The effects of recombinant human IGF-I administration on concentrations of acid labile subunit, IGF binding protein-3, IGF-I, IGF-II and proteolysis of IGF binding protein-3 in adolescents with insulin-dependent diabetes mellitus

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Abstract

The long term therapeutic potential of recombinant human (rh) IGF-I administration in insulin-dependent diabetes mellitus (IDDM) may be determined by changes in the IGF binding proteins (IGFBPs) and thus the bioavailability of IGF-I. We have therefore studied the effects of a single subcutaneous dose of rhIGF-I (40 µg/kg at 1800 h), when compared with an untreated control night, in 17 subjects with IDDM, on serum concentrations of IGF-I, IGF-II, IGFBP-3, acid labile subunit (ALS), and IGFBP-3 proteolysis.

Mean ($\pm$ s.e.m.) IGF-I levels increased from 242 ± 30 ng/ml to 399 ± 26 ng/ml ($P=0.01$) after rhIGF-I whereas IGF-II levels declined from 600 ± 45 ng/ml to 533 ± 30 ng/ml. There was a small overnight reduction in baseline ALS levels from 48 ± 2.8 to 44.5 ± 3.2 µg/ml ($P=0.04$) after rhIGF-I administration. An early fall in IGFBP-3 concentrations on the control night was not seen after rhIGF-I and overall mean levels were increased

(5.2 ± 0.2 µg/ml vs 4.9 ± 0.2 µg/ml, $P=0.04$, on the control night). On the baseline night, IGFBP-3 levels correlated with the sum of IGF-I and IGF-II ($r=0.73$, $P=0.02$) and with levels of the ALS ($r=0.7$, $P=0.002$). However after rhIGF-I, the sum of IGF-I and IGF-II no longer correlated with IGFBP-3, whereas the relationship with ALS was maintained. Immunoblot studies in six subjects indicated that 60%–70% of the IGFBP-3 was detected as a low molecular weight fragment at 1900 h on both study nights, but the amount of fragment declined to approximately 50% at 0100 h and 45% at 0700 h.

In conclusion, despite a slight but significant fall in ALS, IGFBP-3 levels rise after rhIGF-I administration in IDDM. This cannot be explained by alterations in IGFBP-3 proteolysis, and may relate to the relative stability of ALS/IGFBP-3 when complexed principally with IGF-I rather than IGF-II.

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Introduction

The insulin-like growth factors (IGF)-I and -II circulate bound to a series of six high affinity IGF binding proteins (IGFBP-1 to -6) which regulate their bioavailability and bioactivity (Ballard *et al.* 1989, 1992). IGFBP-3, combined with either IGF-I or IGF-II and an acid labile subunit (ALS), provides a circulating reservoir of IGFs which is retained in the circulation (Baxter *et al.* 1989). The half-life of unbound IGF-I is around 10 to 12 min whereas the half-life of the ternary complex is approximately 15 h (Guler *et al.* 1989). The complex has a molecular mass of approximately 150 kDa and contains the majority of circulating IGFs with the remainder circulating as binary complexes with IGFBP-3 and other binding proteins (Binoux & Hossenlopp 1988, Baxter & Martin 1989).

Serum proteases identified during pregnancy, severe illness and after surgery may alter the affinity with which the IGFs are bound in these complexes and hence IGF bioavailability (Hossenlopp *et al.* 1990, Davies *et al.* 1991, Cwyfan-Hughes *et al.* 1992, Davenport *et al.* 1992).

