Retinoic acid stimulates IGF binding protein (IGFBP)-6 and depresses IGFBP-2 and IGFBP-4 in SK-N-SH human neuroblastoma cells

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Abstract

Insulin-like growth factors (IGF-I and IGF-II) stimulate proliferation and differentiation in many cell types. In biological fluids, they associate non-covalently with high-affinity binding proteins (IGFBPs) which control their bioavailability and modulate their action. We previously demonstrated that IGFBP-2, -4 and -6 are intimately involved in the growth of cells derived from human neuroblastomas. Here, we have investigated the effects of retinoic acid (RA), which induces differentiation in these cells, on the expression of IGFBPs secreted by SK-N-SH neuroblastoma cells. Analysis of transcriptional activity of the IGFBP-2, -4 and -6 genes in isolated nuclei (run-on experiments) showed that RA increased the transcriptional activity of the IGFBP-6 gene, reduced that of the IGFBP-4 gene and had no effect on that of the IGFBP-2 gene. Northern blot analysis following treatment with actinomycin D showed that RA increased the stability of IGFBP-6 mRNA by a factor of 2·6, decreased that of IGFBP-2 mRNA by a factor of 2·3 and failed to affect IGFBP-4 mRNA. Treatment of cells with cycloheximide indicated the involvement of labile proteins in the stabilization of these mRNAs the expression of which could be under the control of RA. The transcriptional and/or post-transcriptional mechanisms by which RA regulates each of the IGFBPs produced by SK-N-SH cells are therefore different. Such regulation may also reflect the state of differentiation of the neuroblastoma cells. With RA-induced differentiation, IGFBP-6 is strongly stimulated, whereas IGFBP-2 and IGFBP-4 are severely depressed, which would suggest that each IGFBP plays a specific role. Moreover, this regulation seems tissue-specific because it is different in other cell types.

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Introduction

Insulin-like growth factors-I and -II (IGF-I and -II) play an important role in proliferation, differentiation and transformation in a wide variety of cell types (Stewart & Rotwein 1996, Werner & LeRoith 1996) and they are actively involved in the development of the nervous system (Sara 1992, De Pablo & De la Rosa 1995). Neuroblastomas are embryonic tumours derived from neural crest cells giving rise to the sympathetic nervous system and are generally seen in young children. IGFs are known to be directly involved in the proliferation and differentiation of cell lines derived from neuroblastomas, acting via autocrine and/or paracrine mechanisms (El-Badry *et al.* 1989, Burke & Vuk-Pavlovic 1993, Meghani *et al.* 1993) and their effects being mediated via the type 1 IGF receptor (Martin *et al.* 1993). In all biological fluids, IGFs are non-covalently bound to high-affinity binding proteins (IGFBPs) which regulate their bioavailability and modulate their interactions with their target cells (Rechler 1993, Jones & Clemmons 1995). To date, six distinct molecular species, IGFBP-1 to IGFBP-6, have been identified (Shimasaki & Ling 1991, Jones & Clemmons 1995) and four other proteins possessing sequence homologies with the IGFBPs, but binding IGFs with only weak affinity have recently been described (Swisshelm *et al.* 1995, Oh *et al.* 1996). Although they are produced ubiquitously, each IGFBP exhibits characteristic patterns of expression related to tissue of origin, stage of development and hormonal environment. Under basal conditions, human neuroblastoma cells secrete IGF-II (El-Badry *et al.* 1991) and among the IGFBPs, predominantly IGFBP-2, smaller amounts of IGFBP-4 (Bernardini *et al.* 1994, Babajko & Binoux 1996) and traces of IGFBP-6. With the arrest of proliferation in these cells, there is an increase in IGFBP-6 expression, but decreases in IGFBP-2 and IGFBP-4 expression, suggesting that the ratio of IGFBP-2 and -4 to IGFBP-6 influences cell growth (Babajko & Binoux 1996, Babajko *et al.* 1997). Similar association of IGFBP-6 expression and arrest of cell

