Differential effects of 9-*cis* and all-*trans* retinoic acid on the induction of retinoic acid receptor- β and cellular retinoic acid-binding protein II in human neuroblastoma cells

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The objective of this study was to compare the properties of 9-*cis* and all-*trans* retinoic acid with respect to the induction of expression of retinoic acid receptor β (RAR- β) and cellular retinoic acid-binding protein (CRABP) II in human neuroblastoma SH SY 5Y cells. RAR- β and CRABP II mRNA was induced by both all-*trans* and 9-*cis* retinoic acid in SH SY 5Y cells. Induction was rapid, detectable within 2–4 h, and inhibited by actinomycin D. Time-courses of induction for RAR- β and CRABP II differed: RAR- β mRNA levels reached a maximum 4–6 h after adding all-*trans* or 9-*cis* retinoic acid, whereas CRABP II mRNA levels increased over at least 18 h. These differences were attributed to the longer half-life of CRABP II mRNA (20 h) compared with RAR- β mRNA (3.9 h). The dose–response characteristics of all-*trans* and 9-*cis* retinoic acid were different:

all-*trans* was effective at nanomolar concentrations, whereas 10-fold higher levels of 9-*cis* retinoic acid were required to achieve comparable induction of RAR- β and CRABP II. Conversely, at high concentrations, 9-*cis* retinoic acid gave a greater induction of RAR- β and CRABP II than all-*trans*. The induction of RAR- β and CRABP II by all-*trans* retinoic acid was maintained in the subsequent absence of all-*trans* retinoic acid, whereas induction by 9-*cis* retinoic acid was dependent on its continued presence in the culture medium. These results suggest that, at high concentrations, 9-*cis* retinoic acid may produce its transcriptional effects via retinoid X receptor (RXR) homodimers. This has implications for the cellular functions of 9-*cis* retinoic acid and its use as a biological response modifier.

INTRODUCTION

As a regulatory molecule, retinoic acid has wide-ranging importance with respect to development, morphogenesis, carcinogenesis and the maintenance of epithelial phenotypes [1]. *In vitro*, retinoic acid promotes the differentiation and inhibits the proliferation of some tumour cell lines, and this has led to clinical trials of retinoic acid to treat neuroblastoma and other neoplastic diseases [2].

Retinoic acid regulates gene expression by binding to specific nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs) [3,4]. Low-molecular-mass cytosolic proteins, cellular retinol-binding protein (CRBP) and cellular retinoic acid-binding protein (CRABP), are involved in regulating the availability of all-*trans* retinoic acid to the nuclear receptors [5]. RXRs function as auxiliary proteins which enable RARs to bind to specific retinoic acid response elements (RAREs) present in the 5' regulatory sequences of retinoic-acid-regulated genes [6]. RARs thus appear to function predominantly as heterodimers with RXRs (for review see [7]). Structurally, RXRs are similar to RARs, although forming a distinct class of nuclear receptors [4]; their similarity to RARs prompted a search for a retinoic acid-type ligand, culminating in the identification of 9-*cis* retinoic acid as a high-affinity ligand for RXRs [8,9]. Although 9-*cis* retinoic acid promotes the formation of RXR homodimers, it is not clear whether 9-*cis* retinoic acid is important as a ligand in RAR–RXR heterodimers. Furthermore, despite the value of 'mobility-shift' and transient transfection experiments in elucidating interactions between response elements and RARs, there is little quantitative

information on how endogenous genes respond to different retinoids.

The objective of this study was to make a direct comparison between 9-*cis* and all-*trans* retinoic acid with respect to the induction of expression of two genes, RAR- β and CRABP II, in human neuroblastoma SH SY 5Y cells. Retinoic acid induces the expression of RAR- β in many cell types [10–12] including neuroblastoma cells [13]. We show here that CRABP II is also induced by retinoic acid in SH SY 5Y neuroblastoma cells. Phase III clinical trials using 13-*cis* retinoic acid to treat neuroblastoma patients are in progress and the possibility that neuroblastoma cells may give a better response to 9-*cis* retinoic acid is of particular relevance for the use of retinoic acid as a biological response modifier. The results of this study suggest that the regulation of gene expression by all-*trans* and 9-*cis* retinoic acid is achieved by different mechanisms and this has implications for the potential clinical use of 9-*cis* retinoic acid or stable analogues.

