Modulation by retinoic acid of insulin-like growth factor (IGF) and IGF binding protein expression in human SK-N-SH neuroblastoma cells

Sylvie Babajko and Michel Binoux

Institut National de la Santé et de la Recherche Médicale, Unité de Recherches sur la Régulation de la Croissance, Hôpital Saint Antoine, Paris, France


Growth in neuroblastoma cells is regulated by insulin-like growth factors (IGFs) whose action is modulated by IGF binding proteins (IGFBPs). In this study, SK-N-SH neuroblastoma cells were shown to produce IGF-II, IGFBP-2, IGFBP-4 and small quantities of IGFBP-6. We have studied the effects of a natural morphogen, retinoic acid (RA), on growth and IGFBP expression in these cells. In all experiments, cells were cultured in serum-free medium and treated with 1 µmol/l RA for 12 h. Cell number increased by almost 50% during the first 24 h after the beginning of treatment. This stimulation was inhibited by 80% or more in the presence of the anti-type 1 IGF receptor antibody α-IR3 and anti-IGF-II antibody. The IGF-II concentrations in the culture media, measured after acidic gel filtration, increased about 1.5-fold and Northern blotting showed a concomitant increase in IGF-II mRNA levels. The mitogenic effect of RA therefore reflects its stimulation of IGF-II production. The availability of IGF-II to the cells may also be enhanced because of the proteolysis of IGFBP-2 to which it is bound. After this initial phase, proliferation ceased despite continued IGF-II production between 24 and 72 h. Both IGFBP-2 and IGFBP-4 production decreased, whereas that of IGFBP-6 increased. These changes appeared both in the protein quantities and in their mRNAs. Insulin-like growth factor binding protein 6 has a strong affinity for IGF-II, 5–10 times that of IGFBP-2 and at least 10 times that of the type 1 IGF receptor, and the arrested proliferation may result, at least in part, from sequestration by IGFBP-6 of the IGF-II secreted.

Sylvie Babajko, INSERM U142, Hôpital Saint Antoine, 184 rue du Faubourg, St Antoine, 75571 Paris Cedex 12, France

Neuroblastomas are neural crest-derived tumours of early childhood. Among human tumours, they have the highest incidence of spontaneous regression reported to date (1). One possible mechanism of this regression is neuroblast differentiation.

Many types of tumour cell produce IGFs, particularly IGF-II, suggesting that they are involved in malignant transformation (2–4). Known neuroblastoma cell lines secrete large amounts of IGF-II and very little IGF-I. They express the IGF-II/mannose-6-phosphate receptor and the type 1 IGF receptor that mediates IGF action in the cell (5). Insulin-like growth factors play an important role in cell proliferation and survival (6–9) and are also capable of inducing cell differentiation (10–12). The neuroblastoma cell line SK-N-BE(2) has recently been shown to secrete two of the IGFBPs: IGFBP-2 and IGFBP-4 (13). The IGFBPs bind to IGFs with affinities close to those of IGF receptors and modulate their action at cellular level, either inhibiting or potentiating it (14).

Retinoic acid (RA) is a natural morphogen involved in the growth and development of various embryonic tissues and, in particular, nervous tissue (15). For this reason, RA is beginning to be used in the treatment of tumours and especially in children with neuroblastomas (16). It has now been reported to modulate the expression of IGF-II (17, 18) and IGFBPs (13) secreted by neuroblastoma cells. Although it appears that the IGFs and IGFBPs are involved in neuroblast proliferation and differentiation, their specific roles remain unknown. The aim of this study was to determine relationships that may exist between IGF-II and IGFBP expression and growth in neuroblastoma cells treated with RA.

