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# Insulin-like growth factor binding protein-6 inhibits neuroblastoma cell proliferation and tumour development

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**Abstract**

In neuroblastoma cells, survival and proliferation are dependent upon the insulin-like growth factor (IGF) system. IGFs actively participate in cell growth, whereas IGFBP-6, is associated with the arrest of growth. With a view to blocking IGF-II action, we produced recombinant human IGFBP-6 capable of binding IGFs with affinities between 1.23 and $6.36\times10^9$ $M^{-1}$. *Ex vivo* mitogenic activities were tested on two human neuroblastoma cell lines, in which 100 ng/ml IGFBP-6 completely abolished the effects of both endogenous and exogenous IGF-II. *In vivo*, nude mice previously injected with neuroblastoma cells were submitted to either 15 daily injections of 4–20 μg IGFBP-6 or implantation of mini-pumps diffusing 20–100 μg IGFBP-6 over 2 weeks. The result was an average 18% reduction in the incidence and development of tumours. Delivery of the IGFBP-6 via mini-pumps also delayed tumour appearance by 6–15 days. Our results therefore show the involvement of IGFBP-6 in neuroblastoma cell growth, both *ex vivo* in terms of proliferation and *in vivo* in terms of tumour development.


*Keywords:* IGF system; IGFBP-6; Neuroblastoma; Cell proliferation; Tumour growth

## 1. Introduction

The insulin-like growth factors (IGF-I and IGF-II) are powerful mitogens in numerous cell types, including neuroblastoma cells [1,2]. Neuroblastomas are childhood tumours of varying severity, that are formed by neural crest cells and sometimes regress spontaneously without chemotherapy [3]. The implication of IGFs in cancer development is amply documented [4,5]. Their mitogenic effects are mediated by the type 1 IGF receptor (IGF-IR), a membrane receptor with tyrosine kinase activity [6] which is directly involved in cell transformation and proliferation and protection against apoptosis [7–9]. The close association of the IGF system with neuroblastoma cell proliferation (reviewed in Ref. [10]) is reflected in the ability of the N-*myc* oncogene to increase the expression of IGF-IR [11] and activate its signalling pathway [12]. Another receptor, known as type 2, is identical to the cation-independent mannose-6-phosphate receptor which mediates IGF-II clearance [13].

In biological fluids, IGFs are non-covalently bound to high-affinity binding proteins (IGFBPs), of which six distinct molecular species have been identified [14]. By binding IGFs, these IGFBPs prolong their half-lives and modulate their biological action in metabolic regulation, development, cell growth and tumorigenesis [15,16]. The IGFBPs are also known to have intrinsic intracellular activities that are divorced from IGF-binding, by which they may inhibit cell proliferation or transformation, or induce apoptosis [17,18]. Each of the IGFBPs has individual expression profiles and structural characteristics, hence specific properties [19]. In the case of IGFBP-6, it is a 30-kDa, *O*-glycosylated protein whose affinity for IGF-II is 100-fold that of IGF-IR [20]. It was originally identified in cerebrospinal fluid, but is expressed ubiquitously. Its expression is stimulated by retinoic acid [21], cortisol [22], IGF [23], and vitamin D [24], all of which induce cell differentiation and/or the arrest of proliferation [25].

Under basal conditions, neuroblastoma cells produce IGF-II, IGFBP-2 and small quantities of IGFBP-4

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[23,26,27]. When they cease proliferating, IGFBP-6 expression is strongly stimulated [23,26] and in neuroblastoma cells transfected with a vector promoting constitutive IGFBP-6 expression, proliferation and tumorigenic potency are depressed [28].

Since IGF-II is necessary for neuroblastoma proliferation, our aim in the present study was to block IGF-II action with specificity and high affinity. We therefore produced recombinant (r) IGFBP-6 with strong affinity for IGF-II and tested its effects first on the mitogenic action of IGFs in cultured neuroblastoma cells and, more especially, on the development of xenografts in nude mice previously submitted to subcutaneous (s.c.) injection of neuroblastoma cells.

## 2. Materials and methods

### 2.1. Construction of the IGFBP-6-expression vector

The *IGFBP-6* cDNA encoding the mature protein lacking the signal peptide and amplified by polymerase chain reaction (PCR) using the 5′CGCGGATCCCGGTGCCAGGC3′ (forward) and 5′CGCGAATTCGGTTTGACCCCAAGC3′ (reverse) oligonucleotides was inserted in the pGEX-6P-1 expression vector from Amersham Pharmacia Biotech, following digestion with *BamH1* and *EcoR1*. The orientation of the insert was confirmed by restriction mapping using enzymes cutting the insert once and the vector once. The sequencing was carried out by mwg (Les Ulis, France) to confirm that no errors had been introduced during the PCR step.

### 2.2. Preparation and purification of IGFBP-6

rIGFBP-6 or glutathione-S-transferase (GST) were purified from 500-ml cultures of *Escherichia coli* BL21 bacteria induced by overnight treatment with 0.1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) at an optical density (OD) = 1 to 600 nm. The pellet was resuspended in 10 ml of 10 mM Tris–HCl pH 8, 150 mM NaCl, 1 mM ethylene diamine tetra acetic acid (EDTA) containing a cocktail of protease inhibitors (Boehringer, Mannheim, Germany), then incubated for 15 min at 4 °C in 100 μg/ml lysozyme, 5 mM dithiothreitol (DTT), 1.5% sarkosyl. Thereafter, the bacteria were lysed by sonication until the medium became translucid. The supernatant recovered after centrifugation, was incubated overnight at 4 °C with 5% glutathione Sepharose. Thereafter, the glutathione Sepharose beads were rinsed twice in phosphate-buffered solution (PBS) and either incubated with 10 mM glutathione (Boehringer), 50 mM Tris–HCl pH 8, to elute the GST, or resuspended in 1 ml 50 mM Tris–HCl pH 7, 150 mM NaCl, 1 mM EDTA, 1 mM DTT for separation of IGFBP-6 by incubation with 20 units of Precision Protease (Amersham Pharmacia Biotech) for 4 h at 4 °C.