In patients with insulin-dependent diabetes mellitus (IDDM) IGF-I levels are invariably low or in the low normal range (Amiel *et al.* 1984, Taylor *et al.* 1988) even though growth hormone (GH) hypersecretion has been well documented (Edge *et al.* 1990, Batch & Werther 1992). There is little detailed observation of the kinetics of the binding proteins and the ALS in IDDM although circulating levels of IGFBP-3 are low and IGFBP-1 may be increased (Batch *et al.* 1991). There is good evidence to indicate that the reductions in IGF-I and the elevations in IGFBP-1 arise because of inadequate portal delivery of

Table1 Subject characteristics

	Range	Median
Age (years)	13·5–18·9	15·5
Diabetes duration (years)	3·0–13·8	5·9
C-peptide (pmol/l)*	20–270	50
BMI (kg/m ²)	18·8–29·42	22·3
Insulin dose (U/kg)	0·73–1·43	1·05

*Blood glucose >7·0 mmol/l.

insulin (Brismar *et al.* 1994). The hepatic GH receptor is partially insulin-dependent (Baxter *et al.* 1980) and circulating levels of the GH binding protein (GHBP) are reduced in IDDM (Menon *et al.* 1992, Holl *et al.* 1993). Improved insulinisation leads to increased levels of GHBP and IGF-I with reductions in IGFBP-1 (Amiel *et al.* 1984, Brismar *et al.* 1994). The administration of insulin to newly diagnosed patients also affects circulating IGFBP-3 protease activity (Bereket *et al.* 1995). It therefore seems likely that insulin may have an important role as a regulator of IGF-I production and of the kinetics and distribution of IGFBs between the different IGFBPs.

The subcutaneous administration of low doses of recombinant human (rh) IGF-I to adolescents with IDDM has been shown to increase IGF-I levels and to reduce GH hypersecretion and the insulin infusion requirements for the maintenance of euglycaemia (Cheetham *et al.* 1993, 1994a). A corresponding fall in IGF-II levels and an increase in IGFBP-3 concentrations were also observed in these studies (Cheetham *et al.* 1994b). We now report a more detailed examination of the effects of rhIGF-I on IGF-I, IGF-II, IGFBP-3, ALS, and IGFBP-3 proteolysis in overnight studies of rhIGF-I administration.

Subjects and Methods

Seventeen subjects with IDDM took part in these studies. There were 13 females and 4 males aged from 13·5–18·9 years (median 15·5). All had been diagnosed for at least 3·0 years and were in mid-late puberty stages 4 to 5 (Tanner 1962) with body mass indices ranging from 18·8–29·4 kg/m² (median 22·3). Stimulated C-peptide concentrations (blood glucose >7·0 mmol/l) ranged from 20–270 pmol/l (median 50 pmol/l). The daily insulin dose ranged from 0·73–1·43 U/kg (median 1·05 U/kg). Subject characteristics are detailed in Table 1.

Protocol

Subjects were admitted for two separate nights and randomly allocated to a treatment group with rhIGF-I administered in the thigh in a s.c. dose of 40 µg/kg or a control group (control night) when either no injection (*n*=8) or a placebo (*n*=9) was administered. Subjects

stopped all intermediate-acting insulin 36 h before the study began. Between then and admission to hospital blood glucose concentrations were controlled with four daily injections of soluble insulin. The last subcutaneous insulin dose was at lunch-time on the day of admission.

Subjects were admitted to hospital between 1600 h and 1700 h on the day of the study. Cannulae were inserted into distal forearm and antecubital fossa veins following the application of local anaesthetic cream. The forearm was maintained in a heated box to arterialise the venous samples obtained from the forearm cannula. The proximal cannula was used to administer a continuous insulin infusion to maintain euglycaemia overnight.

Samples for IGF-I and IGFBP-3 estimation were taken every hour and every three hours respectively during the single bolus studies. ALS concentrations were determined in samples taken at 1800 h and 0800 h.

IGF-II was determined in samples taken two-hourly on both nights in the nine subjects who were part of the placebo-controlled trial.

Assays

Plasma samples for IGF-I were acid ethanol extracted and IGF-I concentrations determined by radioimmunoassay as described previously (Taylor *et al.* 1990). The intra-assay coefficients of variation were 5·2% and 4·8% at analyte levels of 27·5 and 220 ng/ml respectively. The interassay coefficients of variation were 12·7% and 10·6% at analyte levels of 77 and 242 ng/ml respectively.

