**EXPERIMENTAL STUDY**

**O-glycosylation delays the clearance of human IGF-binding protein-6 from the circulation**

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**Abstract**

**Objective:** The actions of insulin-like growth factors (IGF-I and IGF-II) are modulated by a family of six structurally related, high-affinity binding proteins (IGFBPs 1–6). IGFBP-6, an O-linked glycoprotein, preferentially binds IGF-II and inhibits its actions. The aim of this study was to investigate whether O-glycosylation modulates the pharmacokinetics of IGFBP-6.

**Design and Methods:** The pharmacokinetic profiles of 125I-labelled glycosylated (g) and non-glycosylated (n-g) recombinant human IGFBP-6 were studied following intravenous bolus administration in anaesthetised rats.

**Results:** The redistribution half-life of gIGFBP-6 was 2.3-fold greater than that of n-gIGFBP-6 (14.4 ± 1.2 vs 6.3 ± 1.5 min, *P* = 0.006). The elimination half-life of gIGFBP-6 was 21-fold greater than that of n-gIGFBP-6 (584.2 ± 130.2 vs 28.0 ± 4.2 min, *P* = 0.019). The effect of O-glycosylation on IGFBP-6 pharmacokinetics was not due to inhibition of intravascular proteolysis. Radioactivity was found in stomach, kidneys, lung, spleen, heart and liver but not brain 4 h after injection of g or n-gIGFBP-6.

**Conclusions:** O-glycosylation delays the clearance of IGFBP-6 from the circulation and may therefore contribute to its role as a circulating inhibitor of IGF-II actions.

**Introduction**

Insulin-like growth factors (IGF-I and IGF-II) are important mediators of physiological growth (1). Circulating and tissue IGFs are largely bound to a family of six structurally related high-affinity binding proteins (IGFBPs 1–6) (1, 2). IGFBPs are synthesised by most cell types, and may inhibit or potentiate IGF actions.

Within the circulation, about 75% of IGFs are bound in a 150 kDa ternary complex containing IGFBP-3 or -5 and an acid labile subunit (3, 4). Most remaining IGFs are bound to other IGFBPs, including IGFBP-6, in a 50 kDa binary complex whereas levels of free IGFs are very low (5). IGFs in ternary complexes are retained in the circulation (6), whereas IGFs in binary complexes can cross the capillary endothelial barrier. Whereas the circulating half-lives of free IGFs are ~15 min, this increases to 30–50 min in binary complexes, and 12–15 h in ternary complexes (6). The ternary complex therefore acts as a reservoir of circulating IGFs, whereas IGFBPs in binary complexes may assist in transport and targeting of IGFs to tissues (5).

IGFBP-6 binds IGF-II with higher affinity than other IGFBPs and has a distinctive 20- to 100-fold preference for IGF-II over IGF-I. IGFBP-6 is a relatively specific inhibitor of IGF-II actions (7). Expression of IGFBP-6 is associated with non-proliferative states (7) and is induced by differentiating agents such as retinoic acid (8). These properties suggest that the main role of IGFBP-6 is to inhibit IGF-II-induced proliferation, which may be very important given that IGF-II has been implicated as a tumour growth factor (9). IGFBP-6 is found in human serum at a concentration of ~10 nM, which is similar to that of IGFBP-2 and which exceeds that of IGFBP-1 (5). By contrast, circulating IGFBP-3 levels are ~100 nM (5).

Human IGFBP-6 is O-glycosylated on five Ser/Thr residues within the non-conserved mid region of its amino acid sequence (10, 11). The role of O-glycosylation of IGFBP-6 has not been completely defined. Glycosylation increases the circulating half-life of a number of glycoproteins such as erythropoietin (12), tissue plasminogen activator (13) and human luteinizing hormone (14). We therefore investigated the effect of O-glycosylation on the pharmacokinetics of IGFBP-6 in the rat.

