Pergamon

European Journal of Cancer, Vol. 33, No. 12, pp. 2075–2080, 1997

Printed in Great Britain
0959-8049/97 $17.00+0.00

PII: S0959-8049(97)00242-6

Original Paper

Retinoids in Neuroblastoma Therapy: Distinct Biological Properties of 9-*cis*- and All-*trans*-Retinoic Acid

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We investigated the potential for 9-*cis*-retinoic acid in the differentiation therapy of neuroblastoma using an N-type neuroblastoma cell line, SH SY 5Y, as an experimental model. In these cells, 9-*cis*-retinoic acid is more effective than other isomers at inducing the expression of RAR-β. An RAR-α-specific antagonist inhibited the induction of RAR-β in response to all-*trans*-but not to 9-*cis*-retinoic acid. This indicates that the mechanism of gene induction by 9-*cis*-retinoic acid differs markedly from all-*trans*-retinoic acid. 9-*cis*-retinoic acid is also better than all-*trans* at producing sustained morphological differentiation and inhibition of proliferation of SH SY 5Y cells. Although N-type neuroblastoma cells are not thought to undergo apoptosis in response to all-*trans*-retinoic acid, we observed a significant degree of apoptosis in SH SY 5Y cells treated with 9-*cis*-retinoic acid for 5 days and then cultured in the absence of retinoid, an effect not observed in cells treated with the all-*trans* isomer. These results suggest that 9-*cis*- and all-*trans*-retinoic acid have distinct biological properties and that 9-*cis* retinoic acid may be clinically effective in neuroblastoma by inducing both differentiation and apoptosis under an appropriate treatment regimen. © **1997 Elsevier Science Ltd.**

Key words: retinoids, differentiation, receptors, RAR, 9-*cis*-retinoic acid, neuroblastoma, apoptosis
Eur J Cancer, Vol. 33, No. 12, pp. 2075–2080, 1997

INTRODUCTION

AN IMPORTANT goal of cancer research is to understand the molecular and biochemical mechanisms controlling cell behaviour *in vivo* sufficiently well to be able to cure malignant disease. A crude but effective approach to treatment is to kill proliferating cells using DNA damaging agents which uncouple S and M phases of the cell cycle [1]. An alternative strategy is to target the malignant cell type specifically with 'biological response modifiers' which act on cellular control pathways to induce differentiation (and hence quiescence) or programmed cell death (apoptosis) in specific cell types. Retinoids have been used clinically for the treatment of some malignant and hyperproliferative diseases for many years and continue to hold out promise as effective biological response modifiers, perhaps in combination with other agents. In neuroblastoma, 13-*cis* and all-*trans*-retinoic acid have been used clinically on the basis of *in vitro* data demonstrating their ability to induce neuroblastoma differentiation [2]. However, sustained remission rates in clinical trials have so far been disappointing [3–5].

The biological effects of retinoic acid are apparently mediated primarily through retinoic acid receptors (RARs) and retinoid X receptors (RXRs), subgroups of the family of steroid and thyroid hormone receptors, which bind as receptor dimers to specific response elements, formed by a direct repeat (DR) of the sequence (A/G/T)GGTCA separated by 1–5 nucleotides (DR1-DR5) [6], and regulate gene transcription. All-*trans*-retinoic acid binds with high affinity to RARs and these receptors function in a ligand-dependent manner as heterodimers with ligand-independent RXRs. Although 9-*cis*-retinoic acid binds with high affinity to both RARs and RXRs [7], direct evidence that 9-*cis*-retinoic acid can activate an RAR–RXR heterodimer as a result of binding to either or both partners is hard to obtain. However, 9-*cis*-retinoic acid can promote the formation of ligand-dependent RXR homodimers [8] and activate the RXR partner in heterodimers between RXRs and other nuclear receptors such as

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LXRs [9] and NGFI-B [10]. Since 9-*cis*-retinoic acid-dependent RXR homodimers and heterodimers between RXRs and other nuclear receptors may regulate gene transcription from different DR response elements to all-*trans*-retinoic acid-dependent RAR–RXR heterodimers, biological responses to 9-*cis*-retinoic acid may also differ.