Serum IGFBP-3 levels were determined using a double antibody RIA. IGFBP-3 antiserum (SCH-2/5) was used at a final concentration of 1:8000. Recombinant glycosylated IGFBP-3 (Celtrix Pharmaceuticals, Santa Clara, CA, USA) was used for standards, giving a range of 5–500 ng/ml. Bound and free ¹²⁵I-labelled glycosylated IGFBP-3 were separated using a donkey anti-rabbit SAC-CEL second antibody (I.D.S. Ltd, Bolden Business Park, Bolden, Tyne and Wear, UK). The inter-assay coefficient of variation was 4·28% at 5 µg/ml and the intra-assay coefficient of variation was 5·14% at 5 µg/ml.

The ALS was measured by a specific RIA using an antiserum raised in New Zealand white rabbits following the injection of purified human ALS. Serum samples were incubated with antiserum, ¹²⁵I-labelled ALS and RIA buffer (Baxter 1990). The standard curve was derived from incubations containing pure alpha-subunit. Bound and free ¹²⁵I-labelled ALS were separated and counted after the addition of goat anti-rabbit immunoglobulin and normal rabbit serum. The intra-assay coefficients of variation were 3·4% at 5·1 µg/ml, 3·3% at 20·8 µg/ml and 3·4% at 42·7 µg/ml. The interassay coefficients of variation were 10·5% at 5·3 µg/ml, 5·4% at 24·0 µg/ml and 6·5% at 57·5 µg/ml.

IGF-II concentrations were determined after acid ethanol extraction in the laboratories of Prof. W Blum (University Children's Hospital, Tübingen, Germany) using a specific radioimmunoassay which utilises excess IGF-I to block interference from IGFBPs (Blum *et al* 1988). The interassay coefficient of variation at 50% of maximum binding capacity was 12.2% and the minimum detection limit was 0.018 ng.

Immunoblot studies

Immunoblot studies were undertaken to assess protease activity in samples collected at 1900 h, 0100 h and 0700 h in the six subjects who demonstrated the largest changes in IGFBP-3 overnight when rhIGF-I treatment and control nights were compared. Western immunoblotting was performed as described before (Mason *et al.* 1996). Serum samples (2.5 µl) were separated through a 12.5% SDS-polyacrylamide gel and then blotted onto a Hybond C-super membrane (Amersham International, Amersham, Bucks, UK). This was blocked with a 5% powdered milk solution and then probed with the same IGFBP-3 antiserum used in the RIA (SCH-2/6 at 1:15 000 dilution). At this dilution this antiserum did not cross-react with recombinant IGFBP-1, IGFBP-2, IGFBP-4, IGFBP-5 or IGFBP-6 loaded onto immunoblots at supraphysiological levels. After washing this was reprobed with secondary (anti-rabbit) antibody conjugated to horseradish peroxidase and the signal visualised by the use of an enhanced chemiluminescence kit according to the instructions supplied by the manufacturer (Amersham International) and exposed to autoradiographic film. Bands on the film were quantitated by laser densitometry. A stack of three films was simultaneously exposed for each blot in order to ensure obtaining an autoradiographic signal within the linear range.

Statistics

Changes in IGFBP-3, IGF-II and Western immunoblot data were analysed by repeated measures analysis of variance. Parameters at the beginning and end of the study night were compared by paired *t*-tests and separate variables by linear regression. Data are expressed as means $\pm$ S.E.M. and significance has been set at the 5% level.

