Neuroblastoma is a childhood neural crest tumor. Fenretinide, a retinoic acid analogue, induces accumulation of mitochondrial reactive oxygen species and consequent apoptosis in neuroblastoma cells. The p75 neurotrophin receptor (p75NTR) enhances the antineuroblastoma cell efficacy of fenretinide in vitro. We examined the role of the retinoid binding protein, CRABP1, in p75NTR-mediated potentiation of the efficacy of fenretinide. Knockdown and overexpression, respectively, of either p75NTR or CRABP1 were effected in neuroblastoma cell lines using standard techniques. Expression was determined by qRT-PCR and confirmed at the protein level by Western blot. Metabolic viability was determined by Alamar blue assay. While protein content of CRABP1 correlated roughly with that of p75NTR in the three neuroblastoid or epithelioid human neuroblastoma cell lines studied, manipulation of p75NTR expression resulted in cell line-dependent, variable change in CRABP1 expression. Furthermore, in some cell lines, induced expression of CRABP1 in the absence of p75NTR did not alter cell sensitivity to fenretinide treatment. The effects of manipulation of p75NTR expression on CRABP1 expression and the effects of CRABP1 expression on fenretinide efficacy are therefore neuroblastoma cell line-dependent. Potentiation of the antineuroblastoma cell effects of fenretinide by p75NTR is not mediated solely through CRABP1.

1. Introduction

The p75 neurotrophin receptor (p75NTR) has been implicated in both development and disease and, in its interactions with its cognate ligands and binding partners, regulates cell fate [1]. The signaling pathways transduced by p75NTR are complex and each can lead to several alternative downstream outcomes dependent on cell type and environment. The p75NTR is a transmembrane cell surface receptor, the intracellular domain (p75ICD) of which is cleaved off and functions as a transcription factor to modulate protein expression. Parkhurst et al. [2] have shown that p75ICD can translocate to the nucleus, associate with the cyclin E1 promoter, and increase mRNA levels of cyclin E1 in PC12 and HEK293 cells. We have previously demonstrated that p75NTR affects cellular response to oxidative stress [3] and upregulates the enzymes that mediate cholesterol biosynthesis [4]. More recently, it was demonstrated that knocking down p75NTR expression in neuroblastoma cell lines attenuates the cellular response to the chemotherapeutic drug, fenretinide, while p75NTR overexpression has the opposite effect [5]. However, the mechanisms that underlie the potentiation of the effects of fenretinide by p75NTR are incompletely understood.

Neuroblastoma is the most common extracranial solid tumor of childhood. It derives from the neural crest and commonly presents clinically in the adrenal gland or sympathetic chain. Fenretinide, a retinoic acid derivative, is under active clinical investigation for the treatment of neuroblastoma. Unlike all-trans retinoic acid (ATRA), which is used to induce cellular differentiation in the treatment of cancer, fenretinide is known to cause apoptosis through generation of mitochondrial reactive oxygen species thought to “leak” from Complex II [5, 6] and differs structurally from ATRA by only a hydroxyphenyl group. In a recent phase II trial of 65 patients, fenretinide did not meet criteria for clinical efficacy [7] due to low bioavailability of the drug. However, there are now ongoing
The human CRABP1 cDNA was cloned into the pGIPZ CMV-MCS-ires-Tomato-F vector and the lentivirus was produced in 293T cells by the calcium phosphate method. SK-N-AS or SH-SY5Y cells were transduced with lentiviral particles and fluorescent cells sorted by gating for RFP positivity.

2.5. Northern and Western Blotting. Northern and Western blots were performed as we have previously described [5, 10]. In each relevant figure, a representative blot is shown of at least three independent cell preparations and blots performed. Where indicated on the figures, the optical densities of Western blot bands were determined using Image J software (National Institutes of Health, Bethesda, MD).

