The IGF system in neuroblastoma xenografts: focus on IGF-binding protein-6

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Abstract

With a view to investigating the implication of IGF-binding protein-6 (IGFBP-6) in the growth of neuroblastomas, nude mice were injected with IGFBP-6-expressing or control IGR-N-91 human neuroblastoma cells and the resulting xenografts examined.

Expression of IGFBP-3, IGFBP-4 and type 1 and type 2 IGF receptor messengers was similar in control tumours and equal-sized IGFBP-6-expressing tumours that had developed. IGF-II was more strongly expressed in control tumours, and IGFBP-6-expressing tumours contained less IGFBP-2 than controls. In both populations, there was a significant positive correlation between IGF-II and IGFBP-2 expression. In small IGFBP-6-expressing xenografts where tumour development had apparently been arrested, haematoxylin-eosin and TUNEL staining revealed numerous apoptotic cells. *In situ* hybridization indicated homogeneous distribution of the IGFBP-6 signal in test tumours.

In cell culture, IGFBP-6-expressing cells expressed similar amounts of IGFBP-2, IGF-II and N-myc mRNAs as control cells; but media conditioned by IGFBP-6-expressing cells contained less intact IGFBP-2 protein, with no increase in its proteolytic fragment. In media treated with plasminogen, in which IGFBP-2 was proteolysed, IGFBP-6 was increased.

With its especially strong affinity for IGF-II and its resistance to proteolysis, IGFBP-6 would act by sequestering IGF-II, hence inhibiting its mitogenic and anti-apoptotic effects. In excess, IGFBP-6 would displace IGF-II from IGFBP-2 whose potentiation of IGF-II action would cease and whose susceptibility to degradation would be increased. This study therefore shows that IGFBP-6 plays a role in neuroblastoma cell growth *in vivo* and *in vitro* and that stable overexpression of IGFBP-6 leads to alteration of the initial balance between the IGFBPs.

Journal of Endocrinology (2002) **172**, 467–476

Introduction

The insulin-like growth factors, IGF-I and IGF-II, are intricately involved in the proliferation and differentiation of a large number of cell types, and particularly neuroblastoma cells (El-Badry *et al.* 1991, Stewart & Rotwein 1996). Their mitogenic effects are transmitted via a type 1 membrane receptor that has tyrosine kinase activity (De Meyts *et al.* 1994, LeRoith *et al.* 1995). Recent studies have underlined the importance of this system in protection against apoptosis (Werner & LeRoith 1996, Butt *et al.* 1999). In biological fluids, the IGFs are non-covalently bound to high-affinity binding proteins, the IGFBPs, six molecular species of which have been characterized to date (Shimasaki & Ling 1991). Proteins with some homology with the N-terminal domain of IGFBPs have been identified, but the affinities of these proteins are 10- to 100-fold weaker than those of the six IGFBPs. These had been classified as IGFBP-related proteins (Baxter *et al.*

1998), but their functions have proved to be different from those of the IGFBPs and their original names have been re-instituted. IGFBPs are synthesized ubiquitously, although some exhibit tissue specificity. Apart from acting as IGF carrier proteins in the bloodstream, they modulate the half-lives and cellular bioavailability of the IGFs and may either potentiate or inhibit the mitogenic effects of their ligands (Jones & Clemmons 1995). Some have also proved to possess intrinsic activities that are divorced from their ability to bind IGFs (Villaudy *et al.* 1991, Oh *et al.* 1993, Baxter 2001).

Most human neuroblastoma-derived cell lines produce large amounts of IGF-II and IGFBP-2, smaller quantities of IGFBP-4 and sometimes traces of IGFBP-6 (Bernardini *et al.* 1994, Babajko & Binoux 1996, Cianfarani *et al.* 1996). We previously demonstrated that IGFBP-6 is associated with the arrest of proliferation in SH-SY5Y cells derived from human neuroblastomas (Babajko & Binoux 1996, Babajko *et al.* 1997) and

similar effects have been noted in colon cancer cells, keratinocytes and myoblasts (Ewton & Florini 1995, Kato *et al.* 1995, Leng *et al.* 2001). IGFBP-6 has strong affinity for IGF-II, 10 to 100 times stronger than that for the type 1 IGF receptor, which means that IGFBP-6 can sequester bioavailable IGF-II and hence inhibit proliferation. In addition, its expression is blocked by the *N-myc* oncogene, which induces proliferation in neuroblastoma cells (Chambéry *et al.* 1999).

