 39]. Although the function of the enzyme was clearly impaired in the liver of the diabetic animals, the activities between the diabetic and control skin extracts showed no significant difference. This may be due to the small sample studied. However it should be noted that the liver is a highly vascularized organ and that the glucose content of the skin was reported to be $\approx 30\%$ lower than the blood glucose level [40] and the enzyme may be more or less inactivated depending on the localization. A partially deficient biosynthetic machinery may explain the properties of the various HS and heparin-like species isolated from the skin. The D-HSP1 fraction, isolated in this study, devoid of affinity for the FGFs, may represent a fraction of the glucosaminoglycans which almost escaped completely the polymer modification steps. By contrast, the heparin-like D-HSP3 and HS-like D-HSP2 fractions retain full or some biological properties. The motives mediating the interaction with the FGF or the FGFR, which are presumably repeated a number of times along the highly sulfated polysaccharide chain (HSP3), may be still present in sufficient amounts to allow the formation of active high affinity receptor complexes. Such motives are less abundant in the HS-like fraction (HSP2) and the impaired biosynthesis in the diabetics is likely to generate non-functional polysaccharide chains.

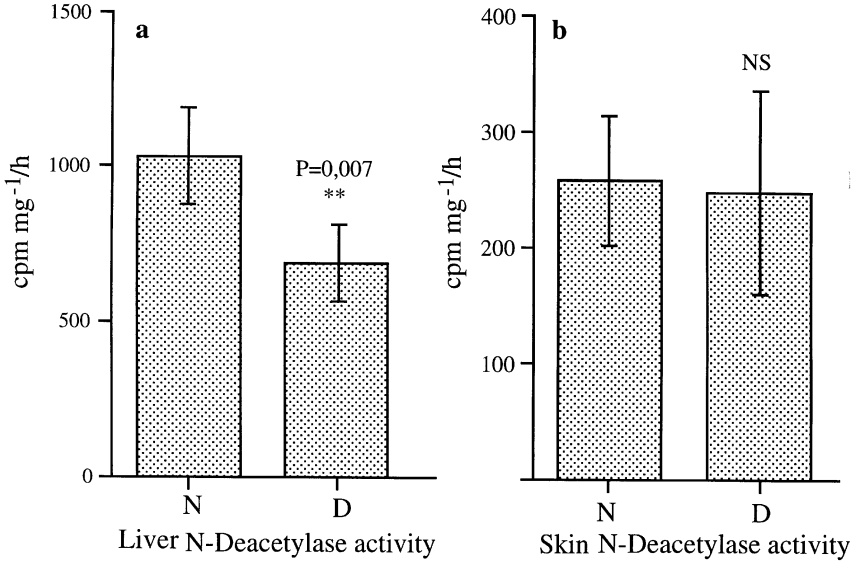

Figure 4. Glucosaminyl *N*-deacetylase assay. The activity of the enzyme was determined in microsomal fractions prepared from liver (a) and skin (b) extracts. The difference between the normal (N) and the diabetic (D) groups was investigated with a *t*-test. A significant difference is indicated by stars. NS: non-significant.

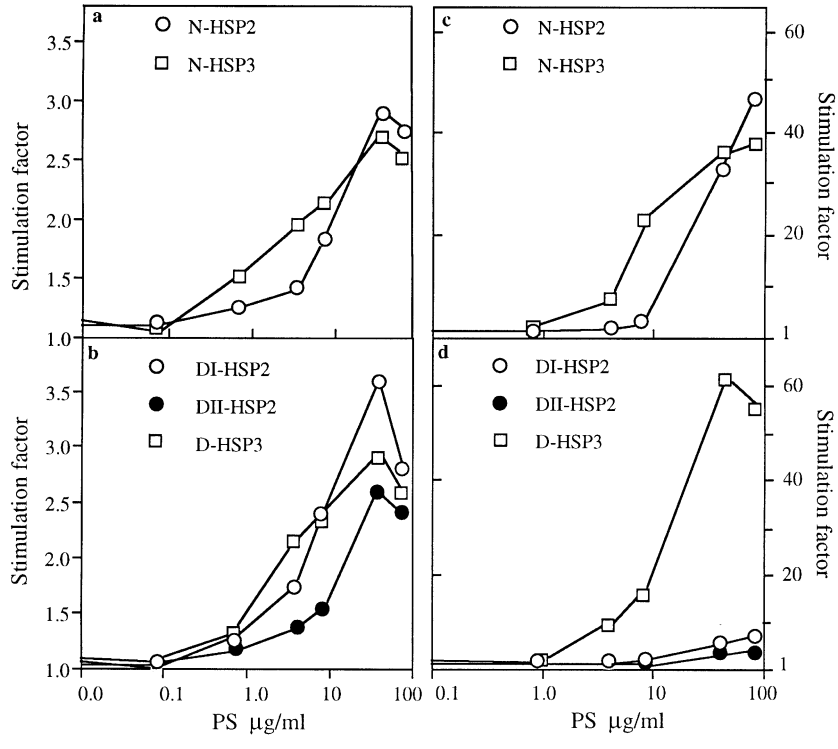

Figure 5. Biological activity of the various heparan sulfate and heparin-like species isolated from normal and diabetic rat skin. The ability of the different fractions (polysaccharide, PS) isolated to promote a cellular response in heparinase treated CCL39 fibroblasts was determined in the presence of either 2.5 ng ml^{-1} of FGF-2 (a and b) or 5 ng ml^{-1} of FGF-1 (c and d). The stimulation factor was calculated from the ratio between the [^{3}H]-thymidine incorporated by the cells in the presence of the FGF and the HS fraction at the dose indicated and the amounts incorporated in the presence of the FGF alone. Each point represents the mean of duplicate determinations of two separate experiments. The pools isolated from rat skin are named as in Figure 1.

A striking result was the absence of cellular response to FGF-1, but not to FGF-2, in the presence of the HS-like fractions isolated from the diabetic rat skin, despite the high affinity binding of D-HSP2 species to the FGFs. Different possibilities may explain the lack of FGFR signalling in the presence of FGF-1 and the D-HSP2 fractions. The oligosaccharides synthesized by the diabetics may be too short to provide binding sequences for both the FGF and the

tyrosine kinase receptor. This is unlikely since the D-HSP2 species emerged on DEAE-ion exchange chromatography at the elution position of the N-HSP2 and of standard HS and these species promoted the biological activity of FGF-2. Alternatively, the oligosaccharide sequences may not allow FGF dimerization, a mechanism proposed to induce receptor dimerization and cellular signalling [41–43]. Although this hypothesis cannot be completely ruled out, it seems improbable in view of the high affinity binding of the D-HSP2 fractions on immobilized FGF-1. Finally the polysaccharides may lack sulfate groups essential for HS-FGFR interaction. In this respect, a high 6-O-sulfate content in the N-sulfated glucosamine residues was reported to be necessary in order to promote the biological activity of FGF-1, but not that of FGF-2 [24, 26, 44]. The present results show that the high affinity binding of the HS to FGF-1 and FGF-2 is not sufficient to promote their biological activities. They support the idea that the structural requirements involved in the HS-FGFR interaction are different depending on the FGF.

Both FGF-1 and FGF-2 are expressed in normal and in wounded skin [45, 46]. Moreover, in diabetic mice wounds the expression of FGF-1 precedes that of other growth factors [47]. The biological activity of FGF-1 is dependent on heparin or cellular HS and is greatly enhanced by the polysaccharide. Thus, the inability of the major HS species isolated from diabetic rat skin to mediate the biological activity of FGF-1 may be an important factor in impaired wound healing in diabetic skin.

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