2.6. Effects of Fenretinide on Cellular Metabolic Viability. Fenretinide was applied to cells in culture at varying concentrations as noted in the text and figure legends. The Alamar blue assay was performed as we have described [5, 10] to discern the time course of cellular metabolic viability for each concentration of fenretinide. All determinations were performed in triplicate and, in each case, a representative study of at least three performed is shown. Plots depict the mean and SEM within a representative study and between-condition comparisons were deemed statistically significant (Student’s t-test) if at the P ≤ 0.05 level.

3. Results

3.1. Correlation of p75NTR and CRABPI Levels in Neuroblastoma Cell Lines. If p75NTR expression affects fenretinide efficacy by inducing coordinate expression of CRABPI, then the two proteins should vary in concentration coordinately with one another. Formulation of this hypothesis followed our observation that p75NTR and CRABPI protein levels vary coordinately with one another in neuroblastoid and epithelioid human neuroblastoma cell lines; the neuroblastoid line that has low p75NTR protein levels (SH-SY5Y) has undetectable levels of CRABPI protein while epithelioid lines with higher p75NTR protein levels (SK-N-AS, SH-EPI) have higher levels of CRABPI protein. IMR-32 is an “intermediate” neuroblastoma cell line with characteristics of neuroblastoma stem cells and mixed characteristics of neuroblastoid and epithelioid lines; IMR-32 cells have comparable levels of p75NTR protein to SK-N-AS cells. Although IMR-32 cells do not have detectable CRABPI protein, they also express CRABPI mRNA like SK-N-AS and SH-EPI cells, suggesting CRABPI mRNA expression appropriate to p75NTR expression with a block at the level of translation (Figure 1).

3.2. Regulation of CRABPI Expression by p75NTR in SH-EPI Cells. Our initial observation and cell signaling studies on p75NTR-induced potentiation of fenretinide efficacy were performed in SH-EPI epithelioid human neuroblastoma cells [5, 10]. We therefore performed our studies of CRABPI in this cell line. Commensurate with the coordinate variation of p75NTR and CRABPI in native neuroblastoma cells, SH-EPI cells induced to overexpress p75NTR (p75OE cells) had higher levels of CRABPI protein than mock-transfected control cells (Figure 2(a)). This is supported by qRT-PCR data showing that the p75OE cells had higher levels of CRABPI mRNA (Figure 2(b)).

Transient knockdown of p75NTR using siRNA resulted in transient knockdown of CRABPI in the p75OE SH-EPI cells, with levels returning to baseline upon recovery of p75NTR expression (Figure 2(c)).

3.3. Effects of Altered Expression of CRABPI on Impairment of Cellular Metabolic Function by Fenretinide. To assess whether
manipulation of CRABP1 expression alone modulates fenretinide efficacy, CRABP1 was knocked down in native SH-EP1 cells using siRNA. Mock- and siRNA-transfected cells were then incubated for 60 h with 4, 10, 13, or 15 μM fenretinide. The knockdown cells showed significantly greater metabolic viability after fenretinide treatment than mock-transfected controls (Figure 3(a)) and cell number (Figure 3(b)) in the absence of a difference in culture growth rate of untreated cells (Figure 3(c)).

3.4. Generalizability to Other Epithelioid Human Neuroblastoma Cell Lines of p75NTR-CRABP1 Coordinate Regulation as a Mechanism for p75NTR-Induced Potentiation of Fenretinide Efficacy. Demonstration of coordinate regulation of p75NTR and CRABP1, and CRABP1 knockdown-induced decrease in fenretinide efficacy in SH-EP1 cells caused us to examine whether these effects are also seen in the SK-N-AS epithelioid human neuroblastoma cell line. We previously demonstrated that SK-N-AS neuroblastoma cells exhibit the same p75NTR-induced enhancement of fenretinide efficacy as seen in SH-EP1 cells [5].