Materials and methods

Cell culture

The neuroblastoma cell line SK-N-SH, kindly provided by Dr J Bénard, was established from human metastatic neuroblastoma tissue and comprised two morphologically distinct cell types—epithelial cells and neuroblasts—for which phenotypic interconversion was clearly demonstrated (19).
Cells were grown in DMEM (GIBCO-BRL) supplemented with 10% heat-inactivated fetal calf serum in the presence of 100 IU/ml penicillin, 10 µg/ml gentamicin and 1 µg/ml amphotericin. Cultures were carried out in a humidified incubator at 37°C, with 5% CO2 atmosphere. At the end of the exponential growth phase, cells were trypsinized using 0.05% trypsin-EDTA (DIFCO) and seeded in 10-cm diameter petri dishes at 2 × 10^6 cells/dish, i.e. 25,500 cells/cm^2 (for analysis of IGFs. IGFBPs and their mRNAs) or in 96-well plates at 15,000 cells/well, i.e. 45,000 cells/cm^2. After 24 h, the medium was discarded and culture continued in serum-free medium for a further 24 h. Thereafter, medium was renewed (time 0 (T) of the experiment) with or without 1 µmol/l all-trans-retinoic acid (Sigma Chemical Company), 40 ng/ml IGF-I, 160 ng/ml IGF-II (Ciba Geigy Ltd) and 5 µg/ml α-IR3 (Oncogene Sciences) or 10 µg/ml monoclonal anti-rat-IGF-II antibody specific for rat and human IGF-II (Upstate Biochemical Corporation). Incubation under these conditions was continued for 12 h, after which the media were replaced by fresh media without RA or IGFs. Thereafter, culture was pursued for 12 h (T + 24 h) to 5 days (T + 120 h).

**Cell proliferation assays**

The number of viable cells was estimated using a colorimetric assay in which mitochondrial dehydrogenase reduction of tetrazolium salt (MTT) (soluble in dimethyl sulphoxide (DMSO)) is detected (20). 3-(4,5-Dimethyylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT or thiazolyl blue) obtained from Sigma Chemical Company was added to a final concentration of 0.5 mg/ml for a 3-h incubation at 37°C, followed by addition of 100 µl of DMSO. The plates were shaken for 5 min and absorbance at 540 nm for each well was measured using a J Bio SA 12-505 ELISA reader. Each time, results for treated cells were corrected for controls (untreated cells).

**Insulin-like growth factor assays**

The methods used have been described in detail elsewhere (21, 22). Lyophiled samples corresponding to 10 ml of culture medium were gel filtered in 1 mol/l acetic acid on columns of Ultrogel AcA 54 (IBF) in order to separate IGFs from their IGFBPs. The eluates were lyophilized and, before being assayed, desalted on Sephadex G-25 disposable columns (Pharmacia-LKB) in assay buffer.

Insulin-like growth factor I was assayed by RIA using the anti-IGF-I antisemum prepared by Dr L Underwood and JJ van Wyk and kindly provided by the Hormone Distribution Program (NIDDK, MD). Insulin-like growth factor II was measured by competitive protein binding assay using IGFBPs extracted from cerebrospinal fluid, which have a selective affinity for IGF-II. Both IGF-I and IGF-II were assayed simultaneously.

**Western ligand blotting**

The conditioned media were desalted on Sephadex G25 columns, lyophilized and analysed by Western ligand blotting (23) as described previously (24). Briefly, a 1.5-ml equivalent of each sample was submitted to 11% SDS-PAGE under non-reducing conditions. The secreted proteins were electrotransferred onto nitrocellulose membranes, which were then rinsed for 45 min in TBS (5 mmol/l TRIS-HCl (pH 7.4) and 150 mmol/l NaCl) containing 0.2% Tween, incubated for 48 h at 4°C with a mixture of [125I]IGF-I and [125I]IGF-II (200,000 cpm each) in TBS and 1 mg/ml BSA, rinsed and finally autoradiographed at -80°C.

**Immunoblotting**

Immunoblotting was carried out as described previously (24). Nitrocellulose membranes were prepared as for ligand blotting. Following transfer, the membranes were saturated and incubated at 37°C for 1 h with anti-human (h) IGFBP-1 (raised in our laboratory), anti-h-IGFBP-2 (kindly provided by Dr J Schwander) or anti-h-IGFBP-3 (raised in our laboratory) at 1/1000 dilution or anti-h-IGFBP-4, anti-h-IGFBP-5 (both kindly provided by Dr T Busby and Dr D Clemmons) or anti-h-IGFBP-6 (kindly provided by Chiron Corporation, CA) antibody at 1/400 dilution. The nitrocellulose membranes were rinsed and then incubated for 45 min with goat polyclonal anti-rabbit immunoglobulin G antibody coupled to horse radish peroxidase (G-HPR) (Sigma Chemical Company) at 1/1000 dilution. The HRP oxidation of luminol (Amersham) gives chemiluminescence, from which the specific IGFBP–antibody complexes can be visualized.