With a view to pinpointing the role of IGFBP-6 in the arrest of cell proliferation, human IGR-N-91 neuroblastoma cells were stably transfected with a plasmid vector coding for human IGFBP-6 (hIGFBP-6), whose expression was placed under the control of the constitutive cytomegalovirus (CMV) promoter (Grellier *et al.* 1998). Injection of hIGFBP-6-expressing cells into nude mice resulted in a lower incidence of xenografts, which also exhibited slower growth than those obtained using control cells (Grellier *et al.* 1998). In the present study, expression of the different components of the IGF system and of the *N-myc* oncogene was analysed in conjunction with the extent of apoptosis in an attempt to understand the mechanisms inhibiting tumour development in the presence of hIGFBP-6.

Materials and Methods

Animals and xenografts

Ten million transfected IGR-N-91 cells were injected into the flanks of 6- to 8-week-old female nude mice (Iffa Credo, Lyon, France). Four series of experiments were carried out, ten mice being injected in each case. Xenografts obtained following s.c. injection of hIGFBP-6-expressing or non-hIGFBP-6-expressing cells were measured twice a week and animals killed either when the tumours became too large ($>10\text{ mm}^3$) or 3–5 months after injection if no tumours had developed. Tumours were divided into two parts; one was frozen and stored at -80°C and the other was fixed in formol buffer for histological examination. Authorization (No. 3300) for experiments on nude mice was obtained from the Ministère de l'Éducation Nationale, de la Recherche et Technologie.

Histological examination and identification of apoptosis in xenograft slices

Apoptotic cells were identified by dichromatic haematoxylin–eosin staining and by the TUNEL technique in previously paraffined $4\text{ }\mu\text{m}$ thick tumour tissue slices. The slices were deparaffined by successive baths in toluene and 100% alcohol, then rinsed in distilled water. Thereafter they were digested for 20 min at room temperature in a 0.05% pepsin solution (Sigma Chemical

Company, St Louis, MO, USA) in 0.1 M HCl, pH 2, rinsed in distilled water, then pre-incubated for 10 min at room temperature in 0.1 M sodium cacodylate–1 mM cobalt chloride (Sigma) buffer, pH 7.2. Elongation of the DNA fragments was achieved in 20 μl of the same buffer using 2 mM Bio-11-dUTP (Sigma) and 0.5 U TdT (Amersham International, Aylesbury, Bucks, UK) containing 0.5 U terminal transferase for 75 min at 37°C in a humid incubator.

The reaction was stopped using $2\times\text{SSC}$ for 10 min and after washing in 10 mM Tris–HCl, pH 7.6, biotin was detected by incubation of the slices with alkaline phosphatase-labelled streptavidin (Boehringer, Mannheim, Germany), diluted 1/1000 in 10 mM Tris–HCl, pH 7.6, 1% BSA for 30 min at room temperature. The slices were washed twice for 3 min in Tris buffer, pH 7.6, then once for 10 min in Tris buffer, pH 8.2, and finally incubated for 60 min in revelation fluid comprising naphthol AS-MX phosphate (Sigma), 10 mg/ml dimethylformamide (Sigma) diluted 1/50 in 10 mM Tris–HCl, pH 8.2, and 1.3 mM levamisole. After washing in running water, the slices were stained with haematoxylin and mounted in glycerol.

In situ hybridization in xenograft slices

Slices of $5\text{ }\mu\text{m}$ were prepared from frozen tissue using a cryostat, placed on superfrost/plus slabs (Menzel Glaser, CML, Nemours, France) and fixed in acetone for 10 min at room temperature, then in 4% paraformaldehyde, $1\times\text{PBS}$ for 20 min at 4°C . The slices were acetylated for 5 min at 4°C in 0.1 M tri-ethanolamine/0.25% acetic anhydride, then hybridized with 10^6 c.p.m. cDNA probe in 30 μl 0.3 M NaCl, 20 mM Tris, pH 7.5, 5 mM EDTA, pH 8, 10 mM phosphate buffer, pH 8, $2\times\text{Denhardt}$, 10 mM dithiothreitol (DTT), 0.5 mg/ml yeast tRNA, 10% dextran sulphate, 40% formamide for 15–16 h at 50°C in a humid incubator.