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growth has been described in keratinocytes (Kato *et al.* 1995) and myoblasts (Ewton & Florini 1995). With exposure to retinoic acid, cells begin to differentiate and changes occur in IGFBP expression: IGFBP-6 is increased (Babajko & Binoux 1996, Gabbitas & Canalis 1996, Zhou *et al.* 1996) and IGFBP-2 and -4 are depressed in neuroblastoma cells (Bernardini *et al.* 1994, Babajko & Binoux 1996) and increased in other cell types (Glantschnig *et al.* 1996). Retinoic acid (RA) is a natural derivative of vitamin A (retinol) and plays a role in the growth and development of various embryonic tissues, particularly in the nervous system. Therapeutic uses for RA and its 9 cis-RA or 13 cis-RA derivatives are under study for the treatment of neuroblastomas (Khan *et al.* 1996) and, since IGFBPs appear to influence neuroblastoma cell growth, it seemed of interest to characterize the changes in their expression provoked by an agent known to induce differentiation.

Materials and Methods

Cell culture

The human neuroblastoma cell line, SK-N-SH (Ross *et al.* 1983), was kindly provided by J Bénard (Institut Gustave Roussy, Villejuif, France). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Paisley, Strathclyde, UK) 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% CO_2 atmosphere. At the end of the exponential growth phase, cells were trypsinized using 0·05% trypsin-EDTA (Difco, Detroit, MI, USA) and seeded in 10 cm-diameter petri dishes at 2×10^6 cells/dish. After 24 h, the medium was discarded and the culture was continued in serum-free medium for a further 24 h with or without 1 μM all-trans-retinoic acid (Sigma Chemical Company, St Louis, MO, USA). Thereafter, medium was renewed with or without 5 μg/ml actinomycin D, and/or 5 μg/ml cycloheximide (Sigma).

Isolation of RNA and Northern blotting

Total RNAs were extracted from frozen cells using the standard CsCl/guanidine isothiocyanate method (Maniatis *et al.* 1982). RNA concentrations were determined by spectrophotometry.

Forty micrograms total RNA were loaded onto 1·2% agarose/2·2 M formaldehyde gels, submitted to electrophoresis, stained with ethidium bromide, transferred to Hybond-C nylon membranes (Amersham, Little Chalfont, Bucks, UK) and covalently bound to the nylon by baking of the membranes at 80 °C for 2 h. After 4 h pre-hybridization at 50 °C in 5 × SSC, 50% formamide, 5 × Denhardt, 50 mM sodium phosphate, pH 6·5, and 250 μg/ml sonicated salmon sperm DNA, the blots were hybridized to 3×10^6 c.p.m./ml ^{32}P-labelled complementary DNA (cDNA) probe (h-IGFBP-2, -4 or -6 cDNA) (Babajko & Binoux 1996) (Multiprime DNA labelling system, Amersham) for 24 h at 50 °C in the same buffer plus 10% dextran sulphate. The Northern blots were washed twice for 15 min in 2 × SSC, 0·1% SDS at room temperature and once for 30 min in 0·5 × SSC, 0·5% SDS at 65 °C and finally autoradiographed at −80 °C with intensifying screens.

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

Nuclear extract preparation

Nuclei were prepared according to Bresnick & Felsenfeld (1993) from cells pre-cultured for 24 h with or without RA. Cells were trypsinized (Difco), then centrifuged for 10 min at 600 ***g***. The pellet was washed twice with 20 volumes PBS phosphate buffer (Difco), then resuspended in 1·5 volumes 10 mM Tris–HCl, pH 7·5, 10 mM NaCl, 3 mM $MgCl_2$, 0·2% Nonidet P-40, 10 mM dithiothreitol (DTT). The cells were lysed in a Type B Dounce potter (12 passages) and the nuclei recovered after centrifugation at 4 °C for 5 min at 1000 ***g***. They were rinsed in 1·5 volumes 10 mM Tris–HCl, pH 7·5, 10 mM NaCl, 3 mM $MgCl_2$, 10 mM DTT, collected by centrifugation for 5 min at 800 ***g*** and immediately resuspended in an equal volume of 20 mM HEPES, pH 7·5, 25% glycerol, 20 mM KCl, 0·2 mM EDTA. Nuclei were stained with trypan blue and counted on a Malassez grid, divided into aliquots and stored in liquid nitrogen.

