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Effect of Retinoic Acid on p21^{ras} and Regulators of its Activity in Neuroblastoma

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p21^{ras} is a membrane-associated guanine nucleotide-binding protein with intrinsic GTPase activity. This protein is important in the regulation of cell growth and differentiation in a number of different cell types. Therefore, the aim of the present study was to examine the role of p21^{ras} and regulators of its activity in the differentiation of neuroblastoma cells induced by retinoic acid (RA). Phosphorylation of p21^{ras} is regulated by the GTPase activity of type I GAP^{120} and neurofibromin. RA-induced differentiation of the two neuroblastoma cell lines SK-N-SH and IMR-32 was closely related to growth inhibition. Differentiation induced by RA resulted in an increase in both type I GAP^{120} and neurofibromin mRNAs. This increase was accompanied by a decrease in the activation of p21^{ras}. These results suggest that, in neuroblastoma, activation of p21^{ras} is not associated with RA-induced differentiation. However, the GTPase activating proteins type I GAP^{120} and neurofibromin may have effector functions in RA-induced differentiation of neuroblastoma.

Key words: neuroblastoma, GTPase activating proteins, p21^{ras}, GTP/GDP binding, differentiation, retinoic acid
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INTRODUCTION

NEUROBLASTOMA IS THOUGHT to arise from neural crest precursor cells that appear to be altered during normal development. The tumour shows a wide range of clinical behaviour from spontaneous regression to aggressive malignant disease [1]. Although these different phenotypes may originate from a single precursor cell, very little is known about the molecular mechanisms regulating neuroblastoma cell differentiation, although they are crucial to our understanding of the disease process.

Products of cellular proto-oncogenes are involved in the regulation of cell growth in both normal and pathological tissues. Amongst the best studied are the p21^{ras} proteins [2, 3]. These are membrane-associated guanine nucleotide binding proteins which belong to the highly conserved *RAS* gene family. They are thought to play an important role in the regulation of signal transduction and have been implicated in transformation *in vitro* and tumorigenesis *in vivo* [5, 6]. Like other guanine nucleotide proteins, p21^{ras} is active when GTP bound and inactive when GDP bound [7]. p21^{ras} is known to have weak intrinsic GTPase activity, which dephosphorylates GTP to GDP [8–10], although phosphorylation is regulated to a greater extent by separate GTPase activities [11, 12]. Three p21^{ras} GTPase activating proteins have been described; type I GAP^{120} (GTPase activating protein^{120}), type II GAP^{120} (an alternatively spliced product of type I GAP^{120}) [13] and more recently neurofibromin, the product of the neurofibromatosis gene, which contains the neurofibromatosis type I GAP related domain (NF1-GRD). Type I GAP^{120} appears to be ubiquitously expressed, whereas type II is expressed only in placental trophoblasts [13–15]. Neurofibromin expression has been described in a number of neural crest derived tissues [16, 17], implying that it may have a specific function in cells of neural crest lineage. However, non-neural crest derived cells, such as neurons and oligodendrocytes, also express neurofibromin [18–20]. Therefore, both type I GAP^{120} and neurofibromin may be important in the regulation of p21^{ras} in different tissue types.

Both type I GAP^{120} and NF1-GRD mRNA have been identified in neuroblastoma [21] suggesting they may have a role in this tumour. Also, high levels of H-ras protein have been found expressed in neuroblastoma tumours with a good prognosis [22], and more recently changes in the expression of GTP-binding proteins [23] and GTPase activating proteins [21] have been reported in advanced neuroblastoma disease. These observations suggest that p21^{ras} and regulators of its activity may be important in the development of neuroblastoma. Since retinoic acid (RA) is one of the most potent differentiation-promoting agents described in human neuroblastoma cells [24], we have used this to examine the role of p21^{ras} and regulators of its activity during neuroblastoma cell differentiation *in vitro*.