Recent *in vitro* and animal studies have shown that 9-*cis*-retinoic acid is more effective than the all-*trans* isomer for inducing cell differentiation, inhibiting proliferation and preventing chemically-induced cancers [11–16]. This is also true for some neuroblast or N-type neuroblastoma cell lines, where 9-*cis*-retinoic acid is better than the all-*trans* isomer at inducing sustained morphological differentiation, inhibiting proliferation and inducing gene expression [17, 18]. Here, we provide evidence that the mechanism of action of 9-*cis*-retinoic acid is distinct from that of all-*trans*-retinoic acid, and demonstrate that the biological effects of the two isomers are also distinct in that, unlike all-*trans*-retinoic acid, 9-*cis*-retinoic acid induces apoptosis of SH-SY-5Y cells after treatment and subsequent washout of the retinoid.

MATERIALS AND METHODS

Cell culture

To study morphological responses to retinoic acid isomers, 0.4×10^4 cells were seeded into 24-well tissue culture plates in 2 ml Dulbecco's modification of Eagles's medium, supplemented with 10% fetal bovine serum (culture medium) and incubated overnight at 37°C in a humidified atmosphere of 5% CO_2 in air. All-*trans*- (Sigma, Poole, U.K.) or 9-*cis*-retinoic acid (Hoffmann-La Roche) was then added in ethanol to a final concentration of 10^{-6} M. An equal volume of ethanol (< 0.1% of culture volume) was used to treat control cells. The culture medium was changed every 2 days, replacing with fresh medium containing the appropriate concentrations of retinoic acid. After 5 days of retinoic acid treatment, the culture medium was replaced with fresh medium without retinoic acid and the cells cultured in the absence of retinoic acid for a further 6 days. The cells were photographed after 1, 3, 5, 7, 9 and 11 days.

Evaluation of apoptosis by flow cytometry

SH-SY-5Y cells were seeded into 25 cm^2 flasks at a density such that a minimum of 1×10^6 cells were available from each flask at the time of harvest. Cells were initially allowed to attach overnight and then treated with 10^{-6} M all-*trans*- or 9-*cis*-retinoic acid or an equal volume of control ethanol. The culture medium was changed every 2 days and at each change, the existing medium was harvested, centrifuged at 800*g* for 5 min and pelleted material, consisting of cells and apoptotic bodies, was resuspended in fresh culture medium plus retinoid or control ethanol as appropriate. At final time points, the medium was harvested and apoptotic bodies and non-adherent cells were collected by centrifugation at 800*g* for 5 min and pooled with cells recovered from the flasks by trypsinisation [19]. Apoptosis was evaluated by flow cytometry of propidium iodide stained cells [20] using a FACScan flow cytometer (Becton Dickinson, California, U.S.A.). Cells suspended in phosphate-buffered saline (PBS) were fixed by adding an equal volume of cold (−20°C) methanol:acetone (4:1 v/v) and stored at 4°C. Fluorescence, resulting from excitation at 488 nm with a 15 mW argon laser, was monitored at 570 nm. Events were triggered by the FSC signal and gated for FSC-H/FSC-A/SSC to avoid aggregates. Five thousand events were evaluated using the lysis II programme.

Northern blotting

SH-SY-5Y cells were seeded into 75 cm^2 flasks (8×10^6 cells per flask) and allowed to attach overnight. All-*trans*- or 9-*cis*-retinoic acid was added in ethanol to concentrations of 10^{-8}–10^{-7} M, in the presence or absence of an RAR-α-specific antagonist, Ro 41-5253 [21], at concentrations from 10^{-9} to 10^{-6} M. After 6 h incubation, total cytoplasmic RNA was extracted [22], size-fractionated by electrophoresis through 1.2% agarose/formaldehyde gels and transferred by vacuum blotting to Nylon membranes [23]. Membranes were then probed consecutively with ^{32}P-labelled cDNA probes for RAR-β and GAPDH (loading control) [19, 23] and autoradiographed.

