Letter to the Editor

Overexpression of RAR γ increases death of SH-SY5Y neuroblastoma cells in response to retinoic acid but not fenretinide

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Dear Editor,

The retinoic acid receptors (RARs) have an important role in cell differentiation and death. Retinoic acid induces differentiation of neuroblastoma cells *in vitro*, a property that has led to the successful use of 13-*cis* retinoic acid, a prodrug for all-*trans* retinoic acid,¹ in the treatment of minimum residual disease of neuroblastoma patients.² All three RAR subtypes (α , β and γ) can be activated by all-*trans* or 9-*cis* retinoic acid, and these receptors function as heterodimers with retinoid-X receptors (RXRs) to enhance or drive the expression of target genes.³ Unlike the all-*trans* isomer, 9-*cis* retinoic acid activates both RARs and RXRs. Retinoic acid analogues (retinoids) with a narrower selectivity for the RAR subtypes are undergoing clinical and laboratory evaluation. One such retinoid is fenretinide (n-(4-hydroxyphenyl) retinamide), which is RAR β - and RAR γ -selective. Unlike retinoic acid, fenretinide is able to induce apoptosis in a range of neuroblastoma cell lines, including some which are resistant to retinoic acid.⁴ This makes fenretinide a potentially powerful agent for the treatment of neuroblastoma.

The induction of caspase-3-dependent death of neuroblastoma cells by fenretinide is linked to the activation of ceramide signalling pathways, 12-lipoxygenase activity and the induction of free radicals.⁵ RAR β/γ -selective antagonists inhibit fenretinide-driven death of neuroblastoma cells⁶ and this suggests that RARs may also be involved. Gel shift assays suggest that heterodimers of RAR γ and RXR β predominate in the neuroblastoma cell model used for our fenretinide studies.⁷ Since retinoic acid induces differentiation of neuroblastoma cells,⁸ fenretinide-induced death of these cells may result from activation of RAR γ in combination with fenretinide-generated free radicals or oxidative stress. This hypothesis leads to two predictions: (1) increased expression of RAR γ should increase cell death in response to fenretinide, and (2) increased levels of RAR γ would not affect cell death in response to retinoic acid since retinoic acid isomers induce differentiation without increasing oxidative stress.⁹ To test these predictions, we developed an SH-SY5Y cell model in which RAR γ is conditionally induced in response to tetracycline antibiotics. Two cell clones, SH-SY5Y^{Tet12-S2} (Tet12-S2) and SH-SY5Y^{Tet12-S4} (Tet12-S4) showing strong induction of RAR γ 24 h after addition of tetracyclines (tetracycline or the more-stable and water-soluble analogue doxycycline) were selected and characterised (Figure 1a). The induction of RAR γ was stable for at least 24 h after washout of the tetracycline inducer (Figure 1a). The Tet12-S2 and Tet12-S4

clones had similar characteristics with respect to the induction of RAR γ and were both used for experiments (Figure 1a). As with the parental SH-SY5Y cells,⁶ the apoptotic effects of fenretinide may be mediated at least in part by RAR γ since the RAR β/γ antagonists CD2848 and CD2665 inhibited the effects of fenretinide in reducing cell culture density (Figure 1b).

Although doxycycline at concentrations up to 4 μ g/ml decreased the density of parental SH-SY5Y cell cultures, at 1 μ g/ml the effect was small and not significantly different ($P > 0.25$) from control cells. In contrast, over a similar time-scale doxycycline reduced the density of Tet12-S4 cell cultures to 75.8% (± 6.2) of vehicle-treated control cells ($t_8 = -7.63$, $P < 0.0001$; Figure 1c). Since the RAR γ -inducible cells were otherwise more resistant to apoptosis-inducing agents such as fenretinide, with IC₅₀ values for fenretinide of around 3.5 μ M compared to 0.5–1 μ M for the parental SH-SY5Y cells, this suggests that overexpression of RAR γ , rather than the doxycycline inducer, reduced the rate of cell proliferation.

