Regulation of cellular processes by PPARγ ligands in neuroblastoma cells is modulated by the level of retinoblastoma protein expression

V.C. Emmans*, H.A. Rodway°, A.N. Hunt† and K.A. Lillycrop*†

*Development and Cell Biology, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, U.K., and †Department of Child Health, University of Southampton, Southampton General Hospital, Tremona Road, Southampton SO16 6YD, U.K.

Abstract

Neuroblastoma is a childhood cancer, which spontaneously regresses. This has led to a search for agents that mimic this process. We show that both natural and synthetic ligands of PPAR (peroxisome-proliferator-activated receptor γ) inhibit the growth of neuroblastoma cells in vitro. The degree of PPAR activation was attenuated however in the presence of the retinoblastoma protein. Addition of trichostatin A, a histone deacetylase inhibitor, abolished retinoblastoma protein repression of PPAR activity. Moreover, enhanced growth inhibition was observed when neuroblastoma cells were treated with a PPARγ ligand and a histone deacetylase inhibitor, suggesting a combination therapy to treat neuroblastoma might prove more effective than using either agent alone.

Introduction

Neuroblastoma is an embryonal tumour of the sympathetic nervous system that arises from the neural crest and is the most common extra-cranial childhood tumour [1]. Most patients present with disseminated disease, which is currently resistant to chemotherapy. However, neuroblastoma has a high rate of spontaneous regression, which has prompted a search for agents which mimic this natural phenomenon.

PPARs (peroxisome-proliferator-activated receptors) are members of the nuclear hormone receptor superfamily, which includes the retinoid X receptor [2]. Nuclear hormone receptors are ligand-activated transcription factors. On binding ligand, PPARs undergo a conformational change and heterodimerize with the retinoid X receptor. The activated heterodimer binds to PPREs (peroxisome-proliferator-response elements) in the promoters of target genes activating their transcription. There are three isoforms of PPAR, α, β and γ, and ligands of PPARγ have been shown to inhibit growth of human breast, prostate and lung cancer cell lines in vitro [3–5]. How PPARγ modulates cell proliferation still remains unclear. However, Fajas et al. [6] have demonstrated that PPARγ control of the cell cycle is dependent on its interaction with the cell-cycle regulator, Rb (retinoblastoma protein).

In our studies, we investigated if PPARγ ligands could also inhibit the growth of neuroblastoma cell lines in vitro. We demonstrate that the natural PPARγ ligand 15dPGJ2 (15-deoxy-12,14 prostaglandin J2) and the synthetic PPARγ agonist, ciglitazone, cause growth inhibition of neuroblastoma cells. Overexpression of Rb in neuroblastoma cells caused repression of PPARγ transactivation, which could be restored by treatment with the HDAC (histone deacetylase) inhibitor, TSA (trichostatin A). Enhanced growth inhibition of the SK-N-AS neuroblastoma cells was observed when they were treated with 15dPGJ2 and ligand, and a histone deacetylase inhibitor, suggesting that a combination therapy for neuroblastoma using PPARγ ligands and TSA might prove more effective than either agent alone.

Methods

The neuroblastoma cell lines, SK-N-AS, IMR-32, SK-N-SH and ND-7, were used in the present study. For cell growth experiments, 4 × 10^4 cells/well were treated with either ligand 15dPGJ2 (Alexis) at 0.1–100 µM or ciglitazone (Tocris) at 25–200 µM. SK-N-AS cells were treated with 5 or 20 µM 15dPGJ2 alone or with 50 ng/ml TSA. Ligand- and vehicle (DMSO)-treated cells were counted over a period of 3 days.

To overexpress Rb, ND-7 cells were transiently transfected with an Rb expression plasmid (a gift from Dr N.S.B. Thomas, Department of Haematological Medicine, King’s School of Medicine and Dentistry, London, U.K.) or pcDNA3.1 (Invitrogen) as a vector-only control. PPARγ transactivation was measured by introducing a PPAR-responsive reporter construct (PPRE-tk-Luc; a gift from Professor R. Evans, Department of Gene Expression, Salk Institute for Biological Studies, San Diego, CA, U.S.A.) into cells. Transfections were performed using the calcium phosphate precipitate method and cells were harvested 24 h later and the luciferase activity was measured.