The hIGFBP-6 cDNA probe was prepared by subcloning the vector, pGEM3zf(+), with a 231 bp fragment of hIGFBP-6 cDNA corresponding to the central domain of the protein, which is the least conserved among the IGFBPs. Following linearization, a ^{35}S -labelled single-strand RNA probe was synthesized by *in vitro* transcription in antisense orientation using T7 RNA polymerase and in sense orientation (background) using SP6 RNA polymerase (Promega Corp., Madison, WI, USA), in the presence of 10 mM DTT, 0.5 mM each of ATP, GTP and CTP, 12 μM UTP, 1 unit/ μl human placental RNasin and 100 μCi ^{35}S -UTP (Amersham).

The slices were washed for 30 min at 42°C in $5\times\text{SCC}$, 1 mM DTT, then for 20 min at 62°C in $2\times\text{SCC}$, 50% formamide, 1 mM DTT, then twice for 30 min at 37°C in 0.4 M NaCl, 5 mM EDTA, pH 8, 10 mM Tris, pH 7.5, twice for 30 min in the same solution with 30–50 $\mu\text{g/ml}$ RNase and twice for 15 min

in $2 \times$ and $0.1 \times$ SCC. Following emulsion coating (Hypercoat emulsion, Amersham) for 3 weeks at 4°C and staining with Mayer's haematoxylin (Merck, Darmstadt, Germany), the slices were autoradiographed.

Cell culture

hIGFBP-6-transfected cell lines were grown in DMEM (Life Technologies, Inc., Paisley, Strathclyde, UK) supplemented with 10% heat-inactivated fetal calf serum, antibiotics and 300 µg/ml selecting agent, G418 (Grellier *et al.* 1998). At confluence, the cells were re-seeded in 10 cm diameter Petri dishes and cultured for 24 h in serum-free medium. The serum-free medium was renewed and the cultures treated with 20 and 120 ng/ml IGF-II or 20 ng/ml des(1–3)IGF-I. In a parallel series of experiments, the same cultures were treated for 48 h with 0.075 mM of the serine protease inhibitor, Pefabloc-SC (4-(2-aminoethyl)-benzene-sulphonyl fluoride) (Chromogenix, Mölndal, Sweden), and with or without 5 µg/ml plasminogen for the last 12 h of culture.

Northern blot analysis of RNA

Total RNA was obtained from cells in culture or frozen tissue using the standard caesium chloride or guanidine isothiocyanate methods (Maniatis *et al.* 1982). Thirty micrograms of total RNA were submitted to 1.2% agarose gel/2.2 M formaldehyde electrophoresis, then transferred to Hybond-C nylon membranes (Amersham). RNA was hybridized for 24 h with 3×10^6 c.p.m./ml human cDNA probe (Multiprime DNA Labelling System; Amersham) as previously described (Grellier *et al.* 1998) and autoradiographed at -80°C . Each membrane was successively hybridized with several probes following de-hybridization by three 30 min washes in 10 mM Tris-HCl, pH 8, 5 mM EDTA, 0.5% SDS at 80°C .

Western immunoblot analysis of IGFBPs

Proteins were extracted from 200–300 mg frozen tissue samples which were rapidly homogenized using a polytron at 4°C in 4 ml 1 M acetic acid and protease inhibitors: 1 mM EDTA, pH 8, 1 mM phenylmethylsulphonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml aprotinin, 1 mM benzamide. The homogenate was maintained at 4°C for 1 h, then centrifuged at 10 000 g for 15 min. The supernatant containing the IGFBPs was recovered and proteins measured by the Bradford method (Bio-Rad, München, Germany). Two hundred microgram samples of protein extract were lyophilized, then taken up in 100 mM phosphate buffer, pH 7.5. In the case of culture media conditioned by IGR-N-91 cells, the media were desalted on Sephadex G25 columns, lyophilized and taken up in the same buffer.

Two hundred micrograms of protein extract or the equivalent of 500 µl conditioned medium were submitted to 11% SDS-PAGE under non-reducing conditions, then electrotransferred to nitrocellulose membranes (Hybond-N), saturated and incubated at 37°C for 1 h with polyclonal antibodies: anti-IGFBP-6 (Austral Biologicals, Tübingen, Germany), anti-IGFBP-2 (UBI, Chicago, IL, USA), anti-IGFBP-3 (prepared in our laboratory by rabbit immunization using recombinant hIGFBP-3 from Celtrix Pharmaceuticals, Santa Clara, CA, USA), diluted to 1/300, 1/1000 and 1/1000 respectively. After rinsing, the membranes were incubated for 45 min with goat polyclonal anti-rabbit IgG antibody coupled to horseradish peroxidase (HRP) (Sigma) at 1/5000 dilution. HRP oxidation of luminol (ECL Western Blotting Detection System; Amersham) yields chemiluminescence from which specific IGFBP-antibody complexes can be visualized.