MATERIALS AND METHODS

Cell culture

Human SH SY 5Y neuroblastoma cells [14] were maintained in Dulbecco's modification of Eagle's medium containing 2 mM L-glutamine, supplemented with 10% fetal bovine serum (FBS) and grown at 37 °C with 5% CO₂ in air. Cells were seeded at a density of 8×10^6 cells per 75 cm² flask (1.07×10^5 cells per cm² growth area) 12–24 h before treatment with either all-*trans* or 9-*cis* retinoic acid. Retinoic acid was added to the cells in ethanol (0.1% of total medium volume) to achieve final concentrations

Abbreviations used: CRABP, cellular retinoic acid-binding protein; CRBP, cellular retinol-binding protein; DR, direct repeat; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); RAR, retinoic acid receptor; RARE, retinoic acid response element; RXR, retinoid 'X' receptor; $t_{1/2}$, half-life.

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of 1×10^{-10} to 2×10^{-7} M, assuming a molar absorption coefficient of $36500 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 343 nm for all-*trans* and 9-*cis* retinoic acid. Fresh retinoic acid solutions were prepared for each experiment. An equal volume of ethanol was added to control cells. For retinoic acid washout experiments, cells were treated with 1×10^{-7} M retinoic acid for 6 h, the medium was then removed and the cells washed twice with 10 ml of PBS before adding fresh medium lacking retinoic acid. Where appropriate, actinomycin D (Sigma, Poole, Dorset, U.K.) was added in ethanol to a final concentration of $5 \mu\text{g} \cdot \text{ml}^{-1}$. All-*trans* retinoic acid was from Sigma; 9-*cis* retinoic acid was a gift from M. Klaus, Basel, Switzerland.

Stability of retinoic acid isomers *in vitro*

To assess the stability of all-*trans* and 9-*cis* retinoic acid in the presence of SH SY 5Y cells, cells were seeded into 24-well plates (approx. 1 cm^2 growth area per well) at a density of 1×10^5 cells per cm^2 . After overnight incubation in 2 ml of medium per well, the medium was replaced with 200 μl of fresh medium containing 1×10^{-7} M all-*trans*-[11,12- ^3H (N)]retinoic acid ($100 \text{ GBq} \cdot \text{mmol}^{-1}$) or 9-*cis*-[11,12- ^3H (N)]retinoic acid ($38\text{--}100 \text{ GBq} \cdot \text{mmol}^{-1}$) and incubated for a further 6 h. These conditions of cell density and culture medium volume were comparable with experiments with larger numbers of cells in 75 cm^2 flasks. At the end of the incubation period, 20 μl aliquots of the medium were added to 50 μl of methanol containing unlabelled all-*trans* or 9-*cis* retinoic acid [$(0.8\text{--}1.4) \times 10^{-5}$ M], centrifuged to remove insoluble material and the supernatant analysed by reversed-phase h.p.l.c. Culture wells containing medium but no cells were used as controls. Remaining cells were scraped off in 100 μl of methanol containing unlabelled all-*trans* or 9-*cis* retinoic acid [$(0.8\text{--}1.4) \times 10^{-5}$ M]; insoluble material was pelleted by centrifugation, the pellet was extracted once with 50 μl of chloroform/methanol/acetic acid (35:15:1, by vol.) and this extract combined with the methanolic supernatant. After evaporation under vacuum, this material was dissolved in methanol and analysed by h.p.l.c.

RNA extraction and Northern blotting

Cells were detached from the culture flasks with 1 ml of 0.05% (w/v) trypsin/0.02% (w/v) EDTA. Trypsin was neutralized with 0.25 ml of FBS and the cells washed once with 0.5 ml of PBS. Cells were resuspended in 0.4 ml of PBS and cytoplasmic RNA extracted by lysis with Nonidet P-40 and sodium deoxycholate as described by Wilkinson [15]. Nuclei were removed by centrifugation at $6000 g$ for 30 s and the supernatant extracted once with 500 μl of phenol/chloroform (4:1, v/v) containing 1% SDS, twice with 500 μl of 1:1 phenol/chloroform and once with 500 μl of chloroform. After ethanol precipitation, RNA samples (20 μg per track) were size-fractionated by electrophoresis through 1.2% agarose/formaldehyde gels, transferred to nylon membranes (Amersham International, Aylesbury, Bucks., U.K.) and probed with ^{32}P -labelled cDNA probes as described previously [12]. Blots were exposed to X-ray film (Amersham International) and the autoradiographic image intensity measured using an image analysis system (UVP, Cambridge, U.K.). The cDNA probes for human RAR- β , human CRABP II and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (loading control) were as described previously [16].

Reversed-phase h.p.l.c.