MATERIALS AND METHODS

Materials

RA (all *trans*) was purchased from Sigma (Poole, Dorset, U.K.). Tritiated thymidine and ^{32}P-orthophosphate were purchased from Amersham International (Bucks, U.K.). Phosphate-free DMEM was made in-house using chemicals from Sigma. The pan-ras antibody (Ab3), goat anti mouse IgG and protein A sepharose were purchased from Oncogene (Cambridge Bioscience, Cambridge, U.K.). Fetal calf serum (FCS) was purchased from Sera lab (Sussex, U.K.) and media from Gibco

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Table 1. *Effect of RA on NF1-GRD and type I GAP^{120} mRNA*

	NF1-GRD 6 h	Type I GAP^{120} 6 h	NF1-GRD 24 h	Type I GAP^{120} 24 h
SK-N-SH, control	0.35 ± 0.1	0.47 ± 0.2	0.33 ± 0.1	0.50 ± 0.2
SK-N-SH, RA	0.78 ± 0.1*	1.84 ± 0.23†	0.87 ± 0.1‡	2.01 ± 0.3‡
IMR-32, control	0.37 ± 0.17	0.93 ± 0.12	0.40 ± 0.15	1.20 ± 0.2
IMR-32, RA	0.82 ± 0.4^{NS}	2.29 ± 0.29*	0.87 ± 0.3*	2.23 ± 0.19§

Results are shown for neuroblastoma cell lines treated with 4 μM RA for 6 and 24 h. Incorporation of radioactivity into RT-PCR products for NF1-GRD, type I GAP^{120} and GAP-DH was measured by scintillation counting (see Methods). Incorporation into NF1-GRD and type I GAP^{120} mRNA was standardised relative to GAP-DH mRNA to generate the ratios given above. *T*-test analysis was made using GraphPAD Instat statistics package. Values given are mean ± S.E.M. for 3 experiments.

* $P < 0.05$; † $P < 0.005$; ‡ $P < 0.01$; § $P < 0.02$; NS, failed to reach significance.

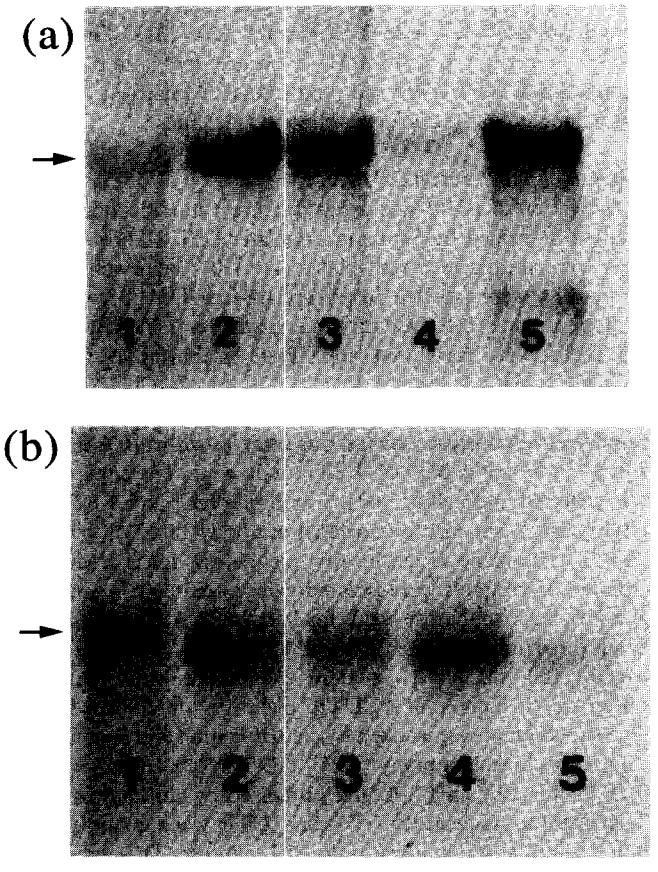

Figure 1. Effect of RA on *C-FOS* expression RNA samples extracted from SK-N-SH and IMR-32 cells before and after treatment with RA (4 μM) were separated on a formaldehyde denaturing gel. These samples were northern blotted and probed with a 40-mer oligonucleotide probe for *C-FOS* (a). The arrow identifies the *C-FOS* signal. The northern blot was hybridised with an 18s cDNA probe to confirm the loading of RNA was equal in each lane (b). 1, SK-N-SH control; 2, SK-N-SH RA for 15 min; 3, SK-N-SH RA for 1 h; 4, IMR-32 control; 5, IMR-32 RA for 15 min.