To our surprise, in contrast to the case for SH-EP1 cells, stable knockdown of p75NTR in native SK-N-AS cells resulted in upregulation of CRABP1 (Figure 4(a)). Furthermore, despite this upregulation of CRABP1, p75NTR knockdown resulted in resistance of SK-N-AS cells to fenretinide (Figure 4(b)), making it likely that the effects of p75NTR knockdown are not mediated through CRABP1 in SK-N-AS cells. In addition, induction of overexpression of CRABP1 without manipulation of or change in expression of p75NTR resulted in a less than 2-fold increase in fenretinide efficacy, statistically significant (P < 0.01) at 4 and 8 μM, in SK-N-AS cells relative to empty vector-transfected cells (Figures 4(c) and 4(d)), suggesting that while CRABP1 is likely not the mediator of p75NTR-induced sensitivity to fenretinide, it may exert similar effects in SK-N-AS cells independent of p75NTR.

3.5. Effects of Manipulation of CRABP1 Expression on p75NTR Expression and Fenretinide Efficacy in Neuroblastoid Human Neuroblastoma Cell Lines. Although the p75NTR content of neuroblastoid neuroblastoma cells is generally lower than that of epithelioid neuroblastoma cells, p75NTR expression does enhance fenretinide efficacy in them, as well [5]. We therefore examined the effects of CRABP1 expression on p75NTR expression and fenretinide efficacy in neuroblastoid human neuroblastoma cells. SH-SY5Y cells do not express CRABP1 in their native state.

Induction of expression of CRABP1 in SH-SY5Y cells did not alter expression of p75NTR (Figure 5). However, CRABP1-expressing SH-SY5Y cells are somewhat more resistant to the effects of fenretinide than empty vector-transfected or Wildtype cells. This is the opposite of the effect of CRABP1 overexpression in native p75NTR-expressing SK-N-AS cells.

4. Discussion

Fenretinide is a retinoic acid analogue originally developed in an attempt to improve the efficacy of retinoic acid as a differentiation-inducing agent for the treatment of retinoic acid receptor-positive cancers. While fenretinide was not, as it turns out, an inducer of differentiation or a particularly avid ligand for retinoic acid receptors, it did induce cancer
cell apoptosis through induction of accumulation of reactive oxygen species in the mitochondria of these cells [6]. Phase II clinical studies of fenretinide that did not establish efficacy in the treatment of neuroblastoma [7], a common solid cancer of childhood that is frequently fatal within five years of diagnosis, have prompted efforts to enhance the bioavailability and efficacy of this drug in vivo [8, 9].

We have previously noted that expression by neuroblastoma cells of the p75NTR enhances the mitochondrial oxidative activity and cytocidal efficacy of fenretinide in vitro [5, 10]. Although the reactive oxygen species that accumulate in the mitochondria of cells treated with fenretinide appear to be generated at the level of Complex II [5, 6], p75NTR expression does not alter expression or activity of Complex II [16].