All Western and Northern blot data shown are representative of at least three separate experiments.

Results

With a view to investigating the implication of IGFBP-6 in the growth of neuroblastomas, nude mice were injected with 10^7 hIGFBP-6-expressing or non-hIGFBP-6-expressing (control) IGR-N-91 human neuroblastoma cells and the resulting xenografts examined. Four separate series of experiments were carried out. Whereas all mice injected with control cells developed tumours, only 50–70% of those injected with hIGFBP-6-expressing cells did so. These tumours were tested for expression of IGF system components (Grellier *et al.* 1998).

Apoptosis in the xenografts

Histologically, the hIGFBP-6-expressing and control xenografts were similar, almost exclusively comprising poorly differentiated or undifferentiated neuroblasts. In hIGFBP-6-expressing tumours that had developed and reached sizes comparable with control tumours, some apoptotic cells were observed, but there was no significant difference between the two types of xenograft. In contrast, in two of three hIGFBP-6-expressing tumours of small volume (below 4 mm^3), where tumour development appeared to have been spontaneously arrested after 15 days, neuroblasts were present (indicating initiation of tumour development), but there were also large areas of apoptotic cells (Fig. 1).

Expression of mRNAs of the components of the IGF system and of the N-myc oncogene in xenografts

Figure 2 shows typical results obtained after Northern blot analysis of total RNA extracted from control

Figure 1 Histological analysis of small-sized ($<4\text{ mm}^3$) xenografts provoked by injection of hIGFBP-6-expressing IGR-N-91 cells into nude mice, where tumour development appeared to have been spontaneously arrested. Cells were identified by haematoxylin–eosin staining (A) ($\times 1000$) and (B) ($\times 2000$)) and the TUNEL technique (C) ($\times 160$ and $\times 400$).

Figure 2 Northern blot analysis of IGFBP-2, -3, -6, IGF-II and N-myc mRNAs in control (C) and hIGFBP-6-expressing xenografts obtained in four series of experiments (E1–E4). RNAs extracted from IGR-N-91 cells were used as reference. Twenty micrograms of total RNA were subjected to electrophoresis, transferred to nitrocellulose membranes and hybridized successively with the different probes.

and hIGFBP-6-expressing xenografts in four sets of independent experiments (E1–E4). The 1 kb mRNA encoding hIGFBP-6 was expressed in all xenografts obtained following injection of IGR-N-91 cells transfected with the pRc/CMV+hIGFBP-6 vector, but in none of the controls. These results were confirmed by *in situ* hybridization (Fig. 3). The distribution of the signal was homogeneous, indicating no spatial specificity of hIGFBP-6 expression, but its intensity varied from one cell to another and from one experiment to another. The signal was stronger in Experiments 2 and 4 where the arrest of growth appeared the most marked (Grellier *et al.* 1998). However, there was no correlation between signal intensity and tumour size.

All the xenografts expressed large quantities of IGFBP-3 mRNA probably produced by fibroblasts and/or endothelial cells, since neuroblastoma cells fail to produce IGFBP-3 *ex vivo*. It is also possible that expression patterns become altered *in vivo* and that IGFBP-3 production is induced in neuroblastoma tumours. IGFBP-4 and type 2 IGF receptor messengers were difficult to detect, in view of the low levels of expression. Type 1 IGF receptor mRNA could be detected only by RNase protection (data not shown).

In general, no significant correlation could be found between expression of the components of the IGF system and either the size of the tumour or its expression of hIGFBP-6. In comparisons between control and hIGFBP-6-expressing xenografts, only IGF-II was more strongly expressed in control tumours. The highest levels of IGF-II expression were found in three control tumours, and the lowest in an hIGFBP-6-expressing tumour. As regards IGFBP-2, there was a significant positive correlation between IGF-II and IGFBP-2 expression in both populations of xenografts (Fig. 4) and IGFBP-2 expression often appeared weaker in hIGFBP-6-expressing than in control tumours.