**Isolation of RNA and Northern blotting**

Total RNAs were extracted from frozen cells using the standard CsCl/guanidine isothiocyanate method (25).

Aliquots of 30 µg of total RNA were loaded onto 1.2% agarose/2.2 mol/l formaldehyde gels, submitted to electrophoresis, stained with ethidium bromide, transferred to Hybond-N nylon membranes (Amersham) and covalently bound to the nylon by baking of the membranes at 80°C for 2 h. After 4 h of prehybridization at 50°C in 5 x SSC, 50% formamide, 5 x Denhardt, 50 mmol/l sodium phosphate (pH 6.5) and 250 µg/ml sonicated salmon sperm DNA, the blots were hybridized to 3 x 10^6 cpm/ml of h-IGF-II, h-IGFBP-2, h-IGFBP-4 or h-IGFBP-6 cDNA probe for 24 h at 50°C in the same buffer plus 10% dextran sulphate.

The IGF-II cDNA probe consisted of a 663-bp fragment containing the entire coding sequence in addition to 15 bp of untranscribed leader exon 5 and 99 bp of 3'-untranslated sequence (26). Partial h-IGFBP-2 (nucleotides 148–1298) cDNA (Hardouin, unpublished) was inserted into the EcoRI site of pT7T3 18U
(Pharmacia). Human IGFBP-4 cDNA, which encompasses the entire 774-bp coding region, was a gift from Dr. S Mohan (27). Human IGFBP-6 cDNA (892 bp containing the entire coding region) was isolated following EcoRI digestion of the pSK vector (gift from Chiron Corporation).

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

Results

Cell proliferation in serum-free medium: effects of IGFs and retinoic acid

In the absence of treatment (controls), cell number as evaluated by the MTT test remained unchanged throughout the 5-day experiments.

After treatment with 40 ng/ml IGF-I, cell number reached a mean of 151% of controls at T + 24 h and 189% at T + 48 h, thereafter remaining stable up to 120 h (not shown). With 160 ng/ml IGF-II, cell number rose to a mean of 153% of controls at T + 24 h and 167% at T + 48 h (Fig. 1A). Preliminary experiments had shown that these doses elicited maximal stimulation of cell proliferation. The lesser potency of IGF-II may reflect its weaker affinity for the type 1 IGF receptor and its stronger affinity for the IGFBPs present in the culture media. Both IGF-I- and IGF-II-induced stimulation was inhibited to approximately 90% when cells were co-incubated with 5 µg/ml α-IR3 antibody (added 30 min before the IGFs), indicating that the effects of both IGFs on SK-N-SH cell proliferation were mediated by the type 1 IGF receptor.

After 12 h of treatment with 1 µmol/l RA, cell number also increased, reaching 131% and 115% of controls at T + 24 and T + 48 h, respectively. Co-incubation with α-IR3 antibody suppressed this stimulatory effect, showing that RA’s mitogenic action involved the type 1 IGF receptor, and consequently the IGFs (Fig. 1B). The same suppression was observed in the presence of 10 µg/ml anti-IGF-II antibody (Fig. 1B), which would indicate that IGF-II secreted by the cells was responsible for the increased proliferation. Either α-IR3 or anti-IGF-II antibody added alone had no effect on cell growth, suggesting that basal production of IGF was insufficient to stimulate proliferation. After 48 h, cell number dropped below that of controls, which may reflect either RA toxicity or reduced bioavailability of the IGF-II secreted by the cells.

Modulation of IGF-II production by RA

In order to demonstrate that the mitogenic effect of RA observed during the 72 h of culture was indeed a reflection of IGF-II production, expression of IGF-II messengers was investigated. Northern blotting showed that SK-N-SH cells produced essentially 6.0- and 4.8-kb IGF-II mRNAs, as do most neuroblastoma cells described to date (7, 17). Retinoic acid stimulated about threefold expression of the 6.0-kb IGF-II mRNA in particular, up to at least 72 h of culture (Fig. 2A, B).