To test the hypothesis that RAR γ is involved in fenretinide-induced cell death, RAR γ -inducible cells were treated with fenretinide in the presence or absence of 1 μ g/ml doxycycline to induce RAR γ . There was a clear effect of fenretinide in reducing culture density with increasing dose (ANOVA, $F_{4,290} = 48.6$, $P < 0.0001$; Figure 1d, upper graph), but, contrary to our prediction that overexpression of RAR γ would decrease the density of fenretinide-treated cultures still further, induction of RAR γ expression resulted in a slight increase in cell density compared to uninduced cells ($F_{1,290} = 4.6$, $P = 0.033$; Figure 1d, upper graph). Flow cytometry of propidium-iodide-stained cells was used to measure apoptosis after treatment with fenretinide in the presence or absence of doxycycline to induce overexpression of RAR γ , and this showed no significant difference between RAR γ -induced and -uninduced cells in their apoptotic response to fenretinide ($F_{1,8} = 3.224$, $P = 0.11$; Figure 1d, lower graph). Therefore, it is clear that overexpression of RAR γ did not increase the apoptotic or antiproliferative effect of fenretinide in the way predicted.

The failure of RAR γ overexpression to increase fenretinide-induced apoptosis was unexpected given evidence from RAR β/γ antagonists that RARs are involved in mediating fenretinide effects.⁶ Since retinoic acid isomers also reduce the rate of proliferation of SH-SY5Y cells, a result of cell

differentiation rather than apoptosis,⁸ we asked whether overexpression of RAR γ would affect the response of these cells to retinoic acid. Tet12-S2 cells were responsive to all-*trans* retinoic acid, and these cells were more elongated with longer cell processes after treatment with 1 μ M all-*trans*

retinoic acid for 3 days (Figure 1e). Fenretinide had a slight effect on cell morphology, producing more-angular cells with less prominent lamellipodia and an increased number of apoptotic bodies. In cultures treated with fenretinide in combination with RAR γ induction, the cells were more similar

Figure 1

to the vehicle control cells (with or without RAR γ induction): less angular and with prominent lamellipodia (Figure 1e). Conversely, treatment with all-*trans* retinoic acid after induction of RAR γ overexpression changed the morphology of these cells to a rounder, flatter phenotype reminiscent of S-type neuroblastoma cells with an increase in the number of apoptotic bodies (Figure 1e). Treatment of the Tet12-S2 cells with all-*trans* or 9-*cis* retinoic acid after induction of RAR γ gave 3.2-fold and 1.85-fold increases in cell death, respectively, relative to retinoic acid-treated cells without overexpression of RAR γ (Figure 1f, upper graph). Similar results were obtained with Tet12-S4 cells where, relative to cells treated with retinoic acid without RAR γ overexpression, a 3.8-fold increase in cell death resulted after treatment of RAR γ -induced cells with 2.4 μ M all-*trans* retinoic acid (Figure 1f, lower graph).

The unexpected finding from these experiments was the morphological change and induction of cell death in response to ligand-activation of overexpressed RAR γ . Treatment of the parental SH-SY5Y cells with all-*trans* or 9-*cis* retinoic acid leads to differentiation without an increase in cell death.⁸ Although the removal of 9-*cis* retinoic acid from differentiated SH-SY5Y cells induces cell death,¹⁰ this may be attributed to an induced dependence on neurotrophic factors conditionally expressed in response to retinoic acid.¹¹ The induction of apoptosis in response to retinoic acid in the context of increased expression of RAR γ could be interpreted in terms of the thymocyte model developed by Szondy *et al.*¹² According to this model, increased activation of RAR γ alters the balance of RAR α :RAR γ activation in favour of pro-apoptotic effects of RAR γ .¹² Therefore, increased expression of RAR γ coupled with the presence of ligand would be predicted to achieve the same effect. Unlike apoptosis in thymocytes,¹³ 9-*cis* retinoic acid was less effective than all-*trans* retinoic acid, suggesting that RXR activation is not involved or has an inhibitory role. Alternatively, the morpho-

logical effects after ligand-activation of overexpressed RAR γ may indicate a transition to a cell phenotype where apoptosis is a consequence of retinoic acid treatment, as has been reported for S-type neuroblastoma cells.¹⁴