Characteristically, IGR-N-91 cells possess an amplified N-myc oncogene, often associated with increased expression (Ferrandis *et al.* 1994). N-myc was expressed *in vitro* in all the xenografts, without any significant correlation between its expression and tumour size or hIGFBP-6 expression (Fig. 2).

Western blot analysis of tissue IGFBPs

Immunoblot analysis for hIGFBP-6 in tumour protein extracts revealed a 31 kDa protein with an electrophoretic profile identical to that of IGFBP-6 in media conditioned by IGR-N-91 cells, which constitutively produce small amounts of IGFBP-6. There were larger amounts of hIGFBP-6 in extracts from Experiments 2 and 4 than in those from Experiments 1 and 3 (not shown).

IGFBP-2 was present in all xenograft extracts and appeared to be the predominant IGFBP in Western ligand blotting (not shown). In each series of immunoblotting experiments, the quantities of hIGFBP-2 were smaller in hIGFBP-6-expressing tumours than in controls (Fig. 5). These smaller quantities did not reflect enhanced proteolysis, since the 16 kDa proteolytic fragment of hIGFBP-2 was equally reduced.

In vitro expression of IGFBP-2 and -6, IGF-II and N-myc in hIGFBP-6-expressing and control IGR-N-91 cells

To examine the relationships seen *in vivo* between IGF-II and IGFBP-2, IGF-II and des(1–3)IGF-I were used to treat the three types of IGR-N-91 cell: untransfected wild-type cells; control cells transfected with vector alone; and cells transfected with the hIGFBP-6-encoding sequence. Expression of hIGFBP-6 had no effect on the amounts of IGFBP-2, IGF-II or N-myc mRNA produced (Fig. 6). Similarly and independently of hIGFBP-6 expression, neither IGF-II nor des(1–3)IGF-I treatment affected the amounts of IGFBP-2, IGF-II or N-myc mRNA expressed (Fig. 6).

To investigate the relationships between IGFBP-2 and IGFBP-6, the same cells were submitted to treatment with plasminogen, the precursor of plasmin, which induces IGFBP-2 proteolysis, and/or with Pefabloc, a serine

Figure 3 *In situ* hybridization of IGFBP-6 mRNA in xenografts obtained using hIGFBP-6-expressing (A and B) or control (C) IGR-N-91 cells. (D) Hybridization with the IGFBP-6 probe in sense orientation. Frozen slices of 5 μ m were incubated with RNA probes synthesized in the presence of 35 S-UTP.

protease inhibitor which blocks proteolysis of endogenous IGFBP-2 (Menouny *et al.* 1997). Plasminogen was activated into plasmin in the cell-conditioned media, stimulating IGFBP-2 proteolysis, Pefabloc inhibited it and co-treatment resulted in intermediary proteolysis. The amounts of IGFBP-2 were smaller in media conditioned by hIGFBP-6-expressing cells, although there was no change in proteolytic profile. Conversely, the amounts of hIGFBP-6 were larger in media conditioned in the presence of plasminogen, where intact IGFBP-2 was reduced owing to proteolysis (Fig. 7).

Discussion

This study shows that IGFBP-6 plays a role in neuroblastoma cell growth *in vivo* and *in vitro*. IGR-N-91 cells

constitutively expressing hIGFBP-6 acquired a differentiated phenotype, exhibited reduced sensitivity to IGFs and serum, and, following s.c. injection into nude mice, developed less frequently and more slowly into tumours (Grellier *et al.* 1998).

An important point in considering comparisons of hIGFBP-6-expressing and non-hIGFBP-6-expressing xenografts is that only hIGFBP-6-expressing xenografts that had developed into tumours could be examined, corresponding to 50–70% of the number of control tumours (Grellier *et al.* 1998). The phenotype of tumours that did not grow would probably have been more pronounced. The incidence and timing of development of hIGFBP-6-expressing tumours were lower and slower in Experiments 2 and 4 where the quantities of hIGFBP-6 and its expression were the most marked. It seems possible that a certain local concentration of IGFBP-6 is required

Figure 4 Correlation between the quantities of IGF-II and IGFBP-2 (BP-2) mRNA identified by Northern blotting in control and hIGFBP-6-expressing xenografts.