In addition, IGFs were assayed in the culture media: IGF-II was present, but IGF-I was at the limit of
Fig. 2. Modulation of IGF-II expression by retinoic acid (RA). Cells were cultured in serum-free DMEM medium for 24 h and, after renewal of the medium, treated either with (RA+) or without (RA−) 1 µmol/l RA for 12 h. Cells were harvested at the times indicated. (A) Aliquots of 30 µg of total RNA were separated by electrophoresis on agarose gel stained with ethidium bromide. Transfer integrity and homogeneity were checked after transfer to nitrocellulose. The RNAs were then hybridized to 32P-labelled IGF-II coding probe. (B) Quantification of hybridization by laser densitometry scanning in three separate experiments (mean ± SEM). (C) Media were concentrated and submitted to acidic gel filtration prior to IGF-II assay (see Methods). The values correspond to cumulative (ng/ml) IGF-II concentrations.

Fig. 3. Effects of retinoic acid (RA) on expression of the IGFBPs secreted by SK-N-SH cells. Cells were cultured in serum-free DMEM medium for 24 h and, after renewal of the medium, treated either with (RA+) or without (RA−) 1 µmol/l RA for 12 h. Cells were harvested at the times indicated for Western blot analysis (1.5 ml eq. medium per slot). The IGFBPs were revealed by their binding to radiolabelled IGFs (A) and by using specific antibodies (B). (C) Laser densitometry scanning of IGFBP-6 immunoblots in three separate experiments (mean ± SEM).
Modulation of IGFBP production by RA

Western ligand blot analysis of the IGFBPs in the culture media showed that under basal conditions SK-N-SH cells produced two major IGFBPs of approximately 34 and 24 kDa and a barely detectable one of 30–32 kDa (Fig. 3A). From immunoblotting experiments done with antibodies specifically recognizing each of the six IGFBPs, the 34-kD form corresponded to IGFBP-2, the 24-kD form to IGFBP-4 and the 30–32-kD form to IGFBP-6 (Fig. 3B). The anti-IGFBP-2 antibody also detected a protein of ~20 kD, indicating limited proteolysis of IGFBP-2. The SK-N-SH cells produced neither IGFBP-1 nor IGFBP-5 and immunoblotting revealed only traces of IGFBP-3 (not shown).

IGFBP-6 clearly increased (Fig. 3B), the stimulation being visible from T + 24 to T + 72 h. Laser densitometry scanning in three experiments revealed between 10- and 25-fold increases.

Both IGFBP-2 and IGFBP-4 mRNAs were clearly reduced after RA treatment, whereas IGFBP-6 mRNA was markedly increased. Both changes were detectable from T + 24 to T + 72 h (Fig. 4).

Discussion

Insulin-like growth factors are known to be involved in the autocrine/paracrine regulation of growth in SH-SY5Y neuroblastoma cells where they interact with the type 1 IGF receptor (5, 8, 28). Because SK-N-SH cells were cloned from SH-SY5Y cells (29), we checked that IGF-I and IGF-II also stimulated their proliferation and that the effect was mediated by the type 1 IGF receptor (inhibition by the α-IR3 antibody that blocks this receptor (30)).

We elected to study the effects of RA on these cells because it is a natural morphogen that regulates cell differentiation (15). Cells cultured in the absence of serum were submitted to 12 h of treatment with 1 µmol/l RA. Unexpectedly, proliferation of SK-N-SH cells was stimulated 24 h after the beginning of treatment. The effect appeared to be attributable to IGF-II secreted by the cells, because it all but disappeared when the cells were treated simultaneously with α-IR3 or anti-IGF-II antibody and, in addition, IGF-II secretion and expression of its mRNA were clearly stimulated by RA. This action of RA on neuroblastoma cell proliferation has not been reported to date, although it is known to induce IGF-II expression (17, 18, 29). Matsumoto et al. (17) found no increased proliferation in conjunction with RA-induced IGF-II expression. The discrepancy with our results could possibly be explained in terms of timing, stimulation of proliferation in our experiments being short-lived. In the other two reports cited, neuroblastoma cells have been seen to continue proliferating in the presence of RA, owing to a resistance to it. It is, however, pertinent that RA fails to stimulate proliferation in the presence of serum.

**Fig. 4.** Modulation of IGFBP-2, IGFBP-4 and IGFBP-6 mRNAs by retinoic acid (RA). Experimental conditions were the same as those described in Fig. 2. (A) The RNAs bound to nitrocellulose were hybridized to [32P]cDNA probes coding for IGFBP-2, IGFBP-4 or IGFBP-6. (B) Quantification of hybridization by laser densitometry scanning in three separate experiments (mean ± s.e.m.).

