CLINICAL STUDY

Glycosaminoglycans increase levels of free and bioactive IGF-I *in vitro*

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Abstract

Background: It is unclear how IGFs become separated from their IGF-binding proteins (IGFBPs) *in vivo*. However, the IGFBPs possess binding sites for glycosaminoglycans (GAGs) and interaction with GAGs alters IGFBP ligand affinity. Accordingly, GAGs may control IGF bioavailability. To test this hypothesis, we investigated the effect of GAGs on serum levels of free and bioactive IGF-I, total IGF-I, and IGFBPs *in vitro*.

Methods: Serum was incubated with increasing concentrations of six different GAGs (heparin, tinzaparin (Innohep[®]), dermatan sulfate, heparan sulfate, non-anticoagulant (nac) heparin, and nac low-molecular weight heparin). To investigate for reversibility, heparin was co-incubated with protamine sulfate (PS). Finally, the effect of heparin was studied in serum from pregnant and *post partum* women, normal subjects and patients with type 1 diabetes.

Results: All GAGs increased free IGF-I in a dose-dependent manner ($P < 0.0001$), whereas total IGF-I and IGFBP levels remained unchanged. However, the potency of the GAGs differed significantly ($P < 0.0001$) and did not relate to their anti-coagulating activity. The effect of heparin on free IGF-I was fully reversed by PS. Heparin increased free and bioactive IGF-I in all tested sera ($P < 0.0001$), but the increase was most pronounced in samples from pregnant women ($P < 0.0001$).

Conclusion: All tested GAGs stimulated the release of free and bioactive IGF-I in several types of serum, most likely by reversible interaction with the IGFBPs. The effect was most pronounced in pregnancy sera, which are characterized by extensive IGFBP-3 proteolysis. Our findings support the view that GAGs localized in the vessel wall and attached to the extracellular matrix control IGF-I tissue accessibility and bioactivity.

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Introduction

The insulin-like growth factors (IGF-I and -II) are multi-potent peptides affecting growth, differentiation, and apoptosis in virtually all cell types through endocrine, paracrine, and autocrine pathways (1, 2). In the circulation, only a minor fraction of IGF-I is free, while the majority is bound to one of six different high-affinity IGF-binding proteins (IGFBPs) forming binary complexes and ternary complexes with the non-IGF-binding glycoprotein acid labile subunit (ALS). The IGFBPs are able to stimulate as well as to inhibit IGF-I-mediated effects, and they also possess IGF-I-receptor (IGF-IR)-independent effects (this particularly applies to IGFBP-3). ALS appears to affect IGF-bioactivity by restraining circulating IGFs-I and -II within the blood stream, hereby limiting the access of the IGFs to the peripheral target tissues (3–8).

Glycosaminoglycans (GAGs) are relatively large molecules composed of polysaccharide side chains attached to a core backbone of protein, forming a proteoglycan. Each polysaccharide side chain consists of a disaccharide repeat unit composed of hexosamine linked to either iduronic or glucuronic acid. The hexosamine is a glucosamine in heparin, and heparin sulfate and a galactosamine in dermatan sulfate (DS). The disaccharide units are heavily modified and the number of modifications allows for a large structural and functional diversity; it is the composition of the disaccharide side chains that defines the individual GAGs (9).

The disaccharides are often heavily sulfated and hence strongly negatively charged, and this may explain the pronounced ability of the GAGs to interact with proteins such as growth factors, enzymes, and chemokines. In the body, GAGs are present in mast cells

(as heparin), in the extracellular matrix (ECM) and attached to cell surfaces (9, 10). Clinically, GAGs are widely used for their anti-coagulating effects. Physiologically, the role of the GAGs has still to be fully determined. However, they are known to interact strongly with several growth factors, and therefore, GAGs are assumed to influence growth of normal as well as neoplastic cells (9, 10).