Run-on experiments

The technique was based on the method of Riol *et al.* (1992) modified according to Babajko *et al.* (1993). Nuclei (5×10^7) were resuspended in 100 μl 20 mM Tris–HCl, pH 7·9, 75 mM NaCl, 0·5 mM EDTA, 40% glycerol, 0·85 mM DTT, 0·125 mM phenylmethylsulphonyl fluoride (PMSF), to which was added a buffer comprised of 100 mM Tris–HCl, pH 7·9, 50 mM NaCl, 0·4 mM EDTA, 0·1 mM PMSF, 1·2 mM DTT, 350 mM $(NH_4)_2SO_4$, 1 mg/ml heparin (Sigma), 4 mM $MnCl_2$, 10 mM creatinine phosphate (Boehringer Mannheim, Indianapolis, IN, USA), 1 mM ATP, CTP and GTP (Boehringer Mannheim). Transcription was initiated in the two-buffer mixture in the presence of 500 U/ml RNase inhibitor (Boehringer Mannheim) and 500 μCi [α-^{32}P]UTP (800 Ci/mmol) (Amersham) and incubation continued for 20 min at 30 °C. The reaction was stopped by incubation in the presence of 10 U/μl DNase I (Boehringer Mannheim) for 30 min at 37 °C, and 10 mg/ml Proteinase K (Boehringer Mannheim) for 30 min at 37 °C. The reaction medium was precipitated overnight at

−20 °C using 8 M guanidine hydrochloride, pH 5, 100 mg/ml tRNA (Boehringer Mannheim) and 100% ethanol. The newly synthesized RNAs were purified three times by washing in 7 M guanidine hydrochloride, pH 7, 2 M sodium acetate and 100% ethanol, then quantified. The labelled RNAs were hybridized for 72 h at 45 °C in 50% formamide, 5 × SSC, 0·1% SDS, 1 × Denhardt with 1 µg cDNA (IGFBP-2, -4 or -6) or pT_7T_3 (controls) covalently bound to nitrocellulose filters (BA 85, 0·45 µm; Schleicher and Schuell, Dassel, Germany). Finally, the filters were washed three times in 5 × SSC for 30 min at room temperature, twice in 50% formamide, 5 × SSC, 0·1% SDS for 1 h at 50 °C, twice in 2 × SSC for 15 min at room temperature, once in 5 × SSC with 10 mg/ml RNase A for 45 min at 50 °C, and lastly, once in 1 × SSC, 0·1% SDS (Boehringer Mannheim) for 30 min at 50 °C. In general, autoradiograms were revealed after 24 or 48 h of exposure, and in some cases up to 7 days.

The intensity of the hybridization signal was determined by laser densitometry scanning. Non-specific hybridization to control filters (pT_7T_3) was subtracted in each case. Results are expressed as percentages of the hybridization of radiolabelled transcripts from untreated cells with the same membrane-bound probe. Data represent the mean ± S.E.M. of five separate experiments. Unpaired Student's *t*-test was used for statistical analysis.

Results

Effects of RA on the transcriptional activity of IGFBP-2, -4 and -6 genes

Earlier results from our laboratory had shown that RA markedly increases the amounts of IGFBP-6 mRNA and reduces IGFBP-2 and -4 mRNAs (Babajko & Binoux 1996). It remained to be determined at what level RA intervenes. The transcription experiments on nuclei isolated from SK-N-SH cells yielded some insight into the effects of RA on the transcriptional regulation of the IGFBP-2, -4 and -6 genes. With the run-on technique, only *in vitro* elongation of nascent IGFBP-2, -4 and -6 RNA transcripts initiated *in vivo* could be measured, since the heparin added to the reaction mixture inhibits binding of additional molecules of RNA polymerase-II to the cap site of these genes (Bresnick & Felsenfeld 1993). Transcription efficiency was strongly reduced by 0·5 µg/ml α-amanitine which at this concentration preferentially inhibits RNA polymerase-II (not shown). Treatment with 1 µM RA for 24 h reduced the transcriptional activity of the IGFBP-4 gene by a factor of 3·3 ± 0·2 (n=5), $P \leq 0·03$, but increased that of the IGFBP-6 gene by a factor of 3·1 ± 0·2 (n=5), $P \leq 0·02$, as compared with those in control cells (treated with only 0·01% dimethyl sulphoxide as solvent). The transcriptional activity of the IGFBP-2 gene was unaffected by RA (1 ± 0·2, n=5), the $P \leq 0·2$ value showing that the two groups of values were not significantly different (Fig. 1).