Proteins were quantified by the Bradford method using the Bio-Rad Protein Assay (BioRad, Hercules, CA, USA).

### 2.3. Competitive binding studies

The techniques used have been described in detail elsewhere in Ref. [29]. Briefly, 1–100 ng rIGFBP-6 were incubated at 4 °C in 0.1 M sodium phosphate buffer pH 7.5, 0.1% bovine serum albumin (BSA) with $^{125}$I-IGF-I or $^{125}$I-IGF-II (2500 counts per minute (cpm)) in a total volume of 0.4 ml/tube in order to determine the amount of IGFBP-6 yielding 25% binding. IGFs provided by Ciba Geigy Ltd (Basel, Switzerland) were iodinated in the laboratory using the chloramine T method. After 18–24 h, free and bound IGFs were separated using albumin-coated charcoal.

The concentration of IGFBP-6 yielding 20–25% binding of labelled IGF was then used in a competitive binding experiment using $^{125}$I-labelled IGFs (2500 cpm per tube) and increasing concentrations of unlabelled IGFs. Conditions of incubation and separation were the same as above. Bound labelled IGF was determined after subtraction of non-specific binding obtained with 400 nM unlabelled IGF, which was 6–8% of the total counts. Equilibrium association constants were calculated from Scatchard analyses.

### 2.4. Cell culture

Cells derived from IGR-N-91 [30] and SK-N-SH [31] neuroblastomas were provided by J. Bénard (Institut Gustav Roussy, Villejuif, France). The cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS) in the presence of 100 IU/ml penicillin, 1 μg/ml amphotericin B and 10 μg/ml gentamicin. Cultures were grown in a humidified incubator at 37 °C, with 5% $CO_2$ atmosphere. At the end of the exponential growth phase, cells were trypsinised using 0.05% trypsin-EDTA (DIFCO, Detroit, MI, USA) and seeded at $2\times10^4$ cells/cm$^2$.

In the experiments to study the mitogenic effects of IGFs, cells were treated simultaneously for 24–72 h with 20 ng/ml IGF-II (Ciba Geigy), or 10 ng/ml des(1–3)IGF-I that does not bind the IGFBPs (Gropep, Adelaide, Australia) or 5 ng/ml basic fibroblast growth factor (bFGF) (Sigma Chemical Company) and 100 ng/ml rIGFBP-6 or 100 ng/ml GST.

### 2.5. [$^4$C]-thymidine uptake

Cells were cultured in 48-well plates and 1 μCi [$^{14}$C]-thymidine added for the final 15 h of culture. They were

then rinsed five times with PBS and lysed using 100 μl 0.6 N NaOH per well for 3–4 h at 37 °C. Liquid scintillation counting was used to determine the amount of radioactivity incorporated into DNA. For each condition, results for IGFBP-6-treated cells were corrected for those obtained with GST-treated cells (controls). Each point is the mean of three independent experiments realised with at least three points.

### 2.6. Analysis of IGFBP-6 by silver staining and western immunoblotting

The conditioned media were desalted on Sephadex G25 columns, lyophilised and analysed by western immunoblotting as previously described in Ref. [32]. Briefly, 1 ml equivalent of each sample, or 10 μl mouse serum, or 100 ng rIGFBP-6 were submitted to 11% sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) under non-reducing conditions.

For silver staining, the gels were fixed twice for 30 min in 10% trichloroacetic acid (TCA), rinsed twice in 50%/MeOH-12% acetate for 30 min, twice for 20 min in water, once for 15 min in 1% glutaraldehyde, twice for 20 min in water, incubated with 0.8% silver for 15 min, water for 15 min, citric acid/formaldehyde for 10 min, then rinsed in 1/100 water/glacial acetic acid for 1 min.

For immunoblotting, the secreted proteins were electrotransferred onto nitrocellulose membranes which were then saturated and incubated at 37 °C for 1 h with anti-human IGFBP-6 polyclonal antibody (Santa Cruz Biotechnology, CA, USA) at a 1/1000 dilution. The membranes were rinsed, then incubated for 45 min with goat polyclonal anti-rabbit immunoglobulin G antibody coupled to horseradish peroxidase (Sigma Chemical Company, St Louis, MO, USA) at a 1/10,000 dilution. Horseradish peroxidase oxidation of luminol yields chemiluminescence, from which the specific IGFBP-antibody complexes can be visualised (enhanced chemiluminescence (ECL) western blotting detection system, Amersham Pharmacia Biotech).