Retinoic acid isomers were analysed by isocratic elution with acetonitrile/methanol/water/acetic acid (23:7:10:0.2, by vol.)

at a flow rate of $1.5 \text{ ml} \cdot \text{min}^{-1}$ from a Beckman (High Wycombe, Bucks., U.K.) Ultrasphere 3 μm ODS column (75 mm $\times$ 4.6 mm). The eluate was monitored at 343 nm and authentic all-*trans* retinoic acid and 9-*cis* retinoic acid used as standards. To prepare tritium-labelled 9-*cis* retinoic acid, all-*trans*-[11,12- ^3H (N)]retinoic acid ($1824 \text{ GBq} \cdot \text{mmol}^{-1}$, Du Pont-NEN, Stevenage, Herts., U.K.) was exposed for 10 s to a 400 W mercury vapour lamp (approx. 20 cm from the light source); unlabelled 9-*cis* retinoic acid (400 pmol) was then added to the photoisomerisate and 9-*cis*-[11,12- ^3H (N)]retinoic acid purified by reversed-phase h.p.l.c. with the u.v. detector turned off. Subsequent re-analysis showed that the 9-*cis*-[11,12- ^3H]retinoic acid was 93% and 77.5% with specific radioactivities of $38 \text{ GBq} \cdot \text{mmol}^{-1}$ and $100 \text{ GBq} \cdot \text{mmol}^{-1}$ respectively. 9-*cis*-[11,12- ^3H (N)]Retinoic acid was dried under vacuum and redissolved in culture medium to a final concentration of 1×10^{-7} M for addition to SH SY 5Y cells.

RESULTS

Induction of RAR- β and CRABP II by all-*trans* and 9-*cis* retinoic acid

We have previously shown that RAR- β mRNA is induced in SH SY 5Y neuroblastoma cells in response to all-*trans* retinoic acid [13]. Of the two transcript sizes detected on Northern blots, the lower (3.1 kb) transcript is predominantly induced [13]. In preliminary experiments we found that CRABP II mRNA (1.2 kb) was also induced by all-*trans* retinoic acid in SH SY 5Y cells. To study the relative potencies of all-*trans* and 9-*cis* retinoic acid in regulating gene expression in SH SY 5Y cells, we compared the expression of the 3.1 kb RAR- β and 1.2 kb CRABP II transcripts in response to a short, 6-h-long exposure to increasing doses of each retinoic acid isomer. While both all-*trans* and 9-*cis* retinoic acid induced RAR- β and CRABP II mRNA (Figures 1 and 2), there were marked differences in their dose-response characteristics. All-*trans* retinoic acid induced RAR- β at low doses of 1×10^{-9} M, with the response reaching a plateau at 1×10^{-8} to 1×10^{-7} M (Figures 1 and 2a). In contrast, 9-*cis* retinoic acid was much less effective at low doses, producing

Figure 1 Dose-dependent induction of RAR- β and CRABP II by all-*trans* and 9-*cis* retinoic acid

Northern blot of RNA extracted from SH SY 5Y cells after treatment with all-*trans* and/or 9-*cis* retinoic acid for 6 h. The blot was probed successively with RAR- β , CRABP II and GAPDH cDNA probes. c, control ethanol.

Figure 2 Dose-dependent induction of RAR-β and CRABP II

Results from densitometric analyses of three experiments in which SH SY 5Y cells were exposed to increasing concentrations of all-*trans* (●) or 9-*cis* (○) retinoic acid, and two experiments in which both isomers (□) were used together. Although each experiment was done with doses ranging over three orders of magnitude from 10^{-10} M, for one experiment, 2-fold higher doses were used at each point and therefore the data from each experiment are plotted separately rather than combined to give a mean and S.E.M. Lines represent logistic curves fitted to the data using Sigmaplot curve-fitting software; the broken lines are for experiments in which both isomers were used together. (a) Induction of RAR-β (lower transcript): after correcting for RNA loading using the GAPDH signal, data are expressed as a percentage of signal obtained with 1×10^{-7} M all-*trans* retinoic acid in each experiment, such that control = 0 and 1×10^{-7} M all-*trans* retinoic acid signal = 100%. (b) Induction of CRABP II, expressed as a percentage of response with 1×10^{-7} M all-*trans* retinoic acid, as in (a).