RESULTS

Receptor-mediated induction of RAR-β

We have previously shown that RAR-β and CRABP II are induced by both all-*trans*- and 9-*cis*-retinoic acid, but with different dose–response characteristics and a differential sensitivity to induction and subsequent washout: (1) 9-*cis*-retinoic acid induces to a lesser extent at low doses but gives a 3-fold higher induction at higher doses of the order of 10^{-7} M; (2) contrary to the case with all-*trans*-retinoic acid, induction of RAR-β by 9-*cis*-retinoic acid is rapidly attenuated once 9-*cis*- is removed from the culture medium [23]. On the basis of these data, we have argued that 9-*cis*- and all-*trans*-retinoic acid have distinct mechanisms of action, and that 9-*cis*- may be working via RXR homodimers [23, 24]. To address this issue, we investigated the effects of an RAR-α-specific antagonist, Ro 41-5253 [21], on the retinoic acid-mediated induction of RAR-β. Initially, SH-SY-5Y cells were treated for 6 h with either 10^{-8} or 10^{-7} M all-*trans*-retinoic acid in the presence of absence of increasing concentrations of Ro 41-5253. The induction of RAR-β was significantly inhibited by the antagonist at 10-fold higher concentrations than the all-*trans*-retinoic acid inducer (Figure 1a). This suggests that all-*trans*-retinoic acid mediates the induction of RAR-β via RAR-α. Conversely, with 9-*cis*-retinoic acid as the inducer, no such inhibition was observed (Figure 1b). This lack of inhibition suggests that 9-*cis*-retinoic acid does not induce RAR-β expression via RAR-α.

Morphological effects of 9-cis- *and all*-trans-*retinoic acid on SH SY 5Y cells*

Although we have previously shown that 9-*cis*-retinoic acid gives a more sustained morphological differentiation over 4 days than other retinoic acid isomers tested [17], it is not known if these effects are reversible. For example, in view of the fact that 9-*cis*-retinoic acid washout produces a rapid reduction in RAR-β mRNA, it is possible that the greater differentiation-inducing effects of 9-*cis*-retinoic acid are not sustained after removal of the retinoid from the culture medium. To examine this question, SH-SY-5Y cells were treated with either control ethanol or all-*trans*- or 9-*cis*-retinoic acid at concentrations of 10^{-6} M for 5 days. Incubation of the cells was then continued in the absence of retinoic acid for a further 6 days. After 3–5 days treatment with retinoic acid, the cells appeared more differentiated as evidenced by the extension of neurite processes, with 9-*cis*-retinoic acid producing a greater effect (Figure 2). The cells in the all-*trans*-

retinoic acid-treated cultures reverted to their original morphology within 2–4 days after withdrawal of the retinoid and regained their proliferative capacity. However, in the 9-*cis*-treated cultures, cell processes or neurites became shorter and took on a clubbed appearance (Figure 2). By 6 days after retinoid washout, these cells had not regained proliferative capacity; in addition, the cell bodies lacked neurite processes and appeared contracted with blebbing of the plasma membrane (Figure 2). These results indicate that neurite processes are not retained once all-*trans*- or 9-*cis*-retinoic acid are removed from the culture medium. The failure of 9-*cis*-treated cells to proliferate and their peculiar morphology indicates that all-*trans*- and 9-*cis*-retinoic acid have distinct biological properties. Although shorter, 3-day treatment with 9-*cis*-retinoic acid does not significantly reduce clonogenicity in these cells [17], one explanation for the morphology of 9-*cis*-retinoic acid-treated cells after a longer exposure and subsequent washout is that the cells are undergoing apoptosis.

FACS analysis of cells treated with all-trans- *or* 9-cis-*retinoic acid*

To determine whether treatment with 9-*cis*-retinoic acid followed by washout and continued incubation in the absence of retinoic acid results in apoptosis, the experiment was repeated and the ethanol-, all-*trans*- and 9-*cis*-retinoic acid-treated cell cultures analysed by flow cytometry at 5 days after treatment and 4 days after washout of the retinoid (day 9). At 5 days (day 0 of washout), no apoptosis was apparent in the cell cultures (Figure 3). This was also the case in the ethanol and all-*trans*-retinoic acid-treated cultures after 9 days (4 days after washout). Conversely, apoptotic cells accounted for 35–40% of cells in the 9-*cis*-retinoic acid-treated cultures at 9 days (4 days after washout) (Figure 3). Apoptosis was not apparent with a continuous culture of cells in the presence of 9-*cis*- or all-*trans*-retinoic acid (data not shown) and was thus specific to the washout experimental design. These data further confirm the view that 9-*cis*- and all-*trans*-retinoic acid have distinct biological effects.