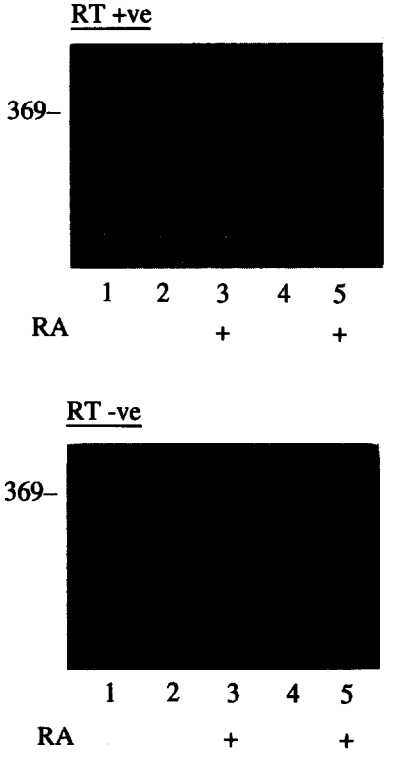

Figure 2. Effect of RA on NF1-GRD RT-PCR products in SK-N-SH cells. RT-PCR products for NF1-GRD in SK-N-SH cells after exposure to RA (4 μM) for 2 and 6 h are shown after separation on an agarose gel and staining with ethidium bromide. 1, control SK-N-SH cell culture; 2, 1 h control untreated cells; 3, 1 h after exposure to RA; 4, 6 h control untreated cells; 5, 6 h after exposure to RA. Corresponding samples (1–5) with no reverse transcriptase enzyme added are shown (RT −ve).

BRL (Paisley, U.K.). Primers were purchased from Oswell DNA Services (Glasgow, U.K.).

Neuroblastoma cell lines

SK-N-SH cells were cultured in DMEM/EMEM plus 10% FCS and IMR-32 cells in DMEM-RPMI plus 5% FCS. SK-N-SH and IMR-32 cultures were purchased from the European Collection of Animal Cell Cultures (PHLS, U.K.).

Expression of C-FOS

Total RNA was extracted from neuroblastoma cell lines before and after exposure to RA (2–8 μM) for 15 min, 1 and 2 h using Ultraspec™ RNA (Biogenesis, Bournemouth, U.K.) as previously described [21]. For each sample, 30 μg of total RNA was run on a formaldehyde denaturing agarose gel and northern blotted on to Hybond N$^+$ (Amersham). These blots were probed

Table 2. Effect of RA on activation of p21^{ras}

	p21^{ras} activation (%)
SK-N-SH, control	8 ± 2
SK-N-SH, RA	2 ± 1
SK-N-SH, post RA	3 ± 2
IMR-32, control	6 ± 1
IMR-32, RA	3 ± 2

The effect of RA (4 μM) on p21^{ras} activation after incubation of cells with RA for 24 h is shown. In the SK-N-SH cells, results are shown 48 h after removal of RA from the culture. *T*-test analysis was made using GraphPAD Instat statistics package; only SK-N-SH cells treated with RA reached significance ($P < 0.05$). Values given are mean ± S.E.M. for 3 experiments.