### 2.7. Nude mouse experiments

$10^7$ IGR-N-91 neuroblastoma cells were injected into the flanks of female nude mice aged 6 weeks (SP Swiss nude mice; origin: K. Hansen, NIH, Bethesda, MD, USA). Two to five weeks later, the mice were subjected either to 15 daily intraperitoneal (i.p.) injections of 4–20 μg IGFBP-6 or GST (control) or to implantation for 15 days of osmotic mini-pumps 1002 (Alzet, CA, USA) diffusing 20–100 μg IGFBP-6 or GST (control). The pumps were removed 2–3 weeks later and the mice inspected for xenografts. Implantation and removal of the pumps was carried out under anaesthesia (0.05% ketamine, 0.2% xylasine, 0.3% pentobarbital). The mice were examined 2–3 times a week for up to 3 months after injection of the cells and xenografts measured using callipers according to the National Cancer Institute formula, $V = L(\text{cm}) \times I^2$ (cm)/2, where $L$ is the largest and $I$ the smallest diameter of the tumour.

Authorisation (no. 3300) for the experiments on nude mice was obtained from the Ministére de l'Education National, de la Recherche et Technologie.

## 3. Results

### 3.1. Purification of a functional IGFBP-6 preparation

Via the GST on the N-terminal end, the fusion protein was purified on glutathione-Sepharose beads (Fig. 1a, lane 2) and proteolysis at the site specifically recognised by the Precision Protease released an intact protein of approximately 30 kDa (Fig. 1a, lane 1). A bacterial culture of 500 ml yielded 0.5–3 mg protein recognised by the anti-IGFBP-6 antibody in immunoblotting. Silver staining of the gels demonstrated the lack of contamination of the preparations (Fig. 1a).

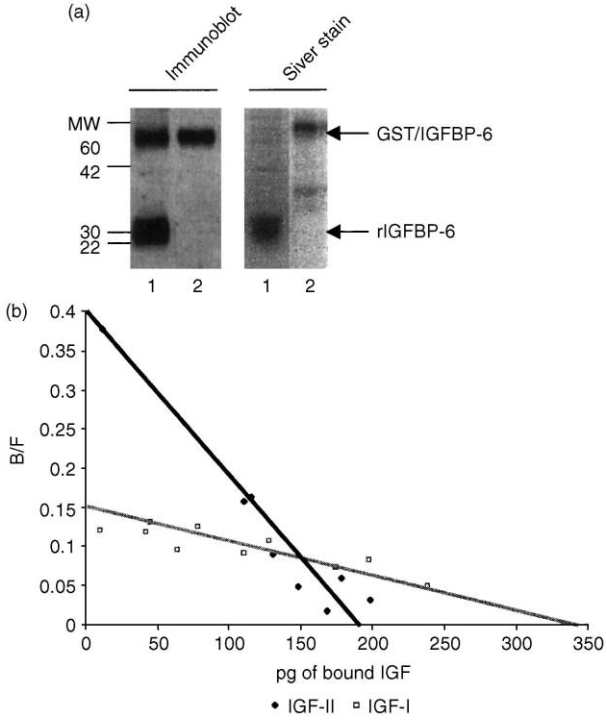


Fig. 1. Qualitative analysis of recombinant insulin-like growth factor binding protein-6 (IGFBP-6). (a) 100 ng rIGFBP-6 (lanes 1) or glutathione-S-transferase (GST)/IGFBP-6 fusion protein (lanes 2) were loaded onto 11% acrylamide gels and immunoblotting carried out using polyclonal antibody that specifically recognises IGFBP-6. All the proteins present in the extracts were revealed by silver staining of the gels. (b) Competitive binding studies were performed using 50 ng rIGFBP-6 and 2500 cpm labelled insulin-like growth factor (IGF). Bound labelled IGF was displaced using increasing quantities of unlabelled IGF. B/F, bound/free.

The affinities of the recombinant protein for IGFs in liquid medium were between $1.23\times10^9$ and $6.36\times10^9$ $M^{-1}$ (Fig. 1b).

### 3.2. *Effects of IGFBP-6 on* ex vivo *growth of neuroblastoma cells*

After 24 h in serum-free medium, the IGR-N-91 and SK-N-SH cells were treated with either 20 ng/ml IGF-II or 10 ng/ml des(1–3)IGF-I that does not bind the IGFBPs, in the presence or absence of 100 ng/ml IGFBP-6 (or 100 ng/ml GST in controls). Cell proliferation was measured by [$^{14}$C]-thymidine incorporation into DNA in three separate experiments where the mitogenic response to IGF-II was reduced by 54±8% in IGR-N-91 cells and by 40±10% in the SK-N-SH cells, whereas des(1–3)IGF-I effect was not inhibited in the presence of IGFBP-6 (Fig. 2a). At 100 ng/ml, IGFBP-6 reduced basal proliferation by 44±5% in IGR-N-91 cells, and 10±2% in the SK-N-SH cells. There was a positive dose–response relationship, 400 ng/ml IGFBP-6 yielding 75% inhibition. Similar results were obtained in simultaneous experiments using 5 ng/ml bFGF to stimulate cell proliferation (Fig. 2b). The above results were obtained for measurements 48 h after the beginning of treatment. Those after 24 and 72 h were similar, but with larger interexperimental variations.

The resistance of rIGFBP-6 to proteolysis was checked between 24 and 72 h and IGFBP-6 found to be intact throughout all experiments (Fig. 3a). Parallel analysis of media conditioned by control (GST-treated) cells for 72 h revealed no IGFBP-6.