Results

Serum IGF-I concentrations were stable on the control night. Following rhIGF-I administration levels rose promptly reaching a peak after 5 h and then declined with a half-life of around 18 h. Mean overnight IGF-I concentrations were 242 ± 20 ng/ml on the control night and 399 ± 26 ng/ml after rhIGF-I administration ($P < 0.01$)

Figure 1 Overnight concentrations of (a) IGF-I, (b) IGF-II, and (c) IGFBP-3 following a single subcutaneous injection of rhIGF-I (○) or placebo (●) given at 1800 h.

(Fig. 1). Glucose and insulin data from this group of subjects have been reported elsewhere (Cheetham *et al.* 1997).

Baseline IGFBP-3 concentrations (1800 h) were similar on the control night and the night of rhIGF-I administration (5.6 ± 0.2 vs 5.3 ± 0.3 µg/ml; $P > 0.1$). On the control night IGFBP-3 levels subsequently fell between 1800 h and 0300 h (from 5.6 ± 0.2 µg/ml to 4.6 ± 0.2 µg/ml; Fig. 1). The decline in IGFBP-3 concentrations was not observed on the night of rhIGF-I administration, and overall IGFBP-3 levels were increased (5.2 ± 0.2 µg/ml) compared with the control night (4.9 ± 0.2 µg/ml) with a significant treatment effect ($P = 0.03$). IGFBP-3 data are summarised in Table 2.

Table 2 IGFBP-3 concentration (µg/ml) in samples from subjects taken over the control night or the night of rhIGF-I administration. Baseline values (at 1800 h) and overall mean values (1800–0800 h) are shown. Values are means ± S.E.M. with range in parentheses

	Control	rhIGF-I	P value
Baseline	5.6 ± 0.2 (4.3–7.0)	5.3 ± 0.3 (4.0–9.4)	NS
Overall mean	4.9 ± 0.2 (3.3–6.8)	5.2 ± 0.2 (3.9–7.5)	0.04

NS, not significant.

Table 3 ALS concentration (µg/ml) in samples taken at 1800 h and 0800 h from control and rhIGF-I-treated subjects. Values are means ± S.E.M. with range in parentheses

	Time (h)		P value
	1800	0800	
Control	47.8 ± 3.2 (19.7–64.4)	45.7 ± 3.1 (27.8–75.1)	0.2
rhIGF-I-treated	48.5 ± 2.8 (24.7–70.7)	44.5 ± 3.2 (21.2–65.1)	0.04

There were no differences in the ALS concentrations at 1800 h on the control and the rhIGF-I treatment nights (47.8 ± 3.2 vs 48.5 ± 2.8 µg/ml respectively). Levels at 0800 h remained unchanged on the control night (45.7 ± 3.1 µg/ml) but fell on the night of rhIGF-I administration from 48.5 ± 2.8 at 1800 h to 44.5 ± 3.2 µg/ml at 0800 h ($P=0.04$) (Table 3).

ALS concentrations at 1800 h and 0800 h correlated with IGFBP-3 levels in individual subjects on both control and rhIGF-I treatment nights. The respective correlation coefficients were $r=0.63$ ($P=0.008$) at 1800 h and $r=0.7$ ($P=0.002$) at 0800 h on the control night and $r=0.52$ ($P=0.034$) at 1800 h and $r=0.68$ ($P=0.003$) at 0800 h on the night of rhIGF-I administration.

IGF-II levels ($n=9$) have been reported previously (Cheetham *et al.* 1994b) and were similar at baseline on the two study nights (626 ± 48 vs 609 ± 40 ng/ml). IGF-II levels tended to decline in both groups during the night to a nadir at 0400 h (Fig. 1). The decline was more pronounced following rhIGF-I administration, however, and at 0400 h levels were 473 ± 32 ng/ml after rhIGF-I administration compared with 559 ± 40 ng/ml on the control night. Overall mean IGF-II levels between 1800 h and 0800 h were 600 ± 45 ng/ml on the control night compared with 533 ± 30 ng/ml after rhIGF-I ($P=0.06$).