**Materials and methods**

**IGFBP-6**

Recombinant human glycosylated IGFBP-6 (gIGFBP-6) was expressed and purified from Chinese hamster ovary
Iodination of IGFBP-6

Both gIGFBP-6 and n-gIGFBP-6 (2 µg) were iodinated to specific activities of 20–40 µCi/µg using chloramine-T. Iodinated IGFBP-6 was separated from free Na[125I] by gel filtration using a P6-DG desalting column (Biorad, Richmond, CA, USA).

Pharmacokinetic studies

Male Sprague-Dawley rats (n = 8, ~300 g) were anaesthetised with sodium bretial (4 mg/kg). Temperature was maintained with a heating pad at 37°C. Both the carotid artery and jugular vein were cannulated using polyethylene tubing (internal diameter 0.58 mm) with 23 gauge needles attached. Animals were allowed to recover for 30 min. Experiments were approved by the Austin and Repatriation Medical Centre Animal Welfare Committee.

Once the animals were cannulated, experiments were performed under anaesthesia. Pharmacokinetics were also assessed once in conscious rats with similar results (not shown). Iodinated IGFBP-6 (3 x 10⁶ c.p.m.) was administered through the jugular vein cannula in a volume of 150 µl heparin saline containing 1% bovine serum albumin. Blood (150 µl) was periodically collected from the carotid artery after 1–180 min. After each blood sample was taken, an equivalent volume of heparin saline was injected to maintain plasma volume. Samples were stored on ice for at least 10 min prior to centrifugation at 3500 g for 10 min at 4°C. Sera (100 µl) were collected and precipitated with trichloroacetic acid (final concentration 15%), and radioactivity in the pellet was counted in a γ-counter. In one experiment, radioactivity in serum was counted. At the end of the study, rats were killed by injection of sodium pentobarbital, and brain, heart, kidney, liver, lungs, spleen and stomach were removed and washed in phosphate-buffered saline, weighed and radioactivity was counted as above.

To determine whether intravascular proteolysis contributed to clearance of IGFBP-6, serum samples were separated by sodium dodecyl sulfate-15%-polyacrylamide gel electrophoresis (SDS-15%-PAGE) followed by gel fixation and exposure to X-ray film.

Data obtained for gIGFBP-6 and n-gIGFBP-6 were analysed using Prism (GraphPad Software Inc, San Diego, CA, USA). The calculated second-order kinetic curves for both gIGFBP-6 and n-gIGFBP-6 fitted the acquired data with r values of ~0.99. Elimination and redistribution half-lives were calculated from these curves. The apparent volume of distribution at steady state (Vss) was calculated using the following equation: Vss = (Dose/AUMC)/(AUC)² where AUC and AUMC are the area under the concentration–time curve extrapolated to infinity and the area under the first moment curve respectively.

Statistics

Results are shown as means ± S.E.M. Pharmacokinetic parameters of gIGFBP-6 and n-gIGFBP-6 were compared using unpaired t-tests.

Results

O-glycosylation prolongs the circulating half-life of IGFBP-6

The disappearance curves of 125I-labelled gIGFBP-6 and n-gIGFBP-6 from serum following intravenous administration into rats were indicative of a two-compartment model (Fig. 1). n-gIGFBP-6 was cleared more rapidly from serum than gIGFBP-6 in both the redistribution and elimination phases. The redistribution half-life (t1/2r) of gIGFBP-6 was 2.3-fold greater than that of n-gIGFBP-6 (Table 1, P = 0.006), whereas the elimination half-life (t1/2e) was 21-fold greater (P = 0.019). The volumes of distribution of gIGFBP-6 and n-gIGFBP-6 were both ~250 ml/kg (Table 1, P = 0.88), consistent
with both forms of IGFBP-6 being distributed throughout the extracellular space.

SDS-PAGE and autoradiography of serum samples indicated that iodinated gIGFBP-6 and n-gIGFBP-6 remained intact, since fragments were not detected (Fig. 2). Intravascular proteolysis therefore does not underlie the faster clearance of n-gIGFBP-6.