### 3.3. *Effects of IGFBP-6 on xenograft development and growth in nude mice*

$10^7$ IGR-N-91 cells were injected s.c. into nude mice (day 0), earlier experiments having shown that these cells are tumorigenic [28,30]. In three series of experiments, osmotic mini-pumps were implanted s.c. in the anaesthetised mice 2 weeks after the injection of the cells. With 20 μg protein in the mini-pumps, 100% of control (GST-treated) and test (IGFBP-6-treated) mice developed tumours, but the time required for tumours to reach the same size was 6–8 days longer in the test animals. With 100 μg protein in the mini-pumps, 10–20% fewer test mice developed tumours. When the pumps were implanted 5 weeks after injection of the cells, at a time when xenografts should become palpable, there were again 20% fewer test mice than controls that developed tumours. Here, the time difference to attain the same size of tumour was 10–15 days. Fig. 4 shows a graphical representation of mean tumour volume over time, showing that once the tumours had developed, their growth was the same in the test and control animals.

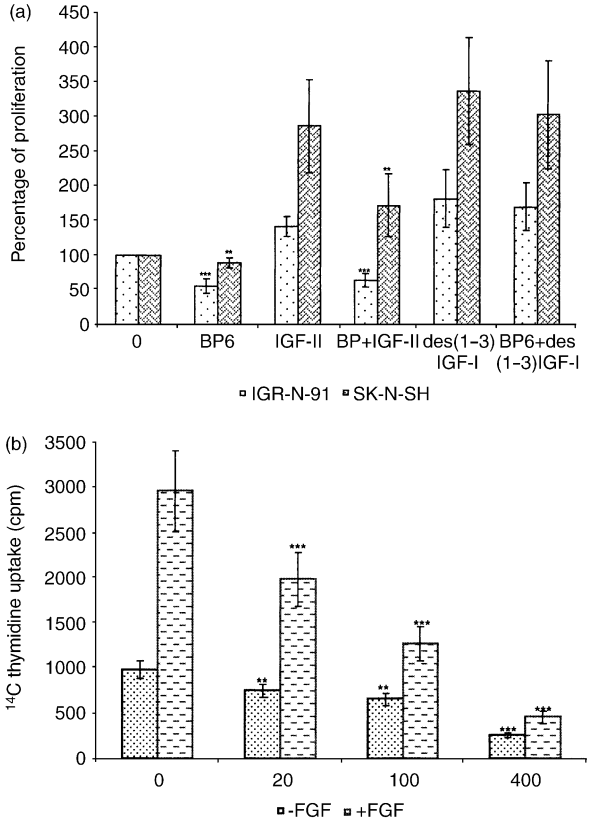


Fig. 2. Effects of insulin-like growth factor binding protein-6 (IGFBP-6) on neuroblastoma cell proliferation. (a) Cells were deprived of serum for 48 h, then treated with 20 ng/ml IGF-II or 10 ng/ml des(1–3)IGF-I in the presence or absence of 100 ng/ml rIGFBP-6 (BP6) (or 100 ng/ml glutathione-S-transferase (GST) in controls). (b) The same type of experiment was run using SK-N-SH cells treated with increasing concentrations of rIGFBP-6 with or without 5 ng/ml bFGF. [$^{14}$C]-thymidine incorporation into DNA was measured using a beta counter and results compared with control conditions (=100%). The results represent the means±standard errors of the mean (S.E.M.) for three series of experiments. The unpaired Student's *t*-test was used for the statistical analysis, $^{**}P<0.05$, $^{***}P<0.001$ for IGFBP-6-treated versus untreated cells.

Three sets of experiments were performed in which the mice received 15 daily i.p. injections of 4–20 μg GST or IGFBP-6, starting from 2 weeks after injection of the cells. No difference was seen between test and control animals in either the timing of appearance or growth of tumours, but 80% of IGFBP-6-treated mice as opposed to 100% of controls developed tumours (Table 1).

The sera of i.p. injected mice were checked for rIGFBP-6 which was present 3 h later (Fig. 3b, lane 2), but not detectable after 24 h (Fig. 3b, lane 3). No IGFBP-6 was found in the sera of control animals.

## 4. Discussion

IGFs, and especially IGF-II, are known to be intimately involved in proliferation in many cell types, as in neuroblastoma-derived cells [33–35], and IGFBPs are

Table 1
Percentages of mice developing tumours

| Delivery method | Intraperitoneal injection | | | | | | Implanted osmotic mini-pump | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Weeks after injection of cells | 2 | | | | | | 2 | | | | | | 5 | |
| Experiment | 1 | | 2 | | 3 | | 1 | | 2 | | 3 | | 4 | |
| Protein dosage (μg) | 4 | | 20 | | 20 | | 20 | | 100 | | 100 | | 100 | |
| Protein | C | BP6 | C | BP6 | C | BP6 | C | BP6 | C | BP6 | C | BP6 | C | BP6 |
| Number of mice with tumours | 10 | 8 | 10 | 8 | 10 | 8 | 10 | 10 | 7 | 6 | 8 | 6 | 8 | 6 |
| Lag time (days) | None | | None | | None | | 6–8 | | 6–8 | | 8–10 | | 10–15 | |

Mice without tumours 3.5 months after injection of IGR-N-91 cells were counted as negative. Mean lag times were determined for tumours to develop to the same size in the insulin-like growth factor binding protein-6 (IGFBP-6)-treated mice and controls. IGFBP-6 (BP6) or glutathione-S-transferase (GST) (C) were delivered either by daily intraperitoneal (i.p.) injection for 15 days, or via s.c. implanted osmotic mini-pumps diffusing the dosage over 15 days.