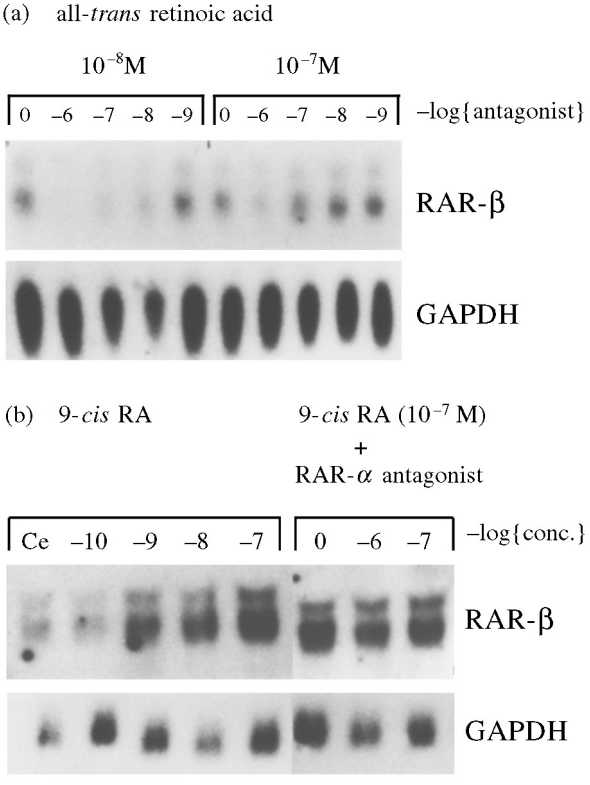

Figure 1. Induction of RAR-β by all-*trans*- and 9-*cis*-retinoic acid and the effects of an RAR-α-specific antagonist. Northern blots of total RNA extracted from SH-SY-5Y cells treated for 6 h with either all-*trans*- (a) or 9-*cis*-retinoic acid (b) in the presence or absence of the RAR-α antagonist Ro 41-5253 [21]. In (a), all-*trans*-retinoic acid was used at concentrations of 10^{-8} and 10^{-7} M and the antagonist at concentrations from 10^{-9} to 10^{-6} M. In (b), SH-SY-5Y cells were treated with 10^{-10}–10^{-7} M 9-*cis*-retinoic acid alone, or at 10^{-7} M 9-*cis*-retinoic acid with antagonist concentrations of 10^{-7}–10^{-6} M. Ce, control ethanol; GAPDH, blot probed with a glyceraldehyde-3-phosphate dehydrogenase cDNA as a loading control.

DISCUSSION

Both retinoic acid isomers induced morphological differentiation of SH-SY-5Y cells which was maintained in the continuous presence of retinoid, with 9-*cis*-retinoic acid inducing a more pronounced increase in neurite length. This is in agreement with previous data for SH-SY-5Y and LA N-5 human neuroblastoma cells showing that 9-*cis*- is more effective than all-*trans*-retinoic acid at promoting morphological differentiation at concentrations greater than 10^{-7} M [17, 18]. After removal of the retinoids from the culture medium, there were significant differences between the two isomers and, in contrast to all-*trans*-retinoic acid, cells pretreated with 9-*cis*- developed morphological changes consistent with apoptosis [25] and confirmed by flow cytometry. These data imply that the continued presence of retinoic acid is required for the maintenance of a differentiated phenotype in SH-SY-5Y cells but indicate that the two retinoic acid isomers have distinct biological effects on SH-SY-5Y neuroblastoma cells.

The dependence of cells treated with 9-*cis*-retinoic acid on the continued presence of 9-*cis*-retinoic acid in the culture medium raises interesting questions concerning the mechanism of apoptosis under washout conditions and the fact that all-*trans*-retinoic acid does not induce apoptosis under similar conditions.