very early to prevent or check tumour development, in the absence of which xenograft development was the same as in control tumours. In order to confirm this notion, tests were done on particularly small tumours with volumes below 4 mm³, in which growth appeared to have been prematurely arrested. Neuroblasts were present, indicating initiation of tumour development, but unlike the larger tumours, these small tumours had wide expanses of apoptotic cells. Apart from their mitogenic action, IGFs are now known to have a protective, anti-apoptotic action in a variety of cell models (Sell *et al.* 1993, Zumkeller & Schwab 1999). With its especially strong affinity for

IGF-II and its resistance to proteolysis, IGFBP-6 could conceivably act indirectly by reducing the bioavailability and hence the biological action of IGF-II. This diminished anti-apoptotic effect of IGF-II once sequestered by excess IGFBP-6 may represent one of the mechanisms inhibiting or retarding tumour development. This hypothesis would be consistent with the results of Leng *et al.* (2001), who showed that in colon carcinoma cells, IGFBP-6 inhibits IGF-II-induced proliferation and colony formation in soft agar. Our results also agree with those of Damon *et al.* (1998), who performed similar analyses of IGFBP-4 overexpression in prostate cancer cell growth. Stable overexpression of IGFBP-4 reduces cell sensitivity to the mitogenic action of IGFs and increases the percentage of apoptotic cells following hydroxyurea treatment. The incidence of tumour development following injection of these cells to nude mice is half that following injection with control cells and tumour development is delayed (Damon *et al.* 1998). These results strongly resemble our observations for hIGFBP-6-expressing xenografts. Acquisition of resistance to the inhibitory effects of IGFBPs on cell proliferation may explain the similar growth of IGFBP-6- and IGFBP-4-expressing tumours and controls, as has been suggested in the case of breast cancer cells transfected with IGFBP-3 cDNA (Firth *et al.* 1998). In this model, inhibition of cell proliferation and serum-induced DNA synthesis disappear after several passages, despite the fact that the concentrations of intact IGFBP-3 in conditioned media remain high. Possible mechanisms in this resistance may be a parallel increase in IGF-I or IGF-II concentrations in the conditioned media, such as that seen in C6 rat glioma cells stably overexpressing IGFBP-2 (Bradshaw *et al.* 1999), or constitutive activation of a signalling pathway leading to cell proliferation (Firth *et al.* 1998).

Figure 5 Western immunoblot analysis of IGFBP-2 secreted by hIGFBP-6-expressing (E1–E4) and control (C) xenografts. Fifty microliters of cerebrospinal fluid (CSF) or 200 µg xenograft protein extract obtained in the four series of experiments (E1–E4) were submitted to 11% SDS-PAGE, transferred to nitrocellulose membranes and revealed using a specific anti-IGFBP-2 polyclonal antibody.

Figure 6 Northern blot analysis of IGFBP-2, IGFBP-6 and N-myc oncogene mRNAs expressed by wild-type (NT: not transfected), mock transfected (CMV) or hIGFBP-6-expressing (BP-6) IGR-N-91 cells treated with 20 or 100 ng/ml IGF-II or 20 ng/ml des(1–3)IGF-I for 24 h.

Analysis of N-myc oncogene expression in xenografts and cells revealed no regulation by either IGF-II or IGFBP-6. These findings are at odds with the recently reported IGF-I-stimulated N-myc expression in an N-myc-amplified neuroblastoma cell line, KP-N-RT, implicating the MAP kinase signalling pathway (Misawa *et al.* 2000). The discrepancy could arise from the differences between the cell models used (compared with IGR-N-91 cells, KP-N-RT cells express very little N-myc in the basal state) and the observation times selected (4 h maximum in the KP-N-RT cell study, 24 h in ours). Conversely, Chambéry *et al.* (1999) found that N-myc expression is accompanied by increased IGF-II, IGFBP-2 and type 1 IGF receptor expression and reduced IGFBP-6 expression. The amplification and overexpression of N-myc in IGR-N-91 cells may at least in part account for the high levels of IGF-II and IGFBP-2 synthesis. The lack of IGFBP-6 expression in this cell line may explain its greater tumorigenic potency than other neuroblastoma-derived lines. For instance, we have never succeeded in obtaining xenografts after injection of SK-N-SH human neuroblastoma cells, which constitutively express IGFBP-6.

There was no correlation between tumour size and the quantities of IGF-II mRNA expressed. These results corroborate those of Sullivan *et al.* (1995) who analysed the presence of IGF-II and its mRNA in 56 childhood neuroblastomas and found no relationship between IGF-II expression and tumour stage, histological appearance or N-myc amplification despite the role of IGF-II in tumour development (Christofori *et al.* 1994, Rogler *et al.* 1994).