known to modulate their action [15,16]. Numerous *in vivo* and *in vitro* studies have demonstrated that in certain cancers IGFBP-2 expression and IGF-II expression increase in parallel and the two peptides have been cited as markers for colorectal adenoma [36]. These two components of the IGF system are further linked in that IGF-II induces IGFBP-2 expression at the transcriptional level [37]. It has been proposed that IGFBP-2 is associated with cell proliferation not only via IGF-independent mechanisms, but also directly by binding or releasing IGF-II and promoting its action [38,39]. In neuroblastoma cells, previous studies showed a shift between IGFBP-2 and IGFBP-6 expression during the transition from proliferation to differentiation. In most studies, IGFBP-6 is described as an inhibitor of cell proliferation, blocking the mitogenic effects of (endogenous and exogenous) IGF-II, essentially through

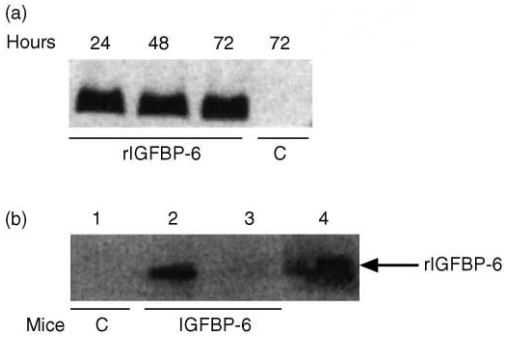


Fig. 3. Immunoblot analysis of rIGFBP-6 resistance to degradation. (a) 1 ml equivalent of medium conditioned by cells treated with 100 ng/ml rIGFBP-6 or glutathione-S-transferase (GST) (C) was submitted to electrophoresis and insulin-like growth factor binding protein-6 (IGFBP-6) revealed by its binding to a specific polyclonal antibody. (b) 10 μl nude mouse serum was submitted to electrophoresis 3 h (lanes 1 and 2) and 24 h (lane 3) after intraperitoneal (i.p.) injection of 20 μg GST (lane 1) or rIGFBP-6 (lanes 2 and 3). In lane 4, 100 ng rIGFBP-6 was loaded to check migration.

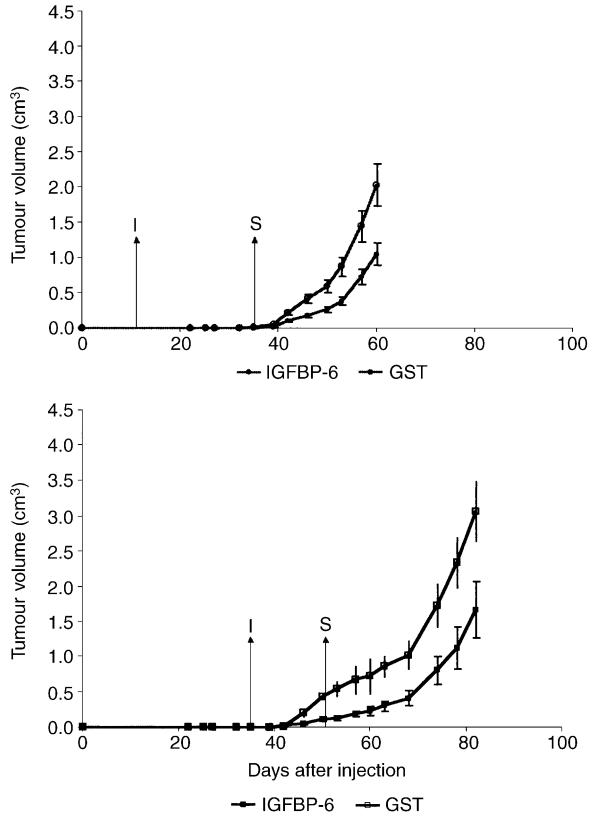


Fig. 4. Xenograft development in nude mice after injection of IGR-N-91 cells. In each set of experiments, $10^7$ IGR-N-91 neuroblastoma cells were injected subcutaneously (s.c.) into 10 nude mice. An osmotic mini-pump diffusing 100 μg rIGFBP-6 (shaded symbols) or glutathione-S-transferase (GST) (open symbols) over 14 days was implanted under the skin near the point of injection. In three sets of experiments, pumps were implanted 2 weeks after injection (=I), then left in place for three weeks (upper graph, dotted lines, round symbols). Insulin-like growth factor binding protein-6 (IGFBP-6) diffusion was stopped by removing the pumps (=S). In one set of experiments, pumps were implanted 5 weeks after injection and left in place for 2 weeks (lower graph, full lines, square symbols). Tumour volume was measured using callipers (see Materials and methods).

sequestration [23,40,41]. We have also shown that in stably transfected IGFBP-6-producing neuroblastoma cells, proliferation and tumorigenicity are reduced [28]. This is consistent with the findings of Sueoka and colleagues that the IGFBP-6 secreted by adenovirus-transfected non-small carcinoma lung cells potently induces programmed cell death and that IGFBP-6 inhibits proliferation in human bronchial epithelial cells [42,43].