Figure 3 Time course of induction of RAR-β and CRABP II after treatment with 1×10^{-7} M all-*trans* or 9-*cis* retinoic acid

(a) Northern blot. The smaller transcript of RAR-β is arrowed. Abbreviations: c, control; at, all-*trans* retinoic acid; 9c, 9-*cis* retinoic acid. (b) Densitometric analysis of data for RAR-β (lower transcript) from three experiments. After correcting for variations in RNA loading using the GAPDH signal, the data were expressed as a percentage of RAR-β signal obtained with 1×10^{-7} M all-*trans* retinoic acid after 18 h incubation (see legend to Figure 2). (c) Densitometric analysis of data for CRABP II from three experiments. Data are expressed as percentages of RAR-β signal obtained with 1×10^{-7} M all-*trans* retinoic acid after 18 h incubation, as above. Error bars on (b) and (c) are ± S.E.M.

comparable induction only at 10-fold higher concentrations (Figures 1 and 2a). However, at 9-*cis* retinoic acid concentrations of 1×10^{-7} M or above, the level of induction of RAR-β was considerably greater than with a comparable concentration of all-*trans* retinoic acid. CRABP II showed similar induction characteristics, 9-*cis* retinoic acid inducing a better response than all-*trans* at 1×10^{-7} M but being less effective at 1×10^{-9} M (Figures 1 and 2b).

Figure 4 The effect of actinomycin D on induction of RAR- β and CRABP II mRNA

SH SY 5Y cells were treated with either all-*trans* (at) or 9-*cis* (9c) retinoic acid for 6 h in the presence (D) or absence of $5 \mu\text{g} \cdot \text{ml}^{-1}$ actinomycin D. One experiment with 9-*cis* retinoic acid was carried out in the presence of the protein synthesis inhibitor cycloheximide (H, final concentration $10 \mu\text{g} \cdot \text{ml}^{-1}$). The blot was probed for RAR- β , CRABP II and GAPDH as before. c, control.

Figure 5 The stability of RAR- β and CRABP II mRNA and the effect of removal of retinoic acid on RAR- β and CRABP II mRNA levels

SH SY 5Y cells were treated with 1×10^{-7} M all-*trans* retinoic acid for 6 h (at, tracks 3,5,6,9,10,13 and 14) or 9-*cis* retinoic acid for 6 h (9c, tracks 4,7,8,11,12,15 and 16). The medium was then removed and replaced (see Materials and methods section) with medium lacking retinoic acid (tracks 6,8,10,12,14 and 16) or lacking retinoic acid and containing actinomycin D (D, tracks 5,7,9,11,13 and 15). The cells were then incubated for 2 h (tracks 5–8), 5 h (tracks 9–12) or 8 h (tracks 13–16) before extracting RNA. The Northern blot was probed successively for RAR- β , CRABP II and GAPDH as before. c, control (tracks 1 and 2). The induced, pre-washout tracks are 3 (all-*trans*) and 4 (9-*cis*). RAR- β transcripts in actinomycin D-treated cells seem to be consistently smaller in size than in non-treated cells. The reason for this is not clear.

The time course of response after exposure of SH SY 5Y cells to 1×10^{-7} M all-*trans* or 9-*cis* retinoic acid is shown in Figure 3: induction of CRABP II or RAR- β was detectable within 4 h of addition of either retinoic acid isomer. However, the induction of RAR- β was faster than CRABP II and detectable only 2 h after treatment with either all-*trans* or 9-*cis* retinoic acid (Figure 3). Furthermore, RAR- β mRNA levels reached a steady state after 4–6 h induction with either all-*trans* or 9-*cis* retinoic acid, whereas CRABP II transcripts continued to increase in abundance, reaching a maximum after 13–18 h. Again, RAR- β and CRABP II mRNA were induced to higher levels with 9-*cis* retinoic acid than with the all-*trans* isomer (Figure 3).

Differential stability of RAR- β and CRABP II mRNA

It has been shown previously that the induction of RAR- β mRNA by all-*trans* retinoic acid in human Hep G2 cells occurs

Figure 6 The half-life of induced RAR- β and CRABP II mRNA

Signal intensities, relative to GAPDH and expressed as a percentage of the signal at time 0, for RAR- β (●, lower transcript) and CRABP II (□) are plotted on a natural log scale. Error bars: $\pm$ S.E.M. The half-lives relative to GAPDH were calculated from the fitted regression line. Inset: decrease in GAPDH mRNA signal intensity with increasing time of actinomycin D treatment. These data were used to estimate the GAPDH mRNA half-life in order to derive absolute half-life values for RAR- β and CRABP II (see text).

at a transcriptional level [10]. This is also the case for the induction of CRABP II in human fibroblasts [17]. To provide indirect confirmation that increased RAR- β and CRABP II mRNA in SH SY 5Y cells resulted from an increased rate of transcription, cells were incubated for 6 h with all-*trans* or 9-*cis* retinoic acid in the presence or absence of actinomycin D. The induction of RAR- β and CRABP II was completely abolished by actinomycin D (Figure 4). The induction of RAR- β by all-*trans* retinoic acid in SH SY 5Y cells is unaffected by the protein synthesis inhibitor cycloheximide [18], and this was also true for the induction of RAR- β and CRABP II by 9-*cis* retinoic acid (Figure 4).