It is becoming increasingly clear that cell death, cell survival and growth are in a state of equilibrium [26] with a number of proteins involved in programmed cell death and maintaining survival. Additionally, specific neurotrophic factors have been identified which are responsible for maintaining the viability of neuronal cells. For example, insulin-like growth factors (IGF) are important survival factors for neuroblastoma cells [27, 28] and are induced by retinoic acid in neuroblastoma cells [29–32]. High levels of Bcl-2 in cells prevent cell death in response to a variety of stimuli [33] and all-*trans*-retinoic acid acts as a survival factor for sympathetic neurones [34]. Furthermore, the differentiated phenotype in SH-SY-5Y cells occurring in response to all-*trans*-retinoic acid treatment has been associated with a high expression of Bcl-2 [35, 36] which is also involved in the regulation of neural differentiation [37]. Thus, treating SH-SY-5Y cells with all-*trans*-retinoic acid will result in increased Bcl-2 expression, neuronal differentiation and an inhibition of cell death. In contrast to N-type neuroblastoma cells, all-*trans*-retinoic acid induces apoptosis in S-type neuroblastoma cells [38]; since these express low levels of Bcl-2 [35], the activities of inducers of cell death may predominate.

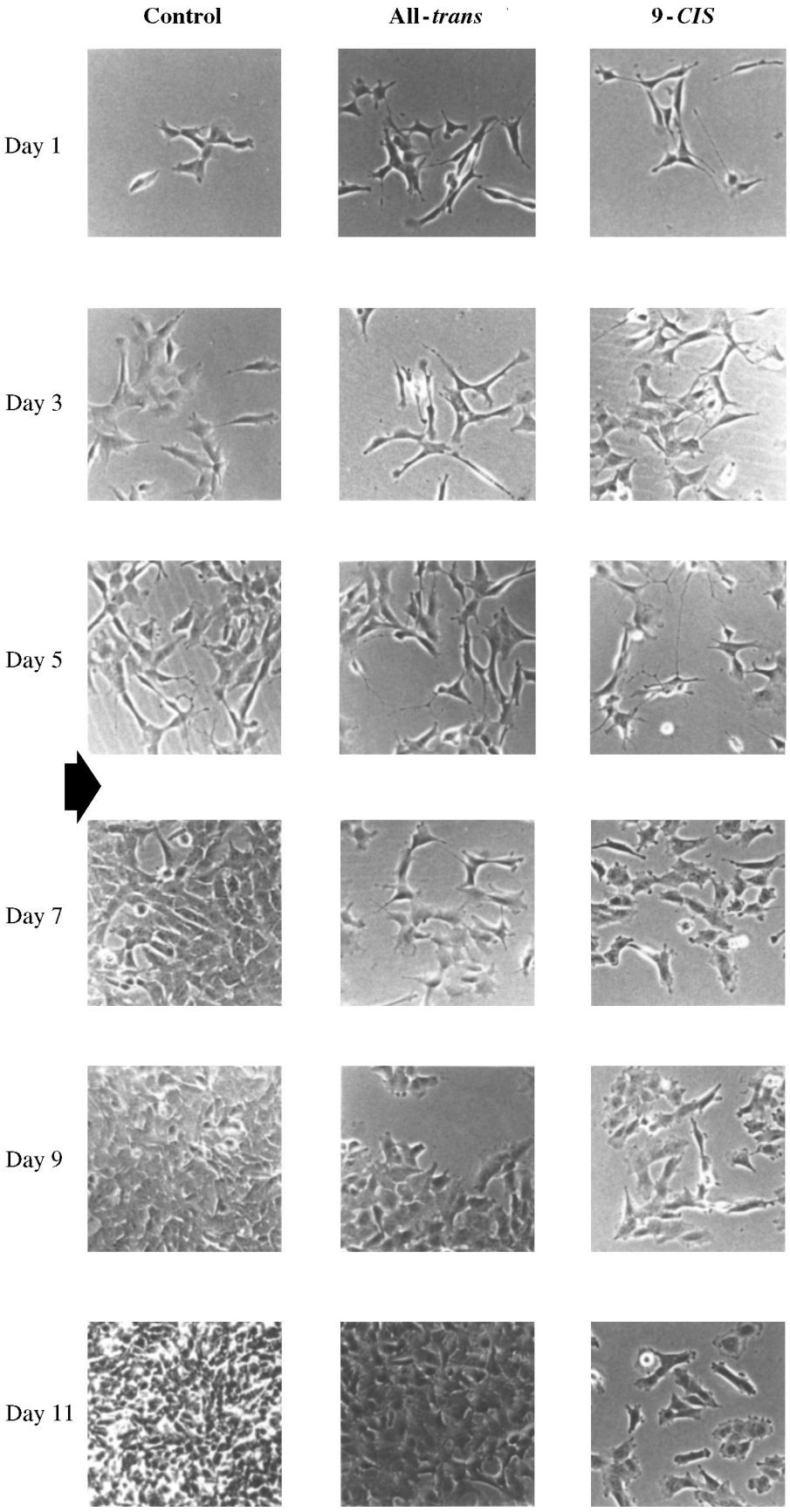

Figure 2. Morphology of SH-SY-5Y neuroblastoma cells treated with ethanol (control), all-*trans*- or 9-*cis*-retinoic acid (10^{-6} M) for 5 days, followed by subsequent culture in the absence of retinoic acid for a further 6 days (marked by the arrow). The cells were photographed at 2-day intervals from day 1.