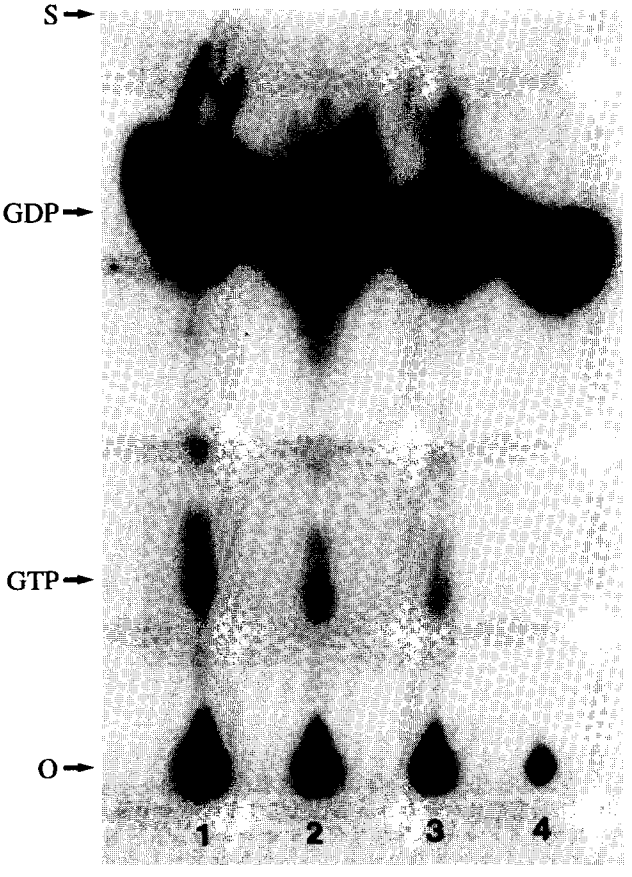

Figure 3. Activation of p21^{ras} in neuroblastoma cell lines and the effect of RA. The activation of p21^{ras} was measured by immunoprecipitation with p21^{ras} antibodies and TLC (see Materials and Methods). TLC plates were exposed to film overnight to produce the shown autoradiograph of control and RA treated samples. The figure shows TLC of SK-N-SH cells treated with RA (1–4 μM) for 6 h. O = origin (where samples are loaded); S = solvent front. 1, control untreated cells; 2, 1 μM RA treated cells; 3, 2 μM RA treated cells; 4, 4 μM RA treated cells.

overnight at 65°C with a ^{32}P-labelled human oligonucleotide probe for the early response gene, *C-FOS* (Oncogene Science). To confirm the amount of RNA loaded in each lane, the *C-FOS* probe was washed from the northern blot and the blot reprobed using an 18s cDNA probe.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of NF1-GRD and Type I GAP^{120} transcripts

Total cellular RNA was extracted from neuroblastoma cell lines using Ultraspec^{TM} RNA and RT-PCR for NF1-GRD and GAP^{120} performed as previously described [21]. Briefly, RNA samples were reverse transcribed to produce cDNA from the target mRNA using a random hexamer primer (Gibco BRL). Primer pairs used for amplification of cDNA by PCR were NF1-GRD 1, TGC GTG CTG CAT CAA AGT TGC TTT TCA C and NF1-GRD 2, CAG AAT TCC CCC CTC AAC TTC GAA GT and type I GAP^{120} 1, GAC AAG ACC GAA CAC TAC TGG and type I GAP^{120} 2, TTG CAG CAA TAG GAG ATG GGG. Both primer sequences for type I GAP^{120} and NF1-GRD were selected to amplify the GTPase-activating domain. Radioactivity was incorporated into the final 10 cycles of the 40 PCR cycles, products separated on agarose gels, stained with ethidium bromide and bands excised to count incorporated radioactivity by scintillation counting. To compare expression of NF1-GRD and type I GAP^{120}, *GAPD* (glyceraldehyde-3-phosphate dehydrogenase) was used as a control gene [21]. The effect of RA (1×10^{-8}–6×10^{-6} M) exposure for 1–24 h on the expression of NF1-GRD and type I GAP^{120} relative to *GAPD* was examined in the two neuroblastoma cell lines.

Analysis of p21^{ras}-bound GDP/GTP

Activation of p21^{ras} was assayed using a modification of the method described by Sato and associates [25]. Neuroblastoma cells were seeded in 6 well plates (1×10^{-4} cells/well). After 24 h, cells were labelled in phosphate-free DMEM with ^{32}P orthophosphate (0.5 mCi/ml; Amersham) for 1 h. After washing, cells were harvested and lysed in buffer A (50 mM Tris–HCl, pH 7.5; 20 mM $MgCl_2$; 150 mM NaCl; 1% aprotinin; 0.1 mmol PMSF) for 10 min. Free ^{32}P-orthophosphate was removed from the lysate by treating samples with a 10% charcoal slurry (in buffer A plus 1% BSA) for 10 min. The supernatant was retained and cellular p21^{ras} immunoprecipitated from the lysates using a mouse monoclonal antibody to p21^{ras}, pan-ras (Ab-3) (Oncogene Science), by incubating for 1 h at 4°C. Immunoprecipitates were isolated using a goat anti-mouse IgG and protein A-sepharose beads for 1 h at 4°C. After washing the beads three times in buffer B (50 mM Tris–HCl, pH 7.5; 20 mM $MgCl_2$; 150 mM NaCl), guanine nucleotides were eluted in buffer C (1% SDS; 20 mM EDTA) by heating at 65°C for 5 min. The supernatant was isolated by centrifugation and analysed by thin layer chromatography (TLC) on PEI plates (Sigma) using 0.75 M KH_2PO_4 (pH 3.4) and visualised by autoradiography. The guanine nucleotides GDP and GTP were quantified by liquid scintillation counting of the PEI cellulose. The percentage of GTP to total amount of GTP plus GDP was calculated; p21^{ras} activation = cpm in GTP/cpm in GTP + GDP × 100. The effect of RA (1–4 μM) on GDP/GTP binding of p21^{ras} was analysed after incubation of cells with RA for 15 min–24 h.