All IGFBPs except IGFBP-1 possess heparin-binding domains (HBDs) located near the C-terminal IGF-binding site. In addition, IGFBPs-3 and -5 possess a HBD near the ALS-binding site (4, 11, 12). It has been hypothesized that GAGs localized within the ECM or at cell surfaces through interaction with the HBD of the IGFBPs are able to liberate bound IGF-I and hence enhance IGF-I bioactivity at the local tissue level (13). It has also been suggested that GAGs localized at the vascular endothelium may regulate the passage of IGF-I to the tissues (14). However, at present, there are no available data on the effect of GAGs on levels of free or bioactive IGF-I.

The aim of the present study was to investigate the effect of several different GAGs with and without anti-coagulating activity on serum levels of free IGFs-I and -II *in vitro*. In addition, we compared the effect of heparin on serum levels of free and bioactive IGF-I in different patient groups characterized by an abnormal IGFBP-profile and IGFBP-3 proteolytic activity.

Materials and methods

Glycosaminoglycans

All GAGs used in the present study were generously provided by LEO Pharma A/S (Ballerup, Denmark). They included unfractionated heparin (Hep, molecular weight (M_w) = 14.0 kDa), unfractionated heparin chemically modified so that it was non-anticoagulant (nac-Hep, M_w = 12.3 kDa), low-molecular tinzaparin (Innohep[®], Tin, M_w = 6.5 kDa), chemically modified tinzaparin devoid of any anti-coagulant activity (nac-Tin, M_w = 5.3 kDa), DS (molecular peak, M_p = 35.5 kDa) and heparan sulfate (HS, M_p = 15.4 kDa).

Assays

The immunological levels of IGFs-I and -II were determined by in-house monoclonal time-resolved immunofluorometric assays as previously described (15). All GAGs were examined for cross-reactivity in the IGF assays. However, none of the GAGs showed any detectable cross-reactivity at concentrations below 400 $\mu\text{mol/l}$, which was the highest concentration used in the present study (a concentration of 400 $\mu\text{mol/l}$ equals 50 U/ml heparin). Serum total IGFs-I and -II were determined in acid ethanol extracted serum with a within assay and between assay coefficient

of variation (CV) averaging 5 and 10% respectively (15). Serum free IGFs-I and -II were determined using ultrafiltration by centrifugation at approximate *in vivo* like conditions (16). Within and between assay CV values averaged 15–20%.

Serum IGF-I bioactivity was determined by an in-house kinase receptor activation assay (KIRA) based on human embryonic renal cells (EBNA 293 from Invitrogen) transfected with the human *IGF-IR* gene (17). In this assay, cultured cells are stimulated with either IGF-I standards or unknown serum samples. After 15 min of stimulation, samples are removed and cells lysed. Then, crude cell lysates are transferred to an assay that detects the concentration of phosphorylated (i.e. activated) IGF-IRs. This assay uses a MAB against the extracellular domain of the IGF-IR for coating and a europium-labeled monoclonal anti-phosphotyrosine antibody (PY20) as tracer. The assay is sensitive (detection limit < 0.08 $\mu\text{g/l}$), specific (IGF-II cross-reactivity is 12%, proinsulin, insulin, and insulin analogs have a cross-reactivity < 1%), and precise (mean within and in-between assay CV values were < 7 and 15%) (17).

Intact IGFBPs were determined by Western ligand blotting (WLB) as previously described (18). The resulting autoradiographs yielded five distinct bands. On the basis of immunoreactivity and/or molecular size, these bands were tentatively identified as representing IGFBP-3, IGFBP-2, IGFBP-1, glycosylated IGFBP-4, and non-glycosylated IGFBP-4 (19).