CRABP1 binds to retinoids and thereby sequesters them in the cytoplasm and prevents their shuttling to the nucleus. In so doing, it enhances the half-lives of retinoids in the cell. While fenretinide does not bind to CRABP1, its more active metabolite 4-oxo-fenretinide does [12]. We therefore hypothesized that induction of enhanced expression of p75NTR enhances the expression of CRABP1. From a therapeutic standpoint, we hoped that this enhancement of CRABP1 expression would increase the cytoplasmic concentration and mitochondrial redox effectiveness of 4-oxo-fenretinide after fenretinide administration.
Our results demonstrate neuroblastoma cell line-dependence of the effects of manipulation of p75NTR expression on CRABP1 expression and the effects of CRABP1 expression on fenretinide-induced cell death. CRABP1 protein concentration does covary with p75NTR protein concentration in native human neuroblastoma cell lines. Furthermore, our initial studies of SH-EPI epithelioid neuroblastoma cells suggested that manipulating p75NTR expression leads to a coordinate change in CRABP1 expression, and manipulating CRABP1 expression alone (i.e., without manipulation of p75NTR expression) mimics the effects of p75NTR manipulation on fenretinide-induced cell death. This suggests that,
Figure 4: p75NTR, CRABP1, and response to fenretinide in SK-N-AS neuroblastoma cells. (a) Western blot of lysates from SK-N-AS cells in their native state (Wildtype), stably transfected with empty vector (Vector), a scrambled construct (Scr), or shRNA for p75NTR (clones NC-1 and NC-2). Knockdown of p75NTR is more efficient in NC-1 cells than in NC-2 cells. Blotting for β-actin serves as a loading control. The graph below the blot depicts the mean optical density and SEM for 3 blots performed. Open bars, p75NTR; solid bars, CRABP1 (b) Alamar blue assay of SK-N-AS cells treated as in (a) after treatment with fenretinide (n = 3 for each point; results for NC-1 (x, solid line; concentration required for growth inhibition by 50% [GI₅₀] = 15) differ from those for Wildtype (gray triangle; GI₅₀ = 7.5), Vector (●, solid line; GI₅₀ = 5), and Scr (○, dashed line; GI₅₀ = 6) with **P < 0.01 and from those for NC-2 (x, dashed line; GI₅₀ = 10) with *P < 0.05; Student’s t-test). Note that while NC-1 cells are more resistant to fenretinide than empty vector- and scrambled construct-transfected cells, NC-2 cells are not. Western blot (c) and Alamar blue assay (d) of SK-N-AS cells transfected with an expression construct for CRABP1 (CRABP1 (black triangle)) or an empty vector (Vector (○)) or examined in their native state (Wildtype (gray triangle)). Results for Wildtype differ from those for Vector (GI₅₀(Wildtype)/GI₅₀(Vector) = 1.5; **P < 0.01), indicating that transfection with an empty construct changes the fenretinide sensitivity of the cells; CRABP1 cells differ from Vector cells (GI₅₀(CRABP1)/GI₅₀(Vector) = 0.8; **P < 0.01) at 4 and 8 μM fenretinide. The top band detected with anti-CRABP1 antibody in the CRABP1 lane is from Flag-CRABP1, the expression of which is induced. The graph below the blot depicts the mean optical density and SEM for 3 blots performed. Expression of p75NTR does not change significantly with induction of altered total expression of CRABP1 (Flag-CRABP1 + CRABP1). α-Tubulin is used as a loading control for Western blotting. Open bars, p75NTR; solid bars, CRABP1.
Figure 5: Western blot (a) and Alamar blue assay (b) of SH-SY5Y cells transfected with an expression construct for CRABP1 (CRABP1 OE (black triangle)) or an empty vector (Vector (◻)) or examined in their native state (Wildtype (gray triangle)). Results for CRABP1 OE differ from those for Vector and Wildtype (GL50(CRABP1)/GL50(Vector) = 2; GL50(CRABP1)/GL50(Wildtype) = 2; *P = 0.05) at 8 and 12 μM fenretinide. β-Actin is used as a loading control for Western blotting.

5. Conclusions

These studies underscore the complexity of neuroblastoma specifically and cancer in general as a therapeutic target. Even among cell lines of common lineage or tumor origin downstream signaling pathways and effects differ. In fact, there is ample evidence to suggest that, within a given patient's tumor at a particular moment in time, there are many genetically divergent subpopulations [17]. The fact that p75NTR and CRABP1 expression differentially affect one another and the impact of treatment with fenretinide in different neuroblastomas makes p75NTR or CRABP1, at best, complex biomarkers for likely responsiveness to that drug. Future studies must focus on downstream effectors in these neuroblastomas, like SH-EPI, in which coordinate regulation of these proteins leads to potentiation of the oxidative and cytotoxic effects of fenretinide.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors’ Contribution

Yaoli Pu Yang and Simeng Wang contributed equally to the work described in this paper.

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