Protein extract preparation and Western blot analysis

Protein samples were prepared from neuroblastoma cell lines before and after treatment with RA. Cells were washed and suspended in 5 volumes of suspension buffer (0.1 M NaCl; 0.01 M Tris.Cl, pH 7.6; 0.001 M EDTA, pH 8.0; 1 μg/ml aprotinin; 100 μg/ml PMSF) and lysed by addition of an equal volume of 2× SDS buffer (100 mM Tris.Cl, pH 6.8; 200 mM dithiothreitol; 4% SDS; 0.2% bromophenol blue; 20% glycerol), heating to 100°C for 10 min prior to sonication (3 × 10 s). Following centrifugation (10000 *g* × 10 min), supernatants

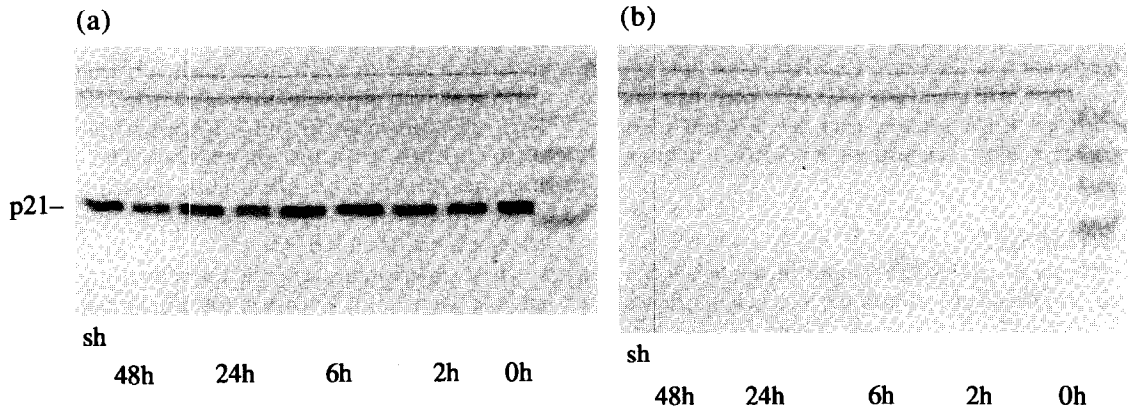

Figure 4. Western blot analysis for p21^{ras} in SK-N-SH cells treated with RA. Western blot was incubated with pan-ras antibody (a); an identical membrane was incubated with a nonspecific antibody (b).

Table 3. RA effect on thymidine incorporation and neurite extension in SK-N-SH and IMR-32 cell lines

	Thymidine (cpm × 10^4) control	Thymidine (cpm × 10^4) RA	Neurite +ve cells control	Neurite +ve cells RA	Neurite +ve cells RA removal*
SK-N-SH	8.0 ± 0.3	2.3 ± 0.2†	30 ± 6	156 ± 5†	145 ± 8†
IMR-32	7.3 ± 0.4	3.9 ± 0.3‡	18 ± 4	89 ± 4†	90 ± 6†

Results are shown for cells treated with RA (4 μM) at 4 days.
* The number of neurite +ve cells 7 days after removal of RA are shown and analysed relative to the control. *T*-test analysis was made using GraphPAD Instat statistics package. Values given are mean ± S.E.M. for 4 experiments; † $P < 0.0001$; ‡ $P < 0.0005$.

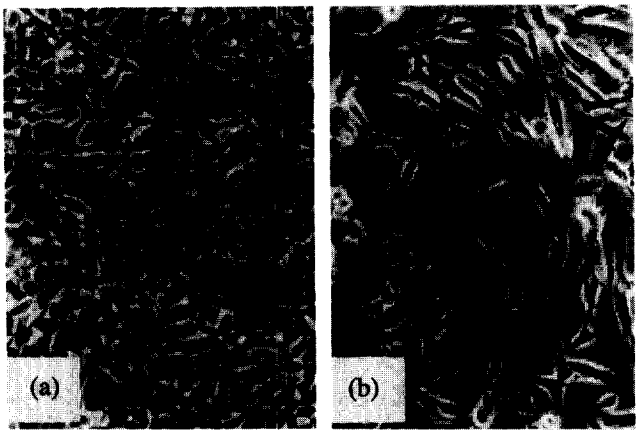

Figure 5. Morphological effect of retinoic acid on SK-N-SH cells. RA-induced neurite extension in the SK-N-SH neuroblastoma cell line after exposure for 24 h. The effect of RA (4 μM) is shown in (b) compared to control (a).