Study 1: effect of increasing doses of GAGs on serum free IGFs-I and -II

To compare the potency of the different GAGs, six non-fasting serum samples from healthy subjects were added in increasing concentrations (0, 4, 40, and 400 $\mu\text{mol/l}$) of one of the six different GAGs mentioned earlier. All GAGs were diluted in Krebs Ringer buffer (KRB) adjusted to pH 7.4 with CO_2 and added 5% (w/v) human serum albumin (ICN Biomedical, Aurora, OH, USA). All serum samples (2 ml) were added into the same volume of buffer (200 μl). After addition of GAG, samples were equilibrated for 30 min at 37 °C, before they were centrifuged. Each sample was centrifuged in triplicates and the ultrafiltrates assayed for free IGFs-I and -II. In addition, samples were assayed for total IGFs-I and -II as well as IGFBPs levels. All samples from each of the six GAGs were assayed within the same run.

Study 2: effect of protamine sulfate (PS) on heparin-treated serum

PS (a polycation) has been shown to reverse the effect of heparin (which is heavily negatively charged) on ALS binding to IGFBP-3:IGF-I complexes (14). Therefore, we found it of interest to investigate whether PS was able to reverse the effect of heparin on serum free IGF-I. Pilot

studies showed that the effect of 400 µmol/l heparin on free IGF-I was fully neutralized by the addition of 5 g/l PS (data not shown). Thus, this dose was used in the next experiment, where heparin (200 µl, 400 µmol/l dissolved in KRB) was added to four non-fasting serum samples (2 ml) which were incubated for 60 min at 37 °C. PS (dissolved in KRB) was added at variable time points; at the beginning of the incubation (0 min), after 15 and 30 min, and immediately before ultrafiltration (after 60 min). Untreated serum samples served as controls; the same volume of KRB was added to all samples. After ultrafiltration, all samples were assayed for free IGF-I.

Study 3: effect of heparin on serum free and bioactive IGF-I in vitro

Before comparing the effect of GAGs on serum levels of free and bioactive IGF-I, pilot studies using six non-fasting serum samples were performed, evaluating different KIRA incubation lengths (15, 30, and 60 min at 37 °C), different GAGs (Hep, Tin, DS, and HS) and increasing doses (0, 4, 10, 40, 100, and 400 µmol/l) (data not shown). In brief, these pilot experiments showed that Hep and HS were the most potent stimulators of IGF-I bioactivity *in vitro*. Furthermore, the KIRA signal was relatively stable within the tested time interval, and therefore it was decided to incubate the KIRA for 30 min. Finally, addition of 40 µmol/l heparin gave a signal well within the range of the KIRA calibration curve. Thus, all further measurements of serum-free and bioactive IGF-I were carried out with a heparin concentration of 40 µmol/l and a KIRA cell incubation length of 30 min.

The effect of heparin on levels of free and bioactive IGF-I was investigated in four different study groups, comprising ten healthy women studied twice; during pregnancy (gestation week 24–28) and at 5 weeks *post partum*, ten type 1 diabetic patients (T1DM) and ten controls matched with the diabetic patients (Table 1). All sera were diluted 1:20 in KRB with or without heparin (40 µmol/l) prior to the assessment of free and bioactive IGF-I. The serum samples used in this study originate from previously described larger study cohorts

(20, 21). All samples were collected in accordance with the Declaration of Helsinki after informed consent.

Statistical analysis

Data are given as mean $\pm$ S.E.M. Paired differences within more than two groups were tested by repeated measures one-way ANOVA followed by Student's Newman–Keuls's test for multiple comparisons or Bonferroni's *t*-test when appropriate. Unpaired differences within more than two groups were tested by ANOVA followed by Student's Newman–Keuls's test for multiple comparisons. Comparisons between two groups were performed by Student's paired or unpaired *t*-test or by paired (Wilcoxon's) or unpaired (Mann–Whitney's) rank sum test when appropriate. Linear regression analysis was used to assess biological associations between serum levels of total, free, and bioactive IGF-I. All data were log transformed prior to statistical analysis.

Results

Study 1

In this experiment, six non-fasting sera from healthy controls were used. The baseline levels of free IGFs-I, -II, total IGFs-I, and -II averaged 1.71 ± 0.35 , 1.63 ± 0.11 , 222 ± 38 , and 952 ± 81 µg/l respectively.