In contrast to recent results for squamous carcinoma cells,¹⁵ RAR γ overexpression did not significantly affect fenretinide-induced apoptosis, and this suggests that RAR γ activation by fenretinide does not have a major role in apoptosis of neuroblastoma cells. One possibility is that, despite its minor contribution to RAR-RXR heterodimers in these cells,⁷ RAR β may be involved in apoptosis. Alternatively, the ability of RAR β/γ (but not RAR α)⁶ antagonists to block fenretinide-induced apoptosis may not be mediated directly by RARs. Clearly, the role of RARs in fenretinide-induced apoptosis in neuroblastoma and other cell types needs critical re-evaluation. However, apoptosis resulting from the retinoic acid-dependent activation of overexpressed RAR γ has important implications for the potential of RAR-specific ligands in the treatment of neuroblastoma and other cancers. Furthermore, the inhibition of retinoic acid-induced neuritogenesis by RAR γ overexpression in SH-SY5Y and other neuroblastoma cell lines¹⁶ raises fundamental questions concerning the role of RARs in specifying neuronal phenotype.

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Figure 1 (a) Induction of RAR γ in stably-transfected SH-SY5Y clones. Stably-transfected SH-SY5Y cells containing an inducible RAR γ construct were prepared¹⁷ by transfecting SH-SY5Y^{Tet12} cells¹⁷ (an SH-SY5Y derivative stably expressing the tetracycline repressor protein) with a full-length human RAR γ cDNA¹⁸ cloned into pcDNA4/TO. Two clones, Tet12-S2 and Tet12-S4, were selected with Zeocin (350 μ g/ml). These cells were treated with 1 μ g/ml tetracycline (Tet; added in ethanol) or ethanol (control) for 24 h and nuclear extracts¹⁹ prepared either immediately or after washout (w/o) of tetracycline and allowing the cells to grow for a further 24 h. Western blots of nuclear extracts (10 μ g per track), separated through 10% polyacrylamide gels,¹⁹ were stained for RAR γ with a polyclonal anti-RAR γ antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and detected on X-ray film using enhanced chemiluminescence. M, marker track. (b) RAR β/γ antagonists CD2848 and CD2665 inhibited fenretinide-induced apoptosis in Tet12-S4 cells. Cells were treated for 5 days with 5 μ M fenretinide in the presence (FenR + CD2848; FenR + CD2665) or absence (FenR) of RAR β/γ antagonists and the density of cell cultures (Crystal Violet assay)⁸ compared to untreated control cells (C). (c) Doxycycline dose-dependently reduced the density of SH-SY5Y cell cultures (●, expressed as a percentage of control, vehicle-treated cells) after 6 days at concentrations from 0–4 μ g/ml (single experiment, 10 replicates per concentration), but the effect at 1 μ g/ml was small. Doxycycline (1 μ g/ml) had a greater effect on Tet12-S4 cells (○, cell density measurements at 5 days; nine independent experiments each with 10 replicates). (d) Induction of RAR γ did not increase the antiproliferative effect of fenretinide (upper graph) and had no significant effect on cell death (lower graph) in response to fenretinide. In the upper graph, Tet12 S4 cell were treated with fenretinide (6–10 μ M) alone or in combination with doxycycline (Dox) for 5 days; cell culture densities (Crystal Violet assay)⁸ were expressed relative to the appropriate control (no fenretinide, without or with doxycycline). Each point is the mean of three independent experiments, 10 replicates per experiment. In the lower graph, Tet12-S4 cells were treated for 3 days with ethanol (vehicle) or fenretinide (5 μ M; FenR) in the presence (grey bars) or absence (white bars) of 1 μ g/ml doxycycline to induce RAR γ . Cells were pre-treated with doxycycline or control diluent for 6 h prior to addition of the retinoid or vehicle. Flow cytometry was used to measure the percentage of sub-G_{1/0} cells after propidium iodide-staining of ethanol-fixed cells as an estimate of cell death.⁶ The experiment was repeated three times, each time in triplicate. (e) Morphology of Tet12-S2 cells in the absence (control column) or presence (RAR γ column) of 1 μ g/ml tetracycline to induce RAR γ , and treated for 3 days with ethanol (vehicle row), all-*trans* retinoic acid (1 μ M; ATRA row) or fenretinide (10 μ M; FenR row). Bar = 10 μ m. (f) Induction of RAR γ markedly increased cell death in Tet12-S2 and Tet12-S4 cultures in response to retinoic acid. In the upper graph, Tet12-S2 cells were treated for 3 days with ethanol (vehicle), 9-*cis* retinoic acid (1 μ M; 9-*cis*) or all-*trans* retinoic acid (1 μ M; ATRA) in the absence (white bars; control) or presence (grey bars) of 1 μ g/ml tetracycline (added 6 h before retinoic acid or ethanol vehicle) to induce RAR γ . Cell death was measured using flow cytometry as above. The experiment was carried out in triplicate except for the ATRA treatment in the absence of RAR γ induction, which was in duplicate. In the lower graph, Tet12-S4 cell cultures were treated for 3 days with ethanol (vehicle), or 2.4 μ M all-*trans* retinoic acid (ATRA) in the absence (white bars; control diluent) or presence of 1 μ g/ml doxycycline (grey bars), added 6 h before addition of ATRA or vehicle. Experiments with vehicle-treated cells were in duplicate, and experiments with ATRA-treated cells were in quadruplicate. In (b–f), points or bar heights are means; error bars are $\pm$ 95% confidence intervals or ranges in the case of duplicate samples