were removed and retained for Western blot analysis using pan-ras antibody (Ab-3). Amplified alkaline phosphatase (Biorad, Hemel Hempstead, U.K.) was the detection method used. Protein extracts were assayed for protein content using the Bradford assay [26].

The effect of RA (4 μM) on the levels of p21^{ras} protein were analysed after incubation of neuroblastoma cells with RA for 2–48 h.

Effects of RA on cell proliferation and differentiation

The effects of RA on cell proliferation were assessed by tritiated thymidine incorporation experiments. Neuroblastoma cells were plated in 24-well plates (2×10^4). After 24 h, RA (1×10^{-8} to 6×10^{-6} M) was added to the culture medium for up to 5 days. Cells were labelled with ^{3}H-thymidine for 16 h and subsequently harvested by trypsinisation. Radioactive incorporation was measured by liquid scintillation counting.

For neuroblastoma cells, the most commonly measured characteristic of differentiation is neurite extension. In this study, a neurite is defined as a process whose length equals or exceeds the cell body diameter [27]. Cells were scored positive if they possessed one or more neurites. The number of cells positive for neurites in 200 cells was recorded.

RESULTS

Effect of RA on C-FOS *expression*

RA (2–8 μM) increased *C-FOS* expression in both SK-N-SH and IMR-32 cells. Expression of *C-FOS* was increased 15 min after incubation with RA and remained elevated at 2 h (Figure 1a). The level of 18s RNA was equal in each sample (Figure 1b).

RT-PCR for NF1-GRD and Type I GAP^{120}

Following RT-PCR of RNA from SK-N-SH and IMR-32 cells for NF1-GRD the expected two bands were identified, one 366bp (type II) and one 303bp (type I). The 366bp transcript was identical to the 303bp of the lower transcript, with a 63bp insert between nucleotides 621 and 684 as previously reported [21]. RT-PCR for type I GAP^{120} produced the expected product

of 546bp [21]. RA increased the level of both NF1-GRD and type I GAP^{120} mRNA relative to *GAPD* (Table 1), but there was no effect on the ratio of the two NF1-GRD transcripts (Figure 2). The level of NF1-GRD and type I GAP^{120} more than doubled after exposure to 4 μM retinoic acid for 6 h and remained elevated after 24 h exposure to RA (Figure 2; Table 1).

p21^{ras}-bound GDP/GTP

The basal level of p21^{ras}-GTP bound in the SK-N-SH and IMR-32 cells was between 5–10% of the total guanine nucleotide bound p21^{ras} (Table 2). Pretreatment of both cell lines with RA (2–4 μM) for up to 24 h produced a small decrease in the level of p21^{ras}-GTP bound (Figure 3, Table 2). Up to 48 h after removal of RA from the SK-N-SH culture medium, the decrease in p21^{ras} activation was essentially unaltered (Table 2).

Western blot analysis

Protein extracts from SK-N-SH and IMR-32 neuroblastoma cell lines showed equal amounts of p21^{ras} on Western blot analysis. Treatment of both cell lines with RA (4 μM) had no notable effect on the level of p21^{ras} protein (Figure 4).

Effect of RA on cell proliferation and differentiation

Proliferation of both neuroblastoma cells lines was significantly inhibited by RA (1×10^{-7}–6×10^{-6} M) in a dose-dependent manner after exposure to RA for 1–5 days. Neurite outgrowth was seen 24 h after exposure to RA in both IMR-32 and SK-N-SH cells (Table 3), although the effect was most marked on SK-N-SH cells (Figure 5). The non-neuronal-type cells did not produce neurites, but became flattened to the flask surface after exposure to RA. Neurite outgrowth, like growth inhibition, was dose-related (results not shown). In the SK-N-SH cell line, removal of RA from the culture medium did not reverse the effect of RA on growth or on neurite extension (Table 3). Even after subculturing of the RA-treated SK-N-SH cells, they remained highly differentiated.