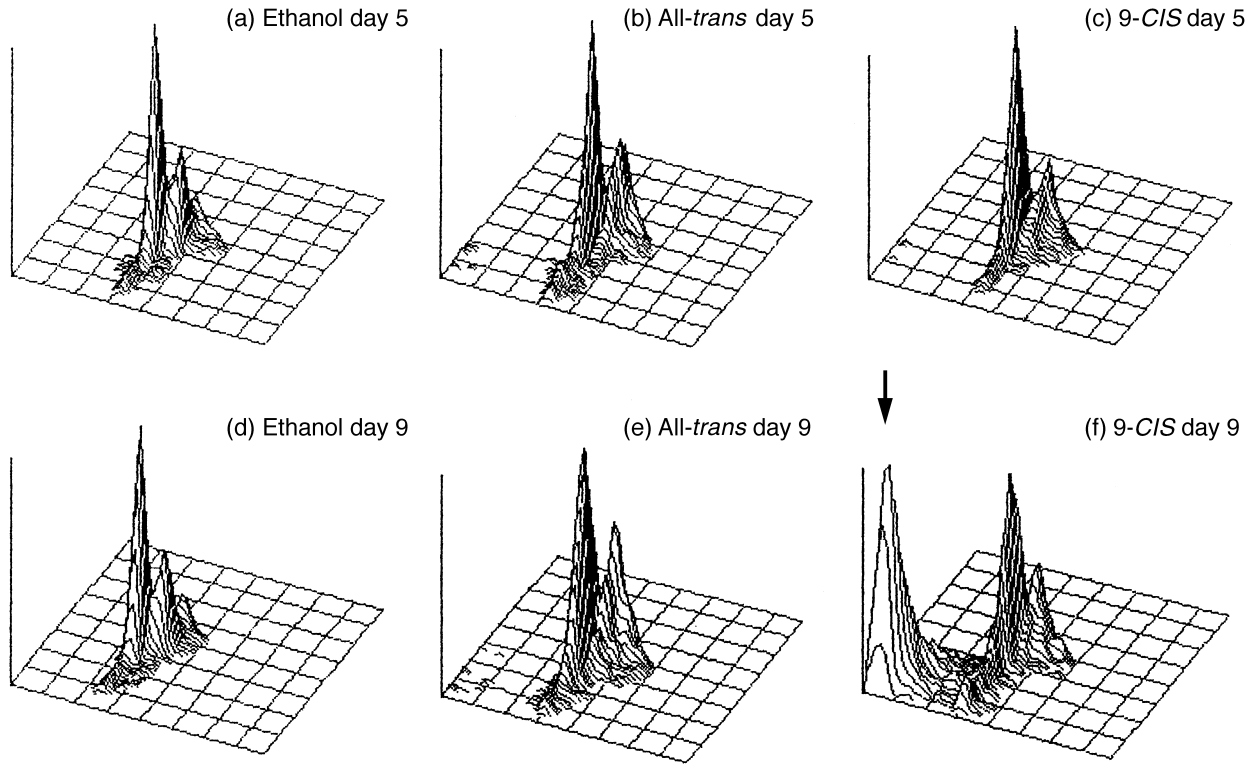

Figure 3. FACS analysis of SH-SY-5Y cells treated with ethanol (a, d), all-*trans*-retinoic acid (b, e) or 9-*cis*-retinoic acid (c, f) at 10^{-6} M for 5 days and then cultured in the absence of retinoic acid for a further 4 days. The cell cultures were analysed by flow cytometry (see Materials and Methods) to estimate the degree of apoptosis at days 5 (the day of washout) and 9. Apoptotic bodies were only detectable in cultures pretreated with 9-*cis*-retinoic acid: the peak of apoptotic cells is marked with an arrow in (f).

The reason why, in this context, 9-*cis*-retinoic acid caused apoptosis of N-type SH-SY-5Y cells after washout is not clear. A possible explanation could be that *Bcl-2* expression is downregulated after withdrawal of 9-*cis*-, allowing cells to enter a programmed cell death pathway. Previous gene induction experiments using short-term (6 h) treatment of SH-SY-5Y cells have shown that 9-*cis*-retinoic acid-induced gene expression is rapidly reduced after washout of 9-*cis*-retinoic acid [23], in contrast to all-*trans*. If this is also true for longer-term (days) treatment with 9-*cis*-retinoic acid, this could explain the apoptosis of SH-SY-5Y cells after 9-*cis*-retinoic acid washout in the context of the induced expression of Bcl-2 and other survival factors. The differential stability of induced gene expression in response to all-*trans*- or 9-*cis*-retinoic acid is evidence to support the view that the mechanism of gene induction differs between these two retinoic acid isomers [23]. The experiments with the RAR-α-specific antagonist provide further support for this concept.