All GAGs increased levels of free IGF-I in a dose-dependent manner ($P < 0.0001$; Fig. 1), however, with different potency ($P < 0.0001$). nac-Hep, HS and Hep were similarly potent, increasing free IGF-I by 933 ± 130 , 628 ± 60 , and $620 \pm 52\%$ respectively, at the highest dose, when compared with baseline. These increases were higher than those observed after addition of Tin ($289 \pm 53\%$) and nac-Tin ($268 \pm 17\%$). DS was the least potent, increasing free IGF-I by $173 \pm 10\%$ above baseline. The GAGs also affected free IGF-II in a dose-dependent manner and with different potencies ($P < 0.0001$; Fig. 2). At the highest dose, the greatest increments in free IGF-II were observed after nac-Hep ($619 \pm 49\%$) and Hep ($532 \pm 89\%$), being significantly higher than those observed after addition of HS ($319 \pm 36\%$), Tin ($245 \pm 23\%$), and nac-Tin

Table 1 The four study groups and their baseline levels of total, bioactive, and free insulin-like growth factor-I (IGF-I). Data are presented as mean $\pm$ S.E.M. or numbers.

Study group	n and gender (♀/♂)	Age (years)	BMI (kg/m ²)	Total IGF-I (µg/l)	Bioactive IGF-I (µg/l)	Free IGF-I (µg/l)
Pregnant women	10/0	27.3 $\pm$ 1.0	22.8 $\pm$ 2.6	192 $\pm$ 17	2.53 $\pm$ 0.31*	1.07 $\pm$ 0.14*
Post partum women	10/0		24.8 $\pm$ 1.0	189 $\pm$ 12	1.14 $\pm$ 0.10	0.52 $\pm$ 0.07
Type 1 diabetes (T1DM)	8/2	34.1 $\pm$ 2.4	24.2 $\pm$ 0.8	89 $\pm$ 15*	0.77 $\pm$ 0.13*	0.29 $\pm$ 0.09*
Controls	8/2	32.8 $\pm$ 2.3	23.0 $\pm$ 0.4	132 $\pm$ 13	1.15 $\pm$ 0.09	0.37 $\pm$ 0.04

* $P < 0.05$ when comparing pregnant women and *post partum* women (paired analyses), or when comparing controls and patients with T1DM (unpaired analyses). For all four study groups, levels of bioactive IGF-I were significantly higher than those of free IGF-I ($P < 0.0001$).

Figure 1 The relative increases in serum-free IGF-I after addition of six different glycosaminoglycans (GAGs). The dotted lines represent baseline levels after addition of buffer (~100%). All GAGs dose-dependently increased levels of free IGF-I ($P < 0.0001$), however, with different potency ($P < 0.0001$): at the highest doses, Hep, nac-Hep and HS (***) increased free IGF-I more potently than Tin and nac-Tin (**), which both increased free IGF-I more potently than DS (*).

Figure 2 The relative increases in serum-free IGF-II after addition of six different GAGs. The dotted lines represent baseline levels after addition of buffer (~100%). All GAGs dose-dependently increased levels of free IGF-II ($P < 0.005$), however, with different potency ($P < 0.0001$): at the highest doses, Hep and nac-Hep (***) increased free IGF-II more potently than HS, Tin and nac-Tin (**), which all increased free IGF-II more potently than DS (*).

($240 \pm 12\%$). Again, DS was the least potent GAG ($142 \pm 7\%$).

In contrast to free peptide levels, total IGFs-I and -II remained unchanged after addition of GAGs (data not shown). Similarly, the levels of intact IGFBPs as estimated by WLB were unaffected by addition of GAGs, when comparing the densities of the different bands. The only exception was nac-Hep, which in the highest dose ($400 \mu\text{mol/l}$) decreased the density of IGFBP-3, when compared with the other doses (0, 4, and $40 \mu\text{mol/l}$, $P < 0.005$) (data not shown).