In the presence of actinomycin D, CRABP II mRNA levels remained similar to control cells, whereas RAR- β mRNA was reduced to undetectable levels (Figure 4). CRABP II and RAR- β mRNA may thus differ markedly in their stability. To confirm this, SH SY 5Y cells were induced for 6 h with either all-*trans* or 9-*cis* retinoic acid (1×10^{-7} M) and then incubated for up to 8 h in fresh medium lacking retinoic acid but containing actinomycin D. Under these conditions, RAR- β mRNA decreased rapidly, with a half-life ($t_{1/2}$) relative to GAPDH of 4.7 h (Figures 5 and 6). Conversely, CRABP II mRNA increased relative to GAPDH (Figures 5 and 6). The GAPDH half-life was estimated to be 7.7 h, assuming equal RNA loading in all tracks (Figure 6, inset), and this value was used to correct the RAR- β and CRABP II data, giving $t_{1/2}$ values of 3.9 and 20.3 h for RAR- β and CRABP II respectively. There was no evidence for differences in mRNA stability between cells treated with all-*trans* or 9-*cis* retinoic acid. Furthermore, since CRABP II mRNA levels in non-retinoic acid-treated cells incubated with actinomycin D for 6 h remained similar to control cells (without actinomycin D, Figure 4), there was no indication of an increase in CRABP II mRNA stability in response to retinoic acid. This adds additional weight to the

Figure 7 The expression of RAR- β and CRABP II after induction with all-*trans* or 9-*cis* retinoic acid and subsequent washout

(a) RAR- β : results are expressed as a percentage of RAR- β signal after treatment with all-*trans* (●) or 9-*cis* (□) retinoic acid for 6 h (washout time zero), i.e. at time zero, the signal for all-*trans* retinoic acid-treated cells is 100% (control is 0%, dashed line). Similarly, for 9-*cis* retinoic acid-treated cells, the time-zero signal (6 h) is taken as 100%. Note that the level of induction by 9-*cis* was higher than for all-*trans* retinoic acid (see Figure 6). (b) CRABP II, details as for (a). Each point is the mean of three or four experiments; error bars: $\pm$ S.E.M.

conclusion that the induction of CRABP II mRNA resulted from increased transcription.

RAR- β and CRABP II mRNA levels after induction and removal of retinoic acid

The previous experiments suggest that the induction of RAR- β and CRABP II mRNA by both all-*trans* and 9-*cis* retinoic acid resulted from transcriptional activation; the longer half-life of CRABP II mRNA may account for the differences in induction time course between RAR- β and CRABP II. The stability of transcriptional activation of both RAR- β and CRABP II was studied by exposing the SH SY 5Y cells to 1×10^{-7} M all-*trans* or 9-*cis* retinoic acid for 6 h, then replacing the medium with fresh medium without retinoic acid after washing the cells extensively

Figure 8 Reversed-phase h.p.l.c. of 9-*cis*-[11,12- 3 H(N)]retinoic acid after incubation with SH SY 5Y cells

The radioactive profile of the starting material is shown in (a). An analysis of the culture medium after a 6 h incubation with cells is shown in (b) and material remaining with the cell pellet in (c). Abscissa: time after injection of material on to the Beckman Ultrasphere 3 μ m reversed-phase column. Ordinate: d.p.m. per fraction; fraction size: 0.2 min. Arrows indicate the elution positions of authentic all-*trans* (1), 9-*cis* (2) and 13-*cis* (3) retinoic acid standards.