In the subjects in whom IGF-II levels were measured ($n=9$) it was possible to examine the relationship between IGF-I, IGF-II, IGFBP-3 and ALS concentrations. In order to determine the relationships between the sum of IGF-I and IGF-II and IGFBP-3, concentrations of these peptides were converted into molar equivalents. There were strong

Figure 2 Western immunoblot showing forms of IGFBP-3 in samples taken over the control night and the night of rhIGF-I administration in one of the subjects. The top two bands represent differentially glycosylated forms of intact IGFBP-3 whilst the lower band represents the major fragment resulting from specific proteolysis.

correlations between the sum of IGF-I and IGF-II concentrations and the levels of IGFBP-3 throughout the control night ($r=0.73$, $P=0.02$), whereas this relationship was perturbed following rhIGF-I administration ($r=0.49$, $P=0.17$).

The sum of IGF-I and IGF-II concentrations was also related to ALS concentrations in the 1800 h sample on both nights ($r=0.8$, $P=0.01$). However this relationship was not observed in the 0800 h sample on either control nights ($r=0.22$, $P=0.5$) or on the morning after rhIGF-I administration ($r=0.24$, $P=0.2$).

Immunoblot studies

Representative immunoblots from one subject are illustrated in Fig. 2. Densitometry analysis of immunoblots from six subjects is illustrated in Fig. 3. At 1900 h on both study nights, 60–70% of the IGFBP-3 was detected as a low molecular weight fragment, but by 0100 h the amount of fragment had declined to around 50% and by 0700 h it had declined further to around 45%. A significant decline in the amount of fragmented IGFBP-3 was observed on both control nights (ANOVA, $P<0.006$) and rhIGF-I treatment nights (ANOVA, $P<0.001$) but with no difference between nights.

Discussion

Recombinant human IGF-I administration to adolescents with IDDM, in a relatively low dose of 40 µg/kg s.c. leads

Figure 3 Densitometry analysis of immunoblots from six subjects represented as mean percentage ($\pm$ S.D.) of IGFBP-3 fragments at 1900 h, 0100 h and 0700 h on nights when rhIGF-I (solid bars) or placebo (open bars) was administered.

to restoration of normal physiological levels of IGF-I and can be given as a single subcutaneous dose (Cheetham *et al.* 1993). In preliminary studies it was demonstrated that small but significant increases in IGFBP-3 concentrations could also be observed (Cheetham *et al.* 1994b) and we have now confirmed this in a larger group of adolescents with IDDM. There are two possible mechanisms whereby this increase in IGFBP-3 could have occurred. There could be increased production of IGFBP-3 or rhIGF-I could alter the relative concentrations of the components of the 150 kDa IGF/IGFBP-3/ALS complex and its stability in the plasma.

Formation of the 150 kDa ternary complex is dependent on the presence of the GH-dependent ALS (Baxter & Martin 1989, Baxter *et al.* 1989). ALS circulates in excess in diabetic rats and normal human subjects (Baxter 1988, Lewitt *et al.* 1993) and baseline ALS concentrations were relatively high in the group of adolescent subjects with IDDM that were studied. Whilst concentrations remained unchanged on the control night, there was a small reduction in ALS overnight following rhIGF-I treatment. This might result from the consistent fall in GH concentrations that we have observed following rhIGF-I (Cheetham *et al.* 1993) and may provide further support for the relationship between GH levels and circulating ALS (Baxter 1990).

Despite the modest fall in ALS concentrations, IGFBP-3 levels tended to rise after rhIGF-I. A clear relationship between IGFBP-3 and the ALS was evident both before and after the administration of rhIGF-I, suggesting that the modest reduction in ALS levels was not limiting the amount of IGFBP-3 which could be retained in the circulation. The relationship between ALS and the sum of IGF-I and IGF-II concentrations was observed on both nights in the 1800 h sample but not in the 0800 h sample, again indicating that complex formation may not be stable overnight in IDDM.