Study 2

PS completely reversed the effect of heparin on free IGF-I levels (Fig. 3). This was true whether PS was added concomitant with heparin or at variable time intervals after addition of heparin.

Study 3

The IGF-I bioassay is believed to determine the sum of free IGF-I plus IGF-I being dissociated from the IGFBPs during incubation of serum with the IGF-IR transfected cells, and accordingly, bioactive IGF-I was significantly higher than free IGF-I in all study groups ($P < 0.0001$; Table 1). Patients with T1DM had lower levels of all three circulating forms of IGF-I, when compared to controls ($P < 0.05$; Table 1). In women, bioactive and

free IGF-I were increased during pregnancy ($P < 0.05$; Table 1), whereas total IGF-I did not differ.

Heparin increased serum levels of free as well as bioactive IGF-I in all study groups ($P < 0.0001$; Fig. 4). In controls, the absolute increase in bioactive IGF-I was larger than that of free IGF-I (2.96 ± 0.37 vs $1.28 \pm 0.11 \mu\text{g/l}$, $P < 0.0006$), and similar findings were made in patients with T1DM (change in bioactive vs free IGF-I: 1.31 ± 0.27 vs $0.99 \pm 0.27 \mu\text{g/l}$, $P < 0.008$) and in pregnant women (change in bioactive vs free IGF-I: 5.35 ± 0.54 vs $2.78 \pm 0.43 \mu\text{g/l}$, $P < 0.003$). In *post*

Figure 3 The effect of protamine sulfate (PS) (5 g/l) on serum treated with heparin ($400 \mu\text{mol/l}$). The gray and black columns show levels of free IGF-I without (baseline) and with (Hep) addition of heparin respectively. The open columns show levels of free IGF-I in heparin-treated serum samples added PS at various time points prior to ultrafiltration. $*P < 0.0001$ when compared with baseline. Each column represents mean $\pm$ S.E.M. of four serum samples.

Figure 4 The effect of heparin on levels of free (upper panel) and bioactive (lower panel) serum IGF-I levels. $*P < 0.0001$ when comparing samples before (white) and after (gray) addition of heparin. a: $P < 0.05$ when comparing the absolute increase in free vs bioactive IGF-I within the same study group. Each column represents mean $\pm$ S.E.M. of ten serum samples.

partum women, bioactive IGF-I tended to increase more than free IGF-I, but the difference did not achieve statistical significance (change in bioactive vs free IGF-I: 2.31 ± 0.24 vs 1.80 ± 0.15 $\mu\text{g/l}$, $P=0.12$). Comparison of the stimulatory effects of heparin on free and bioactive IGF-I respectively in the four study groups showed that the largest change was seen in pregnant women, in whom the increase exceeded those observed in the other groups ($P<0.0001$). In contrast to the observed changes in serum free and bioactive IGF-I, serum total IGF-I remained unaffected by heparin addition (data not shown).

Linear regression analysis was performed on pooled data from pregnant women, healthy controls and patients with T1DM ($n=30$). Data from *post partum* women (which contained the same individuals as the pregnancy group) were not included, because it is not recommended to include more than one observation per individual in a regression analysis. The result of the linear regression analyses showed that serum levels of total, free, and bioactive IGF-I were strongly positively correlated before, as well as after, addition of heparin (Table 2).

Discussion

The present study showed that various GAGs, both with and without anti-coagulating activity, dose-dependently increased serum free IGFs-I and -II, although with different potency, whereas serum levels of total IGFs and IGFBPs remained unchanged (with a single exception). Furthermore, it was shown that PS completely reversed the effect of heparin on free IGF-I. Although we did not measure changes in free IGF-II and heparin was the only GAG studied following co-incubation with PS, the latter finding indicates that the effect of GAGs on IGFBP-IGF interaction in general is fully reversible. Finally, heparin was able to increase in parallel serum levels of free and bioactive IGF-I in various patient samples, irrespective of the endogenous concentrations. However, heparin was particularly

potent in serum from pregnant women, a finding which may relate to the extensive activity of IGFBP-3 proteases during pregnancy.