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1. Armstrong JL, Redfern CPF and Veal GJ (2005) *Biochem. Pharmacol.* 69: 1299–1306
2. Matthay KK *et al.* (1999) *N. Engl. J. Med.* 341: 1165–1173
3. Bastien J and Rochette-Egly C (2004) *Gene* 328: 1–16
4. Reynolds CP *et al.* (2000) *Med. Pediatr. Oncol.* 35: 597–602
5. Lovat PE *et al.* (2004) *J. Natl. Cancer Inst.* 96: 1288–1299
6. Lovat PE *et al.* (2000) *Exp. Cell Res.* 260: 50–60
7. Rana B *et al.* (2002) *J. Cell. Biochem.* 86: 67–78
8. Lovat PE *et al.* (1994) *Neurosci. Lett.* 182: 29–32
9. Lovat PE *et al.* (2000) *Int. J. Cancer.* 88: 977–985
10. Lovat PE *et al.* (1997) *J. Natl. Cancer Inst.* 89: 446–452
11. Feng X *et al.* (2001) *J. Neurosci. Res.* 64: 355–363
12. Szondy Z *et al.* (1997) *Mol. Pharmacol.* 51: 972–982
13. Szondy Z, Reichert U and Fesus L (1998) *Cell Death Differ.* 5: 4–10
14. Melino G *et al.* (1997) *Exp. Cell Res.* 235: 55–61
15. Kabbout M *et al.* (2004) *Mol. Carcinog.* 40: 12–23
16. Cheung B *et al.* (1996) *Biochem. Biophys. Res. Commun.* 229: 349–354
17. Lovat PE *et al.* (2002) *Cancer Res.* 62: 5158–5167
18. Krust A *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86: 5310–5314
19. Rana B, Pearson ADJ and Redfern CPF (2001) *FEBS Lett.* 506: 39–44