IGF-II mRNA and protein probably reflect the different principles on which the techniques of analysis are based. Northern blotting measures RNAs at a specific point in time, whereas the IGF assay measures cumulative concentrations in the media, without recognizing time-related differences in concentration gradient at the cell surface.
Under these conditions, IGF-II mRNA levels are much lower (8) and are not modulated by RA (results not shown).

The action of IGF on target cell proliferation is modulated by the IGFBPs, which have affinities for the IGFs that are equal to or stronger than those of their receptors (14). For this reason we investigated the effects of RA on IGFBP expression. Western ligand blotting and immunoblotting analyses revealed IGFBP-2, IGFBP-4 and IGFBP-6, with predominant expression of the first two under basal conditions. Immunoblotting revealed an additional, lower molecular mass band for IGFBP-2, which reflects limited proteolysis of this protein (which is not affected by RA), whereas IGFBP-4 and IGFBP-6 were intact. This agrees with our earlier observations for cerebrospinal fluid (32). Insulin-like growth factor binding protein 2 has preferential affinity for IGF-II (33) and IGFBP-4 has similar affinities for the two IGFs (34); IGFBP-2 may serve to target IGF-II to the cell membrane, in view of its RGD sequence (14) by which it could bind to an integrin-type receptor on the cell surface, as has been described for IGFBP-1 (35). Limited proteolysis of IGFBP-2 would facilitate dissociation of the IGF-II bound to it, thus increasing IGF-II’s availability to the cells (32).

Beyond T + 24 h after RA treatment, IGFBP-2 and IGFBP-4 expression decreased, IGFBP-6 expression increased and cell proliferation stopped. These changes in IGFBP expression were evident from both protein and mRNA analysis. In Western ligand blotting, the bands corresponding to IGFBP-2 and IGFBP-4 were diminished. This was confirmed by the immunoblotting results, which also revealed a band corresponding to the proteolytic fragment of IGFBP-2 and the absence of proteolysis of IGFBP-4. Similar results have been obtained with two other human neuroblastoma cell lines (SH-SY5Y and UCR-N-91) that express IGFBP-2 and IGFBP-4 (not shown). Secretion of these two IGFBPs was therefore reduced in the neuroblastoma cells, as has been reported recently for the SK-N-BE(2) cell line (13). Because the reduced secretion occurred in concert with the decrease in IGFBP-2 and IGFBP-4 mRNA levels, RA must modulate expression of these genes at the transcriptional or post-transcriptional level. Our results disagree with those reported for the MCF-7 cell line, where RA increases IGFBP-4 expression (36). This difference is probably linked to cell type, because RA reduces IGFBP-2 expression in rat osteoblasts but increases it in hepatocytes (37).

The results for IGFBP-6 were of particular interest in that its expression was considerably increased by RA, as in the case of breast carcinoma cells or human fibroblasts (38). Proliferation of SK-N-SH cells appeared to be arrested when IGFBP-6 concentrations were high, an observation we have also made in SH-SY5Y cells treated with IGFs, occurring at the same time as signs of differentiation (39). It is noteworthy that IGFBP-6 has the highest affinity among the IGFBPs for IGF-II, approximately $10^{11}$M/mol, which is 5–10 times that of IGFBP-2 (33) and at least 10 times that of the type 1 IGF receptor (14). A likely explanation for the arrested proliferation, despite continued IGF-II secretion but in the presence of increased IGFBP-6 levels, would be sequestration of IGF-II by IGFBP-6. It is relevant here that reduced proliferation associated with IGFBP-6 expression has also been reported for breast cancer cells (40) and a keratinocyte cell line where IGFBP-6 has a direct inhibitory effect on spontaneous proliferation (41). Experiments in our laboratory have shown that cells cultured in the presence of IGFBP-6 no longer respond to IGF-II, whereas the mitogenic effects of IGF-I and particularly serum are only partially diminished. These findings support the hypothesis that IGFBP-6 inhibits cells proliferation, probably by sequestering IGF-II (manuscript in preparation).

Both IGFBP-2 and IGFBP-6 have also been seen to have opposite profiles of expression in primary cultures of rat osteoblasts (42). Our observations for neuroblastoma cells are in agreement with reports for these cell models and suggest that their growth regulation involves IGF-II, whose availability to the cells would be increased by IGFBP-2 and decreased by IGFBP-6.

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References


11. Shi DN, Wang C, Li Y. Second messengers mediating gene expression essential to neurite formation directed by insulin-like