In view of these observations, our aim was to prepare recombinant human IGFBP-6 and test the effects of pre-determined amounts of the protein on the growth and tumorigenic potency of neuroblastoma cells. A GST/IGFBP-6 fusion protein was produced in BL21 *E. coli* and purified on glutathione columns via the GST moiety on the N-terminal portion of the fusion protein. The GST was then removed to yield the remaining mature IGFBP-6. Characterisation of this IGFBP-6 showed it migrating at the expected size (around 30 kDa) in immunoblotting and having affinities for the IGFs between approximately 1 and $6\times10^9$ $M^{-1}$, which is of the same order of magnitude as the affinity for IGF-I of IGFBP-6 purified from cerebrospinal fluid or cultured eukaryote cells [20,44]. However, we did not obtain the 100-fold affinity for IGF-II, since our protein was non-glycosylated. It was nevertheless functional *ex vivo*, inhibiting basal proliferation of neuroblastoma cells and blocking the mitogenic action of IGF-II to which the cells clearly responded. It seems highly likely that the inhibition by IGFBP-6 reflects IGF sequestration, because no inhibitory effect was observed when an IGF analogue with no affinity for IGFBPs was used. In addition, the effect on basal proliferation was stronger in IGR-N-91 cells which secrete between 10 and 100 times more IGF-II than SK-N-SH cells (data not shown). The same arguments could be used to explain the inhibitory action of bFGF which could induce IGF-II secretion or could potentiate its effect. Similarly, it has been shown that recombinant IGFBP-6 inhibits the proliferation and growth in soft agar of IGF-II-stimulated and untreated colon cancer cells [45] and blocks the differentiating effect of IGF-II in L6A1 myoblasts [46].

We also investigated the *in vivo* influence of IGFBP-6 on neuroblastoma xenograft development in nude mice submitted to s.c. injection of IGR-N-91 cells. Two protocols were applied 15 days after the injection: either 15 daily i.p. injections of 4–20 μg IGFBP-6 or implantation of osmotic pumps delivering 20–100 μg protein over 15 days. The choice of a 15-day period after injection of neuroblastoma-provoking cells was based on earlier work indicating implication of IGFBP-6 in the initial stages of tumour development [28]. In all experiments where enough protein had been delivered to produce an effect, 10–20% fewer IGFBP-6-treated mice developed tumours than did controls. Therefore, whether exogenous, as in the present study, or endogenous, as in our earlier study [28], IGFBP-6 has the same effect, acting extracellularly and most probably by inhibiting IGF-II. These studies also showed that the development of tumours was delayed by 6–15 days in IGFBP-6-treated mice, depending on the type of experiment. The effect was more marked when mini-pumps were installed 5 weeks after injection of the cells, when xenografts should have become palpable, because of the inhibitory action of IGFBP-6 on cell proliferation by IGF-ll sequestration. However, the delay was not evident in mice injected i.p., possibly because IGFBP-6 is rapidly degraded in the bloodstream. We were unable to detect IGFBP-6 in mouse serum 24 h after injection as the maximal quantity of circulating IGFBP-6 loaded on the gel was 40 ng which is in any case difficult to detect. Moreover, *O*-glycosylation increases resistance to protease attack and decreases clearance 20-fold [47,48]. In contrast, rIGFBP-6 exhibited strong resistance to proteolytic degradation *ex vivo*, since neither its quantity nor its state was altered after 72 h of incubation with neuroblastoma cells. This would suggest that the proteases found in the circulation are not produced by neuroblastoma cells which nevertheless secrete IGFBP-2-degrading proteases [35]. It would also suggest that proteolytic mechanisms may be IGFBP-specific [49].

Our results therefore show that IGFBP-6 inhibits cell proliferation and reduces tumorigenicity by sequestering IGF-II. Since the latter is known to intervene in numerous tumours, it would seem plausible to specifically block its action during chemotherapy by adding r-IGFBP-6 with its particularly strong affinity for IGF-II. Through the use of r-IGFBP-6 in conjunction with other anti-cancer agents, it may also be possible to reduce drug dosages and obviate secondary effects. Interestingly, IGFBP-6 expression has recently been shown to potentiate the effects of etoposide on apoptosis [50]. Another advantage is that IGFBP-6 is documented to be active in a wide variety of cell types, suggesting an absence of tissue specificity. Further investigation will show whether or not IGFBP-6 is more potently inhibitory in cancer cells than in normal cells, in view of its strong dependence on IGF-II.

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## References

1. El-Badry OM, Romanus JA, Helman LJ, Cooper MJ, Rechler MM, Israel MA. Autonomous growth of a human neuroblastoma cell line is mediated by insulin-like growth factor II. *J Clin Invest* 1989, **84**, 829–839.