(see the Materials and methods section). In cells treated with all-*trans* retinoic acid, there was an initial decrease in RAR- β mRNA levels 2 h after washout, but these returned to induced levels within 8 h of washout and remained elevated, relative to control uninduced cells, until at least 24 h after washout (Figures 5 and 7a). A very different result was obtained with 9-*cis* retinoic acid: even though the level of induction of RAR- β was initially higher with 1×10^{-7} M 9-*cis* retinoic acid, by 2 h after washout RAR- β mRNA levels had fallen to near control values and were

Figure 9 Reversed-phase h.p.l.c. of all-*trans*-[11,12-³H(N)]retinoic acid after incubation with SH SY 5Y cells

The radioactive profile of the control medium (no cells) after a 6 h incubation is shown in (a). The radioactive profile of the culture medium of SH SY 5Y cells after a 6 h incubation is shown in (b) and material remaining with the cell pellet in (c). Abscissa: time after injection of material on to the Beckman Ultrasphere 3 μ m reversed-phase column. Ordinate: d.p.m. per fraction; fraction size: 0.2 min. Arrows indicate the elution positions of authentic all-*trans* (1), 9-*cis* (2) and 13-*cis* (3) retinoic acid standards.

lower than control by 5 to 8 h, returning to near control values only at about 24 h (Figures 5 and 7a).

In the same experiments, CRABP II mRNA levels remained elevated after washout of all-*trans* retinoic acid, but continued to increase so that at 24 h the signal intensity was about twice the initial induced level (Figures 5 and 7b). This implies that the initial transcriptional stimulus provided by all-*trans* retinoic acid treatment remained in effect despite the removal of retinoic acid from the culture medium. After washout of 9-*cis* retinoic acid, CRABP II mRNA remained at a steady level for the first 2–8 h but had decreased to near control values by 24 h after washout

(Figures 5 and 7b); this decrease was consistent with a half-life of 20 h for CRABP II mRNA, assuming that the transcriptional stimulus was removed at the time of 9-*cis* retinoic acid washout.

Stability of all-*trans* and 9-*cis* retinoic acid *in vitro*

9-*cis* Retinoic acid is energetically less stable than the all-*trans* configuration [19] and, at photoequilibrium, 9-*cis* is a minor isomer with all-*trans* and 13-*cis* retinoic acid predominating [20]. The induction of RAR- β and CRABP II observed with high concentrations of 9-*cis* retinoic acid could, in part, be due to isomerization to all-*trans* retinoic acid during the experiment. To determine the stability of 9-*cis* retinoic acid *in vitro*, tritium-labelled 9-*cis* retinoic acid was cultured with or without SH SY 5Y cells using similar conditions of cell density and culture medium volume to the induction experiments. At the end of a 6 h-long incubation period with 9-*cis*-[11,12-³H(N)]retinoic acid, 9-*cis* retinoic acid was the major isomer present in the medium, with or without cells, but had decreased to approx. 80% of the amount present at the start of the experiment relative to other isomers (Figure 8). Up to one-third of this decrease could be accounted for by conversion into all-*trans* retinoic acid, and the rest by conversion into 13-*cis* retinoic acid and other isomers. Note, however, that although 9-*cis* retinoic acid was also the most abundant isomer in the material extracted from the cell pellet, all-*trans* retinoic acid was present in significant amounts (Figure 8). In similar experiments with all-*trans*-[11,12-³H(N)]retinoic acid, isomerization during a 6 h incubation was minimal and, unlike 9-*cis* retinoic acid, very little isomerization had occurred in material associated with the cell pellet (Figure 9).

DISCUSSION

The rapidity of induction of RAR- β and CRABP II mRNA by retinoic acid, coupled with its sensitivity to inhibition by actinomycin D, suggests that, as in other cell types [10,17], the induction of these genes in human SH SY 5Y neuroblastoma cells was achieved by a retinoic acid-dependent increase in the rate of transcription. Furthermore, at least for CRABP II, there was no evidence that mRNA induction was brought about by increased mRNA stability. Functional RAREs are present in the 5' regulatory regions of the RAR- β and mouse CRABP II genes. The RAR- β RARE (β RARE) is a DR+5 type, i.e. two (A/G)GTTCA direct repeats (DR) separated by a 5 bp spacer [7,21]. Conversely, mouse CRABP II has two putative RAREs, one of DR+2 type [AG(G/T)TCA] and the other a DR+1 type [TG(A/C)CCT] [22]. The human CRABP II gene has at least one putative D+1-type RARE and is transcriptionally regulated by retinoic acid in human skin fibroblasts [17]. The presence of these elements supports the conclusion that the induction of RAR- β and CRABP II in SH SY 5Y cells is an RAR-mediated transcriptional effect. Although the dose-response curves for RAR- β and CRABP II with each retinoic acid isomer were very similar, the time-course profiles differed considerably: RAR- β mRNA reached a constant level after 4–6 h whereas CRABP II mRNA continued to increase in abundance. This difference may be attributed, at least in part, to the greater stability of CRABP II mRNA relative to RAR- β mRNA.