Nevertheless, interpretation of our results must be tempered by the fact that only tumours that had developed could be examined. From this it can be inferred that the cells injected into nude mice that did not produce tumours (50–60%) had markedly reduced IGF-II expression. This would be supported by the fact that the strongest IGF-II expression was found in control tumours. In addition, among all xenografts investigated, whether hIGFBP-6-expressing or not, there was a positive correlation between IGF-II and IGFBP-2 expression. IGFBP-2 is frequently co-expressed with IGF-II, its expression is associated with cell proliferation and its serum concentrations rise in a variety of malignancies (Reeve *et al.* 1992, Müller *et al.* 1994, Flyvbjerg *et al.* 1997, Fuller *et al.* 1999). The mechanism by which IGFBP-2 is involved in cell proliferation may depend on the IGF system, as suggested by our earlier observation that in the presence of IGFBP-2, IGFBP-3 proteolysis is enhanced, thus increasing IGF bioavailability (Menouny *et al.* 1998). It is also possible that IGFBP-2 possesses intrinsic activity that is independent of IGF binding, as evoked by Hoefflich *et al.* (2000) in an adrenocortical cell model. Our culture media conditioned by hIGFBP-6-expressing cells had little intact IGFBP-2. Damon *et al.* (1998) observed the same phenomenon in prostate carcinoma cells overexpressing IGFBP-4. IGFBP-2 protein was reduced in hIGFBP-6-expressing xenografts and *in vitro* analyses of the proteolytic profile of IGFBP-2 confirmed the reduction in both intact IGFBP-2 and its proteolytic fragment in media conditioned by hIGFBP-6-expressing IGR-N-91 cells. Similarly, in rat glioma cells stably overexpressing IGFBP-2, the quantities

Figure 7 Western immunoblot analysis of IGFBP-2 and IGFBP-6 in culture media conditioned by wild-type (NT: not transfected), mock transfected (CMV) or hIGFBP-6-expressing (BP-6) IGR-N-91 cells treated with 5 µg/ml plasminogen (for 12 h) and/or 0.075 mM Pefabloc (48 h). Five microlitres of adult human serum (AS), 50 µl cerebrospinal fluid (CSF) or 500 µl conditioned medium were submitted to electrophoresis, the proteins transferred to nitrocellulose membranes and revealed using specific anti-IGFBP-2 and anti-IGFBP-6 polyclonal antibodies.

of IGFBP-3 and IGFBP-4 are decreased, without any modification of the proteolytic profile (Bradshaw *et al.* 1999). Also, a recent study has demonstrated an increase in IGFBP-3 and IGFBP-6 expression when IGFBP-4 is reduced in prostate cancer cells (Drivdahl *et al.* 2001). When our IGR-N-91 cells were treated with plasminogen (which, once converted into plasmin, provokes IGFBP-2 proteolysis and thus increases IGF-II bioavailability), not

only did hIGFBP-6 not decrease, owing to its resistance to plasmin, but rather it increased, possibly because of an increase in cell number (as observed in earlier experiments (Menouny *et al.* 1997)). An equilibrium therefore appears to exist between the different IGFBPs in the cellular environment that is not necessarily dependent upon limited proteolysis. Because of its preferential affinity for IGF-II and its resistance to proteolysis, IGFBP-6 in excess would displace IGF-II from IGFBP-2, which would no longer potentiate the mitogenic action of IGF-II. Moreover, IGFBP-2 would no longer be protected from proteolysis by its binding to IGF-II and would be more readily degraded. In the same way, when exposed to plasminogen, IGFBP-2 is proteolysed and releases IGF-II, which would then more readily bind to IGFBP-6. Therefore, stable overexpression of IGFBP-6 leads to sequestration of IGF-II, altering the initial balance between the IGFBPs. Intact IGFBP-2 becomes diminished, as does its ability to potentiate the action of IGF-II (with whose expression its own is linked) or, as is suspected in several laboratories, its intrinsic ability to stimulate proliferation by mechanisms unrelated to IGF binding.

Acknowledgements

We are grateful to Regine Paris for her technical assistance in the evaluation of apoptosis in xenograft slices. This work was supported by INSERM and the Association de la Recherche sur le Cancer. P G was the recipient of a grant from the Ligue contre le Cancer.

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Received 30 October 2001
Accepted 5 November 2001