Results and discussion

To investigate the effect of PPARγ ligands on neuroblastoma cell growth, three human neuroblastoma cell lines were
Figure 1 | Effect of the PPARγ ligands, 15dPGJ2 and ciglitazone on neuroblastoma cell growth
Neuroblastoma cells (4 × 10⁴ cells/well) were plated and treated with DMSO (vehicle control) or 0.1–100 µM 15dPGJ2 (A) or 25–200 µM ciglitazone (B). Results represent means ± S.E.M. of total cell number of two independent experiments 72 h post-initial treatment. Tables show the EC₅₀ values for 15dPGJ2 and ciglitazone for each neuroblastoma cell line.

![Graph A](image1)

![Graph B](image2)

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>EC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-N-AS</td>
<td>34</td>
</tr>
<tr>
<td>IMR-32</td>
<td>2</td>
</tr>
<tr>
<td>SK-N-SH</td>
<td>50</td>
</tr>
</tbody>
</table>

Figure 2 | Effect of overexpression of Rb on PPARγ transcriptional activity
(A) ND-7 cells were co-transfected with a PPAR-responsive reporter construct together with an expression vector containing Rb or pcDNA3.1 as a vector-only control and treated with DMSO (vehicle control) or 5 µM 15dPGJ2 either alone or in combination with 50 ng of TSA for 24 h and then harvested. Luciferase activities, from a representative experiment, are expressed per µg of protein. (B) SK-N-AS cells (4 × 10⁴ cells/well) were treated with 15dPGJ2 (5 or 20 µM) alone or with 50 ng/ml TSA. Results represent the means ± S.E.M. of total cell number of two independent experiments 72 h post-initial treatment.

![Graph C](image3)

![Graph D](image4)

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>EC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-N-AS</td>
<td>98</td>
</tr>
<tr>
<td>IMR-32</td>
<td>55</td>
</tr>
<tr>
<td>SK-N-SH</td>
<td>33</td>
</tr>
</tbody>
</table>

> treated with the PPARγ agonists, 15dPGJ2 and ciglitazone [7], and their growth profiles monitored over a period of 3 days. As shown in Figures 1(A) and 1(B), both 15dPGJ2 and ciglitazone caused a significant growth inhibition of all three neuroblastoma cell lines. In SK-N-AS and SK-N-SH cells, similar concentrations of ciglitazone and 15dPGJ2 were required to induce 50% growth inhibition (see tables in Figures 1A and 1B). This is consistent with the comparable binding affinities reported for ciglitazone and 15dPGJ2 to PPARγ. However, ciglitazone and 15dPGJ2 have been shown to act through both PPARγ-dependent and -independent pathways. The reported PPAR-independent pathways include inhibition of translation initiation and modulation of the nuclear factor κB pathway [8,9]. In contrast, in IMR-32 cells, a 27-fold higher concentration of ciglitazone was required to cause 50% growth inhibition compared with 15dPGJ2, suggesting that the response to this natural ligand is dependent on the genotype of the cell.

Recently, it has been shown that the degree of PPARγ activation induced by PPARγ agonists can be modulated through its interaction with Rb and HDAC3 [6]. Therefore we investigated whether overexpression of Rb in neuroblastoma cells could affect PPARγ activity and, hence, the
response to PPARγ agonists. To test this, cells were co-transfected with a PPAR-responsive reporter construct together with an expression vector containing Rb. We found that 15dPGJ2 induced a 2-fold activation of the PPAR-responsive reporter construct. However, in the presence of transfected Rb, the ability of 15dPGJ2 to stimulate PPAR reporter activity was attenuated (Figure 2A). Since Rb repression of gene transcription usually occurs through the recruitment of HDACs, we next examined whether the ability of Rb to attenuate PPAR activation was dependent on HDAC recruitment. We show that in cells transfected with Rb, induction of the PPAR-responsive reporter construct by 15dPGJ2 was restored by treatment with the potent HDAC inhibitor, TSA (Figure 2A), suggesting that the ability of Rb to attenuate the level of PPAR activity is dependent on recruitment of HDACs.

To determine the effect of HDAC inhibition on the growth of neuroblastoma cells in response to 15dPGJ2, SK-N-AS cells were treated with 15dPGJ2 in the presence of TSA. We found that the growth inhibition of SK-N-AS cells was significantly higher when they were treated with 15dPGJ2 and TSA, when compared with 15dPGJ2 alone (Figure 2B). This shift in the dose response to 15dPGJ2 may in part be due to increased PPARγ activation as a result of inhibition of HDAC recruitment by Rb. This suggests that the Rb status of the cell may be important in determining its response to PPARγ ligands and that a combination therapy, which uses PPARγ ligands and HDAC inhibitors, may prove more effective than either agent alone.

References

Received 23 July 2004