For all-*trans* retinoic acid, half-maximal responses for the induction of both RAR- β and CRABP II were obtained at nanomolar concentrations of retinoic acid; this is consistent with high-affinity binding of all-*trans* retinoic acid to RARs for which K_d estimates range from 0.2 to 0.4 nM [23,24]. Although 9-*cis* retinoic acid is reported to bind RXRs and RARs with comparable affinities (K_d estimates: 1.4–2.2 nM for RXRs and

0.2–0.8 nM for RARs) [24], the dose–response curves for the induction of both RAR- β and CRABP II by 9-*cis* retinoic acid differed considerably from all-*trans*, with 9-*cis* retinoic acid being less effective than all-*trans* retinoic acid at low doses, and much more effective at high concentrations. These results imply that 9-*cis* retinoic acid does not act via RARs, and that its greater effects at high doses result from transcriptional induction via a different mechanism. However, a straightforward interpretation of these differences in dose–response characteristics is difficult for two reasons: (i) 9-*cis* retinoic acid apparently isomerizes readily within cells to form all-*trans* retinoic acid and other isomers, and (ii) the mechanisms by which 9-*cis* and all-*trans* retinoic acid reach the cell nucleus are unknown and not necessarily the same. Isomerization of 9-*cis* to all-*trans* retinoic acid could account for some induction of RAR- β and CRABP II at moderate doses of 9-*cis*, but this cannot explain its greater effects than all-*trans* retinoic acid at high doses unless there is synergism between the two isomers.

Current evidence favours the hypothesis that CRABPs are involved in the metabolic degradation of retinoic acid [25,26], but the possibility that these proteins are also involved in retinoic acid transport to the nucleus [27] cannot be ruled out. Unlike all-*trans* retinoic acid, 9-*cis* does not bind significantly to either CRABP I [28] or CRABP II [28,29], and it is possible that, for cells treated with comparable doses of all-*trans* or 9-*cis* retinoic acid, the effective concentration of 9-*cis* retinoic acid reaching the nucleus could be lower than for the all-*trans* isomer. However, some all-*trans* retinoic acid analogues do not bind to CRABP, yet effectively promote differentiation [30]; in view of the potential metabolic role of CRABPs, the fact that 9-*cis* retinoic acid does not bind to CRABP may enhance rather than limit its access to the nucleus.

At first sight, the strongest evidence from the present study for a different mechanism of induction by 9-*cis* retinoic acid comes from the washout experiments: 9-*cis* retinoic acid induced RAR- β to a higher extent than all-*trans* retinoic acid, yet, unlike RAR- β induction by all-*trans*, the subsequent removal of 9-*cis* retinoic acid resulted in a rapid decrease in RAR- β signal. The same effect was apparent with CRABP II where the resulting decrease in CRABP II mRNA to near control values was consistent with the CRABP II mRNA half-life. These results suggest that transcriptional activation by all-*trans* retinoic acid was maintained in the absence of all-*trans* retinoic acid in the culture medium, but activation by 9-*cis* retinoic acid was dependent on its continued presence. The simplest interpretation of these results is that 9-*cis* may dissociate rapidly from its receptors whereas the dissociation rate of all-*trans* retinoic acid from RARs may be slower. Consequently, 9-*cis* retinoic acid-dependent transcription complexes will be relatively unstable in the absence of free ligand by comparison with all-*trans* retinoic acid-dependent complexes. This interpretation relies on the assumption that intracellular concentrations of free all-*trans* and 9-*cis* retinoic acid are both lowered to similar extents by removal of the respective retinoic acid isomer from the culture medium. However, if CRABP is able to sequester and release free all-*trans* retinoic acid, then this assumption would be invalid. Alternatively, all-*trans* and 9-*cis* retinoic acid may have qualitatively different effects on patterns of gene expression in SH SY 5Y cells: all-*trans* retinoic acid may induce the synthesis of transcription factors that subsequently maintain RAR- β and CRABP II expression.

RARs are thought to act predominantly as heterodimers with RXRs [7]. Since all-*trans* retinoic acid was stable in the culture medium, at least for 6 h, and there was no appreciable isomerization within the cells during this time, the induction of CRABP

II and RAR- β was presumably mediated by the binding of all-*trans* retinoic acid to RAR–RXR heterodimers. 9-*cis* Retinoic acid is the ligand for RXRs, but unless endogenous 9-*cis* retinoic acid levels in SH SY 5Y cells were high, the effects produced with all-*trans* retinoic acid were likely to have been due to all-*trans* retinoic acid alone. If RXRs are indeed involved in the heterodimers, then RXRs can function as ligand-independent auxiliary factors.