DISCUSSION

RA-induced differentiation of neuroblastoma cells was accompanied by growth arrest in both neuroblastoma cell lines. This is in agreement with previously reported effects of RA on neuroblastoma [24], and similar to the effects of RA on leukaemic cells [28] and rat rhabdomyosarcoma cells [29], but in contrast to the effects of RA on human rhabdomyosarcoma cell lines [30]. These differences in tissue response to RA appear not to be due to differential expression of retinoic acid receptors [29]. Removal of RA from the neuroblastoma cultures had no effect on neurite extension or growth inhibition, demonstrating the terminal differentiating effects of RA on neuroblastoma. Growth arrest seen following incubation with RA is presumably due to a block in G1 of the cell cycle [31], typical of that reported in terminally differentiated cells. The effects of RA on different cell populations may reflect characteristics of divergent cell lineages. To investigate this and its relevance to the development of neuroblastoma, we are currently examining the effects of RA on clonal cell populations derived from neuroblastoma.

RA functions are mediated by a family of nuclear receptors, with ligand-dependent DNA binding and transactivating properties [32]. In this study, RA-induced differentiation of neuroblastoma cell lines was accompanied by a rapid increase in expression of the early response gene, *C-FOS*. This increase in expression preceded increases in the level of both type I GAP^{120} and the NF1-GRD (neurofibromin) mRNA. RA also induced hydrolysis of p21^{ras} bound to GTP to GDP, although the decrease in GTP bound to p21^{ras} was small. Hydrolysis of p21^{ras} by GTPase activating proteins may explain the relationship between RA, cell differentiation and GTPase activating proteins; RA disabling the p21^{ras} activation pathway. This suggests that RA functions through other intracellular mechanisms in neuroblastoma. Alternatively, GTPase activating proteins may have an effector function on RA [33].

These observations raise the question as to the role of p21^{ras} and regulators of its activity in the differentiation of neuroblastoma. They also imply that activation of p21^{ras}, through its phosphorylation, may drive cell proliferation and prevent terminal differentiation of neuroblastoma cells. Alternatively, increased levels of the GTPase activating proteins may have an effector function in the differentiation of neuroblastoma. Given the complexity of the effects of RA on cell lines of different lineage, it is likely that RA functions through a number of mechanisms and, therefore, is a useful tool to study the relationship between intracellular regulators of cell signalling and cell proliferation and differentiation.

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Somatostatin and Vasoactive Intestinal Peptide (VIP) in Neuroblastoma and Ganglioneuroma: Chromatographic Characterisation and Release During Surgery

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Neuroblastomas and ganglioneuromas frequently produce somatostatin (SOM) and vasoactive intestinal peptide (VIP), and elevated concentrations in tumour tissue are associated with favourable outcome. Both somatostatin and VIP have been shown to have an autocrine effect on tumour growth and differentiation *in vitro*, and VIP may cause clinical symptoms when released systemically. Using gel-permeation chromatography and specific radioimmunoassays, we further characterised somatostatin-like immunoreactivity (SOM-LI) and VIP-like immunoreactivity (VIP-LI) in neuroblastoma and ganglioneuroma tumour tissue. The major part of SOM-LI and VIP-LI in both neuroblastoma and ganglioneuroma represents the biologically active forms SOM-28, SOM-14 and VIP-28, respectively. 21 children with neuroblastoma and ganglioneuroma were monitored with serial plasma samples during surgery. In 8 children with measurable concentrations of SOM-LI, all showed increased concentrations during tumour manipulation ($P = 0.004$) that subsequently decreased below preoperative levels in all but one case ($P = 0.06$). The only child presenting with diarrhoea showed the highest preoperative plasma VIP-LI in the study (54 pmol/l). 2 children with increased concentrations of VIP-LI preoperatively showed a rapid decrease after surgical tumour removal. These findings indicate a systemic release from the tumours. It is concluded that plasma and tumour tissue from children with neuroblastoma and ganglioneuroma contain biologically active molecular forms of somatostatin and vasoactive intestinal peptide. These peptides may bear significance both for specific symptoms in certain patients as well as influencing tumour growth and differentiation *in vivo*.

Key words: neuroblastoma, ganglioneuroma, vasoactive intestinal peptide, somatostatin, neuropeptide Y, tumour markers, gel-permeation chromatography, radioimmunoassay
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