The six GAGs all significantly increased serum levels of free IGFs-I and -II in a dose-dependent manner, while the extractable peptide concentrations remained unaffected. WLB was used to evaluate the binding capacity of the IGFBPs in serum and with one exception no effect of GAG addition was observed. Thus, it appears that GAGs in general may serve to increase free peptide levels by releasing bound IGFs. The present data were obtained in serum and we cannot therefore conclude whether the increases in free and bioactive IGF levels were caused by liberation of IGF kept in binary and/or ternary complexes. However, both options appear to exist. The proximity of the HBD and the binding sites for IGF and ALS may explain that several different GAGs are reported to affect IGF:IGFBP as well as IGFBP-3:ALS complex formation (13, 14, 22–25). Interestingly, despite their homogeneity, the IGFBPs appear to possess genuine differences in their response to GAGs: IGFBPs-3, -5 and -6 all show a reduced affinity for IGFs following GAG exposure, whereas the ligand affinity of IGFBP-4 appears to be unaffected. Data on IGFBP-2 are contradictory, but at least in some settings, IGFBP-2 has been reported to bind GAGs only in the presence of IGFs, while IGFBP-1 does not bind GAGs at all (23–25). Finally, GAGs appear to exert a larger impact on the binding of ALS to preformed complexes of IGF-I and IGFBP-3 than on the binding of IGF-I to IGFBP-3 (14). Taken together, it seems reasonable to assume that both binary and ternary IGF-complexes contribute to the observed increases in free and bioactive IGF-I as well as free IGF-II, but the relative contribution of the two IGF-fractions remains to be estimated.

The ability of the tested GAGs to increase serum levels of free IGFs-I and -II differed significantly. Apparently, the molecular weight affected the IGF releasing potency as tinzaparin (Innohep®) and nac-tinzaparin were less potent than heparin and nac-heparin DS was the least potent GAG, though its molecular weight is high. This observation is probably related to the low degree of sulfation as the charge density is considerably lower in DS as compared to heparin. In addition, the backbone structure of the GAGs may be as important as galactosamine is present in DS, instead of glucosamine. There was no difference between the IGF-releasing potency of the two low molecular weight heparins (tinzaparin and nac-tinzaparin), and a similar observation was made when comparing the potency of nac-heparin and heparin. As heparin sulfate, which has no anti-coagulant activity, was also quite potent, we conclude that the ability of GAGs to release IGFs-I and -II is not related to their anti-coagulant activity.

It has been suggested that *in vivo*, IGF-I bioactivity is composed of the sum of free IGF-I plus IGF-I being

Table 2 Linear regressions in pooled data from healthy controls, pregnant women and patients with T1DM ($n=30$).

Variables	Heparin addition	r/r^2 -values	P-value
Free IGF-I vs bioactive IGF-I	No	0.88/0.78	<0.0001
Free IGF-I vs bioactive IGF-I	Yes	0.81/0.65	<0.0001
Free IGF-I vs total IGF-I	No	0.77/0.59	<0.0001
Free IGF-I vs total IGF-I	Yes	0.87/0.76	<0.0001
Bioactive IGF-I vs total IGF-I	No	0.89/0.79	<0.0001
Bioactive IGF-I vs total IGF-I	Yes	0.79/0.63	<0.0001
Δ bioactive IGF-I vs total IGF-I	Yes	0.66/0.44	<0.0001
Δ free IGF-I vs total IGF-I	Yes	0.81/0.70	<0.0001
Δ bioactive IGF-I vs delta free IGF-I	Yes	0.59/0.35	<0.0001

Δ , the absolute change in levels of free or bioactive IGF-I following heparin addition.