Since the RAR-α-specific antagonist inhibited the induction of RAR-β in response to all-*trans*-retinoic acid, this suggests that RAR-α is the main receptor mediating the induction of RAR-β by all-*trans*-retinoic acid. SH-SY-5Y neuroblastoma cells thus show some similarity to PC12 cells where mutant RAR-α acts in a dominant negative manner and inhibits RAR-β induction in response to all-*trans*-retinoic acid [39, 40]. Conversely, the inability of the RAR-α-specific antagonist to inhibit the induction of RAR-β by 9-*cis*-retinoic acid indicates that, in these cells, different receptor mechanisms mediate all-*trans*- and 9-*cis*-dependent responses. Although we have previously suggested that RXR homodimers are the primary mediators of 9-*cis*-retinoic acid effects, this is still conjecture and it is not possible to determine whether 9-*cis*-retinoic acid is activating via RXR homodimers, heterodimers between RXRs and other nuclear receptors, or RXR-RAR-γ heterodimers.

Neuroblastoma is a heterogeneous disease, with a spectrum from the primitive neuroblast to the well-differentiated ganglion cell occurring in the same tumour [41]. The response to treatment is likely to vary according to the degree of inherent neuronal differentiation. In recent years, there has been considerable focus directed towards the potential use of drugs able to induce differentiation of neuroblastoma *in vitro*. Since apoptosis is clearly an important mechanism of spontaneous tumour regression in stage 4s disease [42], future direction towards effective therapy should be aimed at producing this biological phenomenon *in vivo*. As retinoids can induce both differentiation and apoptosis in neuroblastoma and phase I trials of 9-*cis*-retinoic acid in the treatment of adult malignancies have now been reported [43, 44], these results suggest that 9-*cis*-retinoic acid may have potential for the treatment of neuroblastoma in children and have implications for the design of treatment schedules.

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Acknowledgements—This research was supported by grants from the North of England Cancer Research Campaign, the North of England Children's Cancer Research Fund and Consiglio Nazionale Delle Richerche, Italy.