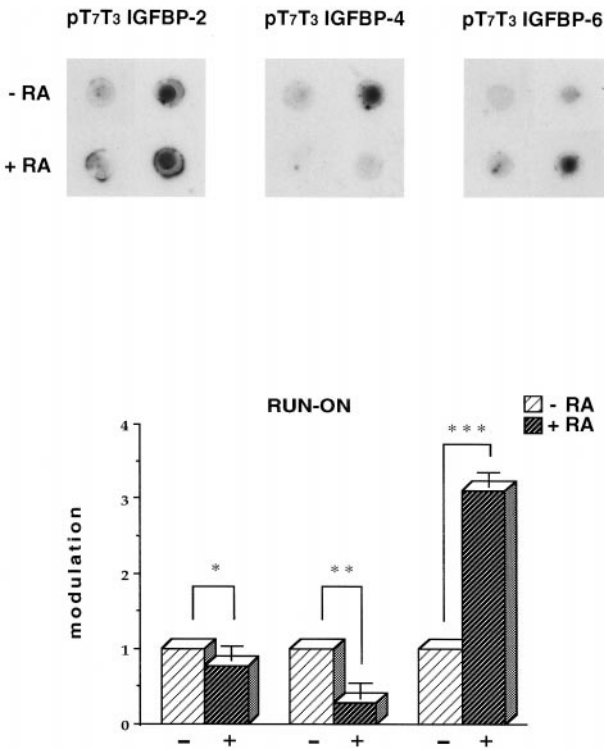

Figure 1 Run-on analysis of the effects of retinoic acid on the transcriptional activities of the genes coding for IGFBP-2, IGFBP-4 and IGFBP-6. *In vitro* transcription was investigated in 5×10^7 nuclei isolated from SK-N-SH cells treated or not with 1 µM RA. Upper panel: autoradiogram of the hybridization spots. Lower panel: the intensity of hybridization signal was determined by laser densitometry scanning. Non-specific hybridization to control filters (pT_7T_3) was subtracted in each case. Results are expressed as percentages of the hybridization of radiolabelled transcripts from untreated cells with the same membrane-bound probe. Data represent the mean ± S.E.M. of five separate experiments. Unpaired Student's *t*-test was used for statistical analysis (*$P \leq 0·2$, **$P \leq 0·03$, ***$P \leq 0·02$).

Effects of RA on the stability of IGFBP-2, -4 and -6 mRNAs

The stability of the IGFBP-2, -4 and -6 mRNAs was investigated by treating the cells for 24 h with 5 µg/ml actinomycin D (a transcription inhibitor). Northern blot analyses showed that pre-treatment with 1 µM RA for 24 h reduced the half-life of IGFBP-2 mRNA from 5·30 to 2·39 h and prolonged that of IGFBP-6 mRNA from 6·06 to 16·06 h (Fig. 2A and Table 1). The half-life of IGFBP-4 mRNA was unaffected by RA, remaining similar (2·51 h) to that under basal conditions (3·16 h) (Fig. 2B and Table 1).