2. Kurihara S, Hakuno F, Takahashi S. Insulin-like growth factor-I-dependent signal transduction pathways leading to the induction of cell growth and differentiation of human neuroblastoma cell line SH-SY5Y: the roles of MAP kinase pathway and PI 3-kinase pathway. *Endocr J* 2000, **47**, 739–751.
3. Suarez A, Hartmann O, Vassal G, *et al*. Treatment of stage IV-S neuroblastoma: a study of 34 cases treated between 1982 and 1987. *Med Pediatr Oncol* 1991, **19**, 473–477.
4. Sullivan KA, Castle VP, Hanash SM, Feldman EL. Insulin-like growth factor II in the pathogenesis of human neuroblastoma. *Am J Pathol* 1995, **147**, 1790–1798.
5. Toretsky JA, Helman LJ. Involvement of IGF-II in human cancer. *J Endocrinol* 1996, **149**, 367–372.
6. De Meyts P, Wallach B, Christoffersen CT, *et al*. The insulin-like growth factor-I receptor. Structure, ligand-binding mechanism and signal transduction. *Horm Res* 1994, **42**, 152–169.
7. Liu X, Turbyville T, Fritz A, Whitesell L. Inhibition of insulin-like growth factor I receptor expression in neuroblastoma cells induces the regression of established tumors in mice. *Cancer Res* 1998, **58**, 5432–5438.
8. Valentinis B, Baserga R. IGF-I receptor signalling in transformation and differentiation. *Mol Pathol* 2001, **54**, 133–137.
9. Peruzzi F, Prisco M, Morrione A, Valentinis B, Baserga R. Anti-apoptotic signaling of the insulin-like growth factor-I receptor through mitochondrial translocation of c-Raf and Nedd4. *J Biol Chem* 2001, **276**, 25990–25996.
10. Zumkeller W, Schwab M. Insulin-like growth factor system in neuroblastoma tumorigenesis and apoptosis: potential diagnostic and therapeutic perspectives. *Horm Metab Res* 1999, **31**, 138–141.
11. Chambery D, Mohseni-Zadeh S, de Galle B, Babajko S. N-myc regulation of type I insulin-like growth factor receptor in a human neuroblastoma cell line. *Cancer Res* 1999, **59**, 2898–2902.
12. Misawa A, Hosoi H, Arimoto A, *et al*. N-Myc induction stimulated by insulin-like growth factor I through mitogen-activated protein kinase signaling pathway in human neuroblastoma cells. *Cancer Res* 2000, **60**, 64–69.
13. Lau MM, Stewart CE, Liu Z, Bhatt H, Rotwein P, Stewart C. Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality. *Genes Dev* 1994, **24**, 2953–2963.
14. Shimasaki S, Ling N. Identification and molecular characterization of insulin-like growth factor binding proteins (IGFBP-1, -2, -3, -4, -5 and -6). *Prog Growth Factor Res* 1991, **3**, 243–266.
15. Cohick WS, Clemmons DR. The insulin-like growth factors. *Annu Rev Physiol* 1993, **55**, 131–153.
16. Grimberg A, Cohen P. Role of insulin-like growth factors and their binding proteins in growth control and carcinogenesis. *J Cell Physiol* 2000, **183**, 1–9.
17. Liu B, Lee HY, Weinzimer SA, *et al*. Direct functional interactions between insulin-like growth factor-binding protein-3 and retinoid X receptor-alpha regulate transcriptional signaling and apoptosis. *J Biol Chem* 2000, **275**, 33607–33613.
18. Mannhardt B, Weinzimer SA, Wagner M, *et al*. Human papillomavirus type 16 E7 oncoprotein binds and inactivates growth-inhibitoryinsulin-like growth factor binding protein 3. *Mol Cell Biol* 2000, **20**, 6483–6495.
19. van Kleffens M, Groffen CA, Dits NF, *et al*. Generation of antisera to mouse insulin-like growth factor binding proteins (IGFBP)-1 to -6: comparison of IGFBP protein and messenger ribonucleic acid localization in the mouse embryo. *Endocrinology* 1999, **140**, 5944–5952.
20. Roghani M, Segovia B, Whitechurch O, Binoux M. Purification from human cerebrospinal fluid of insulin-like growth factor binding proteins (IGFBPs). Isolation of IGFBP-2, an altered form of IGFBP-3 and a new IGFBP species. *Growth Regul* 1991, **3**, 125–130.
21. Gabbitas B, Canalis E. Retinoic acid stimulates the transcription of insulin-like growth factor binding protein-6 in skeletal cells. *J Cell Physiol* 1996, **169**, 15–22.
22. Gabbitas B, Canalis E. Cortisol enhances the transcription of insulin-like growth factor-binding protein-6 in cultured osteoblasts. *Endocrinology* 1996, **137**, 1687–1692.
23. Babajko S, Leneuve P, Loret C, Binoux M. IGF-binding protein-6 is involved in growth inhibition in SH-SY5Y human neuroblastoma cells: its production is both IGF- and cell density-dependent. *J Endocrinol* 1997, **152**, 221–227.
24. Drivdahl RH, Loop SM, Andress DL, Ostenson RC. IGF-binding proteins in human prostate tumor cells: expression and regulation by 1,25-dihydroxyvitamin D3. *Prostate* 1995, **26**, 72–79.
25. Chambery D, De Galle B, Ehrenborg E, Babajko S. Multi-hormonal regulation of IGFBP-6 expression in human neuroblastoma cells. *Growth Horm IGF Res* 2000, **10**, 349–359.
26. Babajko S, Binoux M. Modulation by retinoic acid of insulin-like growth factor (IGF) and IGF binding protein expression in human SK-N-SH neuroblastoma cells. *Eur J Endocrinol* 1996, **134**, 474–480.
27. Bernardini S, Cianfarani S, Spagnoli A, *et al*. Expression and down-regulation by retinoic acid of IGF binding protein-2 and -4 in medium from human neuroblastoma cells. *J Neuroendocrinol* 1994, **6**, 409–413.
28. Grellier P, De Galle B, Babajko S. Expression of insulin-like growth factor-binding protein 6 complementary DNA alters neuroblastoma cell growth. *Cancer Res* 1998, **58**, 1670–1676.
29. Binoux M, Seurin D, Lassarre C, Gourmelen M. Preferential measurement of insulin-like growth factor (IGF) I-related peptides in serum with the aid of IGF-binding proteins (IGF-BPs) produced by rat liver in culture. Estimation of serum IGFBP levels. *J Clin Endocrinol Metab* 1984, **59**, 453–462.
30. Ferrandis E, Da Silva J, Riou G, Benard J. Coactivation of the MDR1 and MYCN genes in human neuroblastoma cells during the metastatic process in the nude mouse. *Cancer Res* 1994, **54**, 2256–2261.
31. Biedler JL, Helson L, Spengler BA. Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. *Cancer Res* 1973, **33**, 2643–2652.
32. Babajko S, Hardouin S, Segovia B, Groyer A, Binoux M. Expression of insulin-like growth factor binding protein-1 and -2 genes through the perinatal period in the rat. *Endocrinology* 1993, **132**, 2586–2592.
33. El-Badry OM, Helman LJ, Chatten J, Steinberg SM, Evans AE, Israel MA. Insulin-like growth factor II-mediated proliferation of human neuroblastoma. *J Clin Invest* 1991, **87**, 648–657.
34. Singleton J, Randolph A, Feldman EL. Insulin-like growth factor I receptor prevents apoptosis and enhances neuroblastoma tumorigenesis. *Cancer Res* 1966, **56**, 4522–4529.
35. Menouny M, Binoux M, Babajko S. Role of insulin-like growth factor binding protein-2 and its limited proteolysis in neuroblastoma cell proliferation: modulation by transforming growth factor-beta and retinoic acid. *Endocrinology* 1997, **138**, 683–690.
36. Renehan AG, Jones J, Potten CS, Shalet SM, O'Dwyer ST. Elevated serum insulin-like growth factor (IGF)-II and IGF binding protein-2 in patients with colorectal cancer. *Br J Cancer* 2000, **83**, 1344–1350.
37. Elminger MW, Bell M, Schuett BS, Langkamp M, Kutoh E, Ranke MB. Transactivation of the IGFBP-2 promoter in human tumor cell lines. *Mol Cell Endocrinol* 2001, **175**, 211–218.
38. Menouny M, Binoux M, Babajko S. IGFBP-2 expression in a human cell line is associated with increased IGFBP-3 proteolysis, decreased IGFBP-1 expression and increased tumorigenicity. *Int J Cancer* 1998, **77**, 874–879.
39. Hoeflich A, Fettscher O, Lahm H. Overexpression of insulin-like growth factor-binding protein-2 results in increased tumorigenic potential in Y-1 adrenocortical tumor cells. *Cancer Res* 2000, **60**, 834–838.