RXRs are reported to form homodimers in the presence of 9-*cis* retinoic acid [31]. In the presence of ligand, RXR homodimers can bind to the β RARE [31,32], the DR + 2 ApoA1-RARE and the DR + 1 CRBP II RARE [31]. The ability of RARs and RXRs to activate transcription has previously been studied by transient co-transfection of receptor and reporter constructs into cell lines. These have shown that in the presence of RXRs, 9-*cis* retinoic acid can promote transcription from β RARE and mouse CRABP II RARE reporters [22,31,32]. Thus, in SH SY 5Y cells, the induction of RAR- β and CRABP II by 9-*cis* retinoic acid could also be mediated via RXR homodimers. Mobility-shift experiments have demonstrated that 9-*cis*, but not all-*trans* retinoic acid, promotes the formation of RXR homodimers in solution and that this interaction is strongly co-operative with respect to binding to specific response elements [31]. Homodimer formation presumably results from the preferential association of ligand-bound RXRs, requiring two molecules of 9-*cis* retinoic acid per homodimer. If homodimer association with the β RARE and CRABP II RAREs is strongly co-operative, this, and a requirement for two molecules of 9-*cis* retinoic acid per dimer, as opposed to one molecule of all-*trans* retinoic acid for a transcriptionally active RAR–RXR heterodimer, could explain the different dose–response curves obtained with all-*trans* and 9-*cis* retinoic acid. The greater response obtained with 9-*cis* than with all-*trans* retinoic acid, suggests that RXR homodimers are more effective transcriptional activators or that lower RAR levels limit a response to all-*trans* retinoic acid.

Evidence from this present study indicates that the effects of all-*trans* and 9-*cis* retinoic acid together are additive rather than synergistic. At low doses, 9-*cis* retinoic acid did not seem to enhance the response to all-*trans* retinoic acid. This would suggest that the potential binding of 9-*cis* retinoic acid to the RXR partner of RAR–RXR heterodimers has little effect on transcriptional function, with the caveat that the relative access of 9-*cis* and all-*trans* retinoic acid to the nucleus may not be the same. Since high concentrations of 9-*cis* retinoic acid can promote RXR-homodimer formation [31], it is likely that 9-*cis* retinoic acid has its effects largely via RXR homodimers. It has been suggested [31] that 9-*cis* retinoic acid does not prevent the formation of RAR–RXR heterodimers, although this may depend on the relative concentrations of 9-*cis* retinoic acid, RARs and RXRs. The potential for 9-*cis* retinoic acid to isomerize suggests that some of the effects of high doses of 9-*cis* could be due to all-*trans* retinoic acid acting via RAR heterodimers, but this is not supported by data from the washout experiments.

Overall, these results support the hypothesis that in SH SY 5Y neuroblastoma cells, 9-*cis* retinoic acid induces RAR- β and CRABP II expression via RXR homodimers. The greater potency of 9-*cis* retinoic acid in inducing RAR- β and CRABP II is also paralleled by its greater potency than all-*trans* retinoic acid in inducing differentiation of SH SY 5Y cells (P. E. Lovat, A. J. Malcolm, A. D. J. Pearson and C. P. F. Redfern, unpublished work). Other studies have also shown that 9-*cis* retinoic acid produces a better biological response than does all-*trans* retinoic acid, particularly with respect to the inhibition of activation-induced apoptosis in thymocytes [33] and the inhibition of vitamin-D-activated expression of osteocalcin [34].

This study highlights a number of important issues concerning the availability of different retinoic acid isomers to nuclear receptors, the function of CRABPs and the molecular interactions between receptors and ligands involved in regulating retinoic acid-dependent gene activity. In particular, the question of the relative contribution of RAR-RXR heterodimer and RXR homodimers to transcriptional responses needs to be addressed. Can 9-*cis* retinoic acid act synergistically with all-*trans* retinoic acid via RAR-RXR heterodimers, or are the greater transcriptional effects of 9-*cis* retinoic acid due to RXR homodimers? Stable analogues of 9-*cis* retinoic acid may be of value in answering these questions. In addition, further studies are required to elucidate the intracellular transport and physiology of different retinoic acid isomers. Answers to these questions will be important for the rational design of new drugs and therapies aimed at retinoic acid-regulated pathways.

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