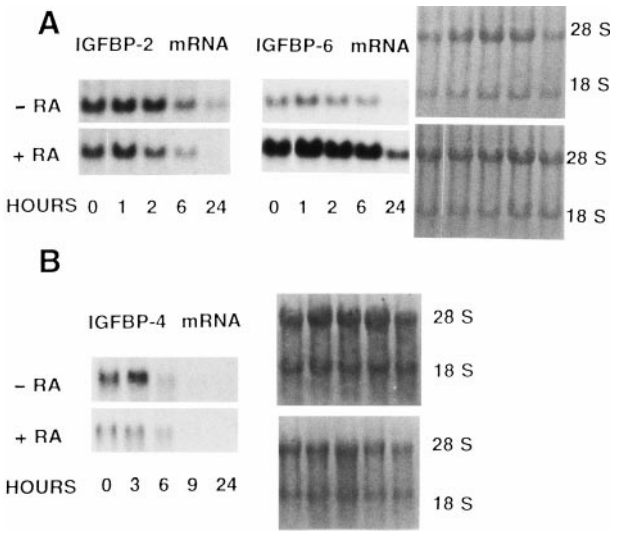

Figure 2 Northern blot analysis of the effects of RA on the half-lives of IGFBP-2, IGFBP-4 and IGFBP-6 mRNAs. SK-N-SH cells pre-treated or not with 1 μM RA were incubated with 5 μg/ml actinomycin D (transcription inhibitor) for 1 to 24 h. Forty micrograms total RNA were separated by agarose/formaldehyde gel electrophoresis, transferred to Hybond-C membranes and hybridized with ^{32}P-labelled cDNA probes specific for each IGFBP. Autoradiograms of the hybridization spots are shown. (A) RA-induced changes in the half-lives of IGFBP-2 and IGFBP-6 mRNAs. (B) Effects of RA on IGFBP-4 mRNA. Three independent experiments were carried out and these data are representative of a typical experiment.

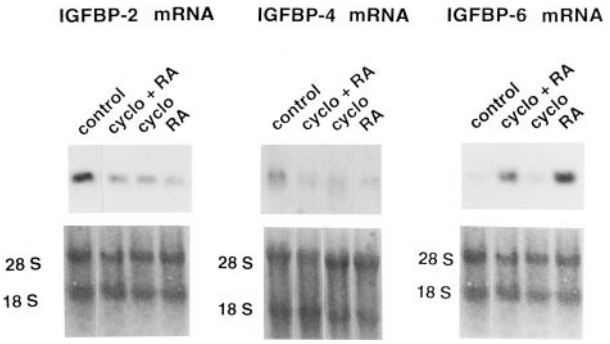

Figure 3 Northern blot analysis of the role of labile proteins in the stability of IGFBP-2, IGFBP-4 and IGFBP-6 mRNAs in the presence of RA. SK-N-SH cells, pre-treated or not with 1 μM RA, were incubated with 5 μg/ml cycloheximide (cyclo) (translation inhibitor) for 3 h. Forty micrograms total RNA were hybridized with ^{32}P-labelled cDNA probes specific to each IGFBP. Three independent experiments were carried out and these data are representative of a typical experiment.

Role of labile proteins in RA-induced modulation of IGFBP-2, -4 and -6 mRNA stability

Following 3 h treatment with 5 μg/ml cycloheximide (a translation inhibitor), the amounts of IGFBP-2 and IGFBP-4 mRNA dropped to 55 ± 12% and 42 ± 3% of those under basal conditions, indicating that labile proteins are involved in the stabilization of these transcripts. IGFBP-6 mRNA was probably unaffected by cycloheximide and remained at very low levels.

Table 1 Half-lives (h) of IGFBP-2, IGFBP-4 and IGFBP-6 mRNAs. The intensity of the hybridization signal was determined by laser densitometry scanning standardized against 28S rRNA and compared with a curve representing signal intensity as a function of duration of actinomycin D treatment. One hundred percent signal was defined as the signal at the beginning of treatment and the estimation of half-life was based on the time when 50% signal remained. Data represent the mean ± S.E.M. of three separate experiments

	IGFBP-2	IGFBP-4	IGFBP-6
−RA	5·30 ± 0·30	3·16 ± 0·32	6·06 ± 0·29
+RA	2·39 ± 0·20	2·51 ± 0·21	16·06 ± 0·37
*P**	0·034	0·57	≤0·001

*Unpaired Student's *t*-test.

With 24 h pre-treatment with RA, there was no additive effect with that of cycloheximide on the quantities of IGFBP-2 mRNA or IGFBP-4 mRNA levels, which remained the same or slightly decreased (− 55 ± 2% versus control). Cycloheximide reduced RA-induced IGFBP-6 mRNA by 48 ± 10% (Fig. 3).

Discussion

It is now well established that the IGFBPs may have IGF-dependent or -independent modulatory effects on cell proliferation. IGFBP-2 appears to be associated with growth, whereas IGFBP-6 expression seems to coincide with the arrest of proliferation (Babajko & Binoux 1996, Babajko *et al.* 1997). No such clear-cut relationship has as yet been determined for IGFBP-4. Recent findings in our laboratory indicate that IGFBP-2 promotes tumorigenesis and that the incidence of neuroblastoma xenografts in nude mice is reduced in the presence of IGFBP-6 (Grellier *et al.* 1998, Menouny *et al.* 1998). RA is increasingly being investigated as a therapeutic agent for cancer and neuroblastomas in particular (Khan *et al.* 1996) and it seemed important to elucidate the mechanisms involved in its regulation of IGFBP expression. Earlier research in our laboratory showed that RA depresses IGFBP-2 and IGFBP-4 expression and increases the amounts of IGFBP-6 secreted by human neuroblastoma-derived cell lines (Babajko & Binoux 1996). In the run-on experiments carried out in this study, RA enhanced the transcriptional activity of the gene coding for IGFBP-6, which would account for the effects seen on accumulation of the corresponding mRNA. The experiments with the transcription inhibitor, actinomycin D, showed that RA prolonged the half-life of IGFBP-6 mRNA which, in