The regulation of IGFBP-3 production is complex. Although originally described as GH-dependent (Baxter & Martin 1989) evidence from *in situ* hybridisation indicates

that in the liver IGFBP-3 is not expressed by hepatocytes but by perisinusoidal cells (Chin *et al.* 1994). As these cells do not appear to express GH receptors this indicates that any effect of GH on IGFBP-3 production should be indirect (Kanety *et al.* 1993). However administration of GH to rats causes a rapid induction of hepatic IGFBP-3 mRNA whereas administration of IGF-I has no such acute effect (Lemmey *et al.* 1994). rhIGF-I administration to normal human subjects leads to a fall in both the levels of ALS and IGFBP-3 (Baxter *et al.* 1993, Kupfer *et al.* 1993) whilst administration of rhIGF-I to subjects with GH insensitivity has not been found to increase circulating IGFBP-3 concentrations in some studies but has in others (Gargosky *et al.* 1993, Kanety *et al.* 1993). Our observation of a small increase in IGFBP-3 following administration of rhIGF-I to adolescents with IDDM occurred despite GH suppression and a fall in ALS levels. Furthermore, the rise in IGFBP-3 that we observed represented, in part, a reversal of the decline in IGFBP-3 levels on the control night. One possible explanation for these observations is that rhIGF-I had led directly or indirectly to an altered stability of the 150 kDa complex.

The circulating concentration of IGFBP-3, like that of IGFs, depends on the proportion which can associate into larger complexes. Uncomplexed IGFBP-3 has a very short half-life but when bound with IGF and ALS its half-life is prolonged. Circulating protease activity may alter the stability of the ternary complex. Our immunoblot studies demonstrated that a large percentage of the IGFBP-3 was fragmented (around 60%) at the beginning of each study night. This is in keeping with previous observations of increased proteolysis of IGFBP-3 in subjects with type 2 diabetes and untreated patients with IDDM (Bereket *et al.* 1995). Recent data have highlighted the importance of insulin in the regulation of IGFBP-3 protease activity in IDDM (Bereket *et al.* 1995). The reduction in the amount of proteolysed fragment overnight on both study days may reflect improved insulinisation, as subjects were clamped in the euglycaemic range with an intravenous insulin infusion. However, the increase in IGFBP-3 on the rhIGF-I treatment night cannot be explained by changes in protease activity. It may relate to differences in the stability of tertiary complexes when formed with IGF-I rather than IGF-II and IGF-I (Holman & Baxter 1996) as IGF-II levels declined after rhIGF-I administration.

The increases in IGFBP-3 during these bolus studies was not sufficient to explain the sustained rises in IGF-I levels that we observed, even when allowance is made for the corresponding fall in IGF-II. Whilst the relationship between the sum of IGF-I and IGF-II with IGFBP-3 was evident on the control nights and at the beginning of the rhIGF-I treatment night, this relationship was perturbed after rhIGF-I administration. It has been shown that, following rhIGF-I administration, although most of the IGF-I is carried in the large molecular weight form, increases can also be observed in the 40–50 kDa range

(Cheetham *et al.* 1994b). Changes in free IGF-I after a dose of 40 µg/kg are relatively small (Cheetham *et al.* 1994b). The IGF-I carried in the 40–50 kDa group may represent IGF bound to fragmented IGFBP-3 but it could also represent significant binding to IGFBP-1, IGFBP-2 or other IGFBPs. Direct measurement of the proportions of IGF-I and IGF-II bound in individual IGFBP complexes will be needed to resolve these issues.

Further study of the effects of rhIGF on IGFBP-1 and IGFBP-2 are clearly indicated. It has yet to be determined whether sustained increases in IGFBP-3 and greater stability of the 150 kDa complex will be observed with long term rhIGF-I therapy but this may be an important factor in determining the therapeutic potential of this peptide in adolescents with IDDM.

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