released from the IGFBPs at the site of the IGF-IR (i.e. readily dissociable IGF-I) (26), and in accordance with this idea, serum levels of bioactive IGF-I were significantly higher than levels of ultrafiltered serum free IGF-I in all four study groups. In addition, heparin caused a significantly larger increase in bioactive than free IGF-I in all groups except non-pregnant women. The reason that non-pregnant women differed in this respect remains unknown, but we speculate that it may be due to pregnancy-related changes in the circulating IGF-system, which may still be present 5 weeks *post partum*. If heparin only increased levels of free IGF-I through competitive binding, we would expect similar increases in free and bioactive IGF-I. Since this was not the case, it may be speculated that heparin not only serves to liberate IGF-I from its binding proteins, but also increase the fraction of readily dissociable IGF-I, most likely by lowering the ligand affinity of the IGFBPs as previously reported (13, 14).

Although levels of free and bioactive IGF-I were strongly correlated before, as well as after, addition of heparin, a more detailed analysis showed that the greatest increases in serum free and bioactive IGF-I were observed in pregnant women. Pregnancy is characterized by an extensive IGFBP-3 proteolytic activity (27), which is easily detectable as early as after 8 weeks after gestation, where ~70% of serum IGFBP-3 has been degraded (20). Enzymatic degradation of IGFBP-3 has been reported to cause a tenfold reduction in its affinity for IGF-I (28), a finding which may explain that pregnant women have increased serum levels of free and bioactive IGF-I, as also observed in the present study (20, 29). Furthermore, IGFBP-3 proteolysis appears to render IGFBP-3 more susceptible to heparin. Accordingly, serum incubated overnight at 37 °C and hence affected by proteolytic enzymes in serum was much more sensitive to the effect of heparin than serum analyzed without overnight incubation (22).

We included patients with T1DM, because their circulating IGF-system is markedly different, when compared to pregnant women. First, T1DM is associated with subnormal levels of total and free IGFs (21, 30); secondly, IGFBP-3 proteolysis does not appear to be a major issue, at least not in the present cohort of patients, who have all been on long-term insulin treatment (21,30); an exception is newly diagnosed and hence poorly controlled patients, in whom IGFBP-3 proteolysis appears to be upregulated (31). Still, despite low endogenous levels of IGF-I, heparin was able to increase serum levels of free and bioactive IGF-I in the diabetic group, and again the absolute increase in IGF-I bioactivity was significantly larger than that of free IGF-I. On the other hand, the observed increases following heparin were less marked than in healthy controls, indicating that the ability of heparin to increase free and bioactive IGF-I is dependent on total IGF-I levels, as also indicated by the regression analyses.

The present data support the hypothesis that GAGs, through their impact on ALS-IGFBP-IGF interaction, participate in the regulation of IGF-I bioactivity (5, 13, 14). This is likely to occur at different sites, affecting the IGF-system in the circulation as well as in the peripheral tissues. GAGs attached to endothelial cells may serve to increase the trans-endothelial transport of IGFs-I and -II, whereas GAGs localized in the ECM may serve to increase free IGF-I levels near the site of the IGF-IR. However, at the time of writing, there are only very sparse data on the effect of GAGs on IGF-I bioactivity *in vivo*, and these observations do not allow us to extrapolate our *in vitro* findings to the *in vivo* situation (32–34).

In conclusion, GAGs possess the ability to increase serum levels of free and bioactive IGF-I *in vitro* without affecting total IGF-I or IGFBP levels. Of note, the IGF-I liberating effect was independent of the anti-coagulating activity of the GAGs, and as judged from the ability of PS to neutralize the effect of heparin, it was fully reversible. We speculate that the non-anti-coagulating GAGs could be used as IGF-I displacers, serving to increase the activity of the endogenous IGF-system in catabolic conditions with upregulated IGFBPs such as in uremia, T1DM, and anorexia nervosa (35, 36).

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