### Localisation of extravascular $^{125}$I IGFBP-6 in the rat

The organ distribution of radioactivity was studied 4 h after intravenous administration of $^{125}$I-labelled gIGFBP-6 and n-gIGFBP-6. The organ with the greatest radioactivity was stomach, which contained 59 ± 6% and 35 ± 4% of iodinated gIGFBP-6 ($n = 3$) and n-gIGFBP-6 ($n = 2$) respectively (Fig. 3). $P = 0.07$ gIGFBP-6 vs n-gIGFBP-6). Other organs with substantial radioactivity were kidney and lungs > spleen > heart > liver. The distributions of gIGFBP-6 and n-gIGFBP-6 were similar except that n-gIGFBP-6 appeared to be localised to a greater extent in the lungs (18 ± 5% vs 6 ± 1%, $P = 0.05$). Neither gIGFBP-6 nor n-gIGFBP-6 crossed the blood–brain barrier as demonstrated by the absence of radioactivity in the brain.

### Discussion

In this study, we have shown that O-glycosylation prolongs the redistribution and elimination half-lives of IGFBP-6, which is consistent with the effects of glycosylation of other proteins (16). This effect may be mediated by a number of mechanisms. The mass contributed by glycans increases the circulating half-lives of glycoprotein hormones (17) and the molecular

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>$t_{1/2r}$ (min)</th>
<th>$t_{1/2e}$ (min)</th>
<th>$V_{ss}$ (ml/kg)</th>
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</thead>
<tbody>
<tr>
<td>Glycosylated IGFBP-6 ($n = 5$)</td>
<td>14.4 ± 1.2</td>
<td>584.2 ± 130.2</td>
<td>240.2 ± 30.6</td>
</tr>
<tr>
<td>Non-glycosylated IGFBP-6 ($n = 3$)</td>
<td>6.3 ± 1.5</td>
<td>28.0 ± 4.2</td>
<td>250.6 ± 60.7</td>
</tr>
<tr>
<td>$P$ value (glycosylated vs non-glycosylated)</td>
<td>0.006</td>
<td>0.019</td>
<td>0.88</td>
</tr>
</tbody>
</table>

$t_{1/2r}$, redistribution half-life; $t_{1/2e}$, elimination half-life; $V_{ss}$, volume of distribution.
Although steady-state concentrations of IGFBP-6 are relatively low compared with those of IGFBP-3 (5), its relatively long elimination half-life together with its IGF-II binding preference may result in its having a significant role in terms of IGF-II physiology. The rate of IGF-II synthesis is similar to IGF-I in man, yet serum levels of IGF-II are three to four times higher, due to free IGF-II levels being tenfold lower than those of free IGF-I (6). IGFBP-6 may therefore have a role in maintaining circulating IGF-II levels.

In contrast to the effects of O-glycosylation on the pharmacokinetics of IGFBP-6, N-glycosylation has no effect on the clearance of IGFBP-3 from the circulation (25). This may be because ternary complex formation is the major determinant of IGFBP-3 pharmacokinetics and binding of IGFBP-3:IGF complexes to the acid-labile subunit to form ternary complexes is unaffected by glycosylation (26).

The volumes of distribution of both gIGFBP-6 and n-gIGFBP-6 were similar to those of IGFBP-1 and IGFBP-2 (24), and indicate that IGFBP-6 is distributed throughout the vascular and extracellular compartments. This result is not surprising as binary IGFBP:IGF complexes are not restricted by endothelial barriers. In fact, IGFBP's may have a role in the specific targeting of IGF's to specific tissues (27–29). The organ distribution of $^{125}$I-labelled IGFBP-3 (30) and IGFBP-6 were similar, with both IGFBPs being predominantly localised to the stomach and kidneys. However, it was not determined in either study whether the radioactivity present in the stomach was due to intact tracer or free iodine. High levels of radioactivity in the stomach may be a result of iodine deposition by the gastrointestinal iodide cycle subsequent to deiodination (31).

In summary, most previous studies of IGFBP-6 have emphasised its properties at the cellular level (7). However, IGFBP-6 may also have an important role in the regulation of circulating IGF-II because of its relatively long half-life due to O-linked glycosylation. O-glycosylation may therefore contribute to maintaining IGFBP-6 as a potent, high-affinity, circulating inhibitor of IGF-II actions.

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