40. Kato M, Ishizaki A, Hellman U, *et al*. Human keratinocyte cell line produces two autocrine growth inhibitors, transforming growth factor-beta and insulin-like growth factor binding protein-6, in a calcium- and cell density-dependent manner. *J Biol Chem* 1995, **270**, 12373–12379.
41. Schmid C, Schlapfer I, Keller A, Waldvogel M, Froesch ER, Zapf J. Effects of insulin-like growth factor (IGF) binding proteins (BPs) -3 and -6 on DNA synthesis of rat osteoblasts: further evidence for a role of auto-/paracrine IGF I but not IGF II in stimulating osteoblast growth. *Biochem Biophys Res Commun* 1995, **212**, 242–248.
42. Sueoka N, Lee HY, Wiehle S, *et al*. Insulin-like growth factor binding protein-6 activates programmed cell death in non-small cell lung cancer cells. *Oncogene* 2000, **19**, 4432–4436.
43. Sueoka N, Lee HY, Walsh GL, *et al*. Insulin-like growth factor binding protein-6 inhibits the growth of human bronchialepithelial cells and increases in abundance with all-trans-retinoic acid treatment. *Am J Respir Cell Mol Biol* 2000, **23**, 297–303.
44. Bach LA, Hsieh S, Sakano K, Fujiwara H, Perdue JF, Rechler MM. Binding of mutants of human insulin-like growth factor II to insulin-like growth factor binding proteins 1–6. *J Biol Chem* 1993, **268**, 9246–9254.
45. Leng SL, Leeding KS, Whitehead RH, Bach LA. Insulin-like growth factor (IGF)-binding protein-6 inhibits IGF-II-induced but not basalproliferation and adhesion of LIM 1215 colon cancer cells. *Mol Cell Endocrinol* 2001, **174**, 121–127.
46. Bach LA, Hsieh S, Brown AL, Rechler MM. Recombinant human insulin-like growth factor (IGF)-binding protein-6 inhibits IGF-II-induced differentiation of L6A1 myoblasts. *Endocrinology* 1994, **135**, 2168–2176.
47. Bach LA. Insulin-like growth factor binding protein-6: the ''forgotten'' binding protein? *Horm Metab Res* 1999, **31**, 226–234.
48. Marinaro JA, Casley DJ, Bach LA. O-Glycosylation delays the clearance of human IGF-binding protein-6 from the circulation. *Eur J Endocrinol* 2000, **142**, 512–516.
49. Shalamanova L, Kubler B, Scharf JG, Braulke T. MDCK cells secrete neutral proteases cleaving insulin-like growth factor-binding protein-2 to –6. *Am J Physiol Endocrinol Metab* 2001, **281**, E1221–E1229.
50. Drivdahl RH, Sprenger C, Trimm K, Plymate SR. Inhibition of growth and increased expression of insulin-like growth factor-binding protein-3 (IGFBP-3) and -6 in prostate cancer cells stably transfected with antisense IGFBP-4 complementary deoxyribonucleic acid. *Endocrinology* 2001, **142**, 1990–1998.