concert with the stimulated transcription, would explain the marked increase in the quantities of IGFBP-6 mRNA. RA-induced modulations of IGFBP-6 expression apparently do not necessitate *de novo* synthesis, since RA was capable of inducing IGFBP-6 expression in the presence of cycloheximide, a translation inhibitor (not shown). Conversely, when cells were pretreated with RA, cycloheximide decreased IGFBP-6 mRNA levels, indicating that labile proteins stabilize IGFBP-6 mRNA whose expression may be controlled by RA. Nevertheless, although the half-life of IGFBP-6 mRNA was increased only 2·6-fold and transcriptional activity 3·1-fold, the amounts of mRNA were enriched by a factor of between 20 and 100. It therefore seems probable that RA affects other regulatory pathways, such as nuclear RNA stability or maturation and/or transport, as has been reported for alkaline phosphatase mRNA (Zhou *et al.* 1994). Our results for IGFBP-6 are similar to those obtained for skeletal cells concerning transcriptional regulation but differ at the post-transcriptional level (Gabbitas & Canalis 1996), suggesting that RA stimulation of IGFBP-6 is widespread among cell types, but that the levels of regulation may vary from one cell type to another.

RA was found to reduce the half-life of IGFBP-2 mRNA, but not to affect the transcriptional activity of its gene, suggesting post-transcriptional regulation. This reduced half-life would account for the diminished expression observed. Labile proteins whose synthesis would be suppressed by RA were found to be involved in stabilizing IGFBP-2 mRNA. Unlike IGFBP-6 whose expression is stimulated by RA whichever the cell model investigated, IGFBP-4 appears to be subject to different types of regulation: RA stimulates its expression in osteoblasts (Glantschnig *et al.* 1996, Zhou *et al.* 1996) and in human breast carcinoma (Adamo *et al.* 1992, Sheikh *et al.* 1993), but depresses it in neuroblastoma cells. This is consistent with reports that RA may have opposite effects on IGFBP-2 expression which is depressed in osteoblasts, but enhanced in hepatocytes (Schmid *et al.* 1992). RA modulation of IGFBP-2 gene expression may therefore be cell type or tissue specific.

RA exerted its effects on IGFBP-4 expression at the transcriptional level, resulting in inhibited expression, although no retinoic acid receptor response element was found on the IGFBP-4 promoter. RA may influence the expression of transcription factors interacting with the IGFBP-4 promoter, exerting an indirect effect. To date, it is only in neuroblastoma cells that RA has been reported to inhibit IGFBP-4 expression (Bernardini *et al.* 1994, Babajko & Binoux 1996). In breast cancer cell lines and osteoblast cells, RA stimulates IGFBP-4 expression (Adams 1993, Zhou *et al.* 1996). It would seem that in this case, RA modulation of IGFBP-4 gene expression is tissue specific, as is RA modulation of IGFBP-2 expression.

In conclusion, the effects of RA on IGFBP expression appear to contribute towards its effects on cell proliferation and differentiation. Our study shows that RA modulates IGFBP expression at both transcriptional and post-transcriptional levels. Under the influence of RA, the considerably stimulated IGFBP-6 would sequester IGF-II whose expression is also enhanced by RA, but for which IGFBP-6 has 100-fold affinity compared with IGF-I. The outcome would be reduced cell growth. Conversely, RA inhibits IGFBP-2 expression which otherwise accompanies proliferation and, in addition, its limited proteolysis releases free IGF-II, which stimulates cell growth (Menouny *et al.* 1997). The combination of stimulated IGFBP-6 and depressed IGFBP-2 and IGFBP-4 would therefore play a role in the arrest of proliferation provoked by RA.

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