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Neuroblastoma: A Malignancy Due to Cell Differentiation Block

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1. Introduction

Neuroblastoma originates from precursor neuroblasts of the sympathetic nervous system. Unlike most other cancer types, neuroblastoma is characterized by a unique capacity for spontaneous complete regression, at least partly through neuronal differentiation, in a proportion of patients, and is therefore regarded as a cancer due to cell differentiation block. The first demonstration of *in vitro* differentiation of human neuroblastoma cells was published 30 years ago, when human SK-N-SH and SH-SY5Y neuroblastoma cells were shown to differentiate morphologically (neurite outgrowth) and biochemically in response to 12-O-tetradecanoyl-phorbol-13-acetate (TPA) treatment (Påhlman et al., 1981). The differentiated cells showed an increased expression of noradrenaline, adrenaline and neuron-specific enolase (NSE), differentiation markers which are employed for the diagnosis of neuroblastoma in patients. In 1982, retinoic acid (RA) was shown to induce concentration-dependent morphologic differentiation and growth inhibition in the LA-N-1 human neuroblastoma cell line (Sidell, 1982). The RA-induced morphologic differentiation and growth inhibition persisted despite removal of the drug. These observations demonstrate that RA promotes the differentiation of LA-N-1 neuroblastoma cells and results in a reduced expression of the malignant phenotype, and suggest that patients with advanced neuroblastoma may be successfully treated by RA to induce their tumour cells to differentiate and to undergo growth inhibition. In the last three decades, a number of factors, such as up-regulation of the expression of retinoic acid receptors, have been identified as crucial for the induction or blockage of neuroblastoma cell differentiation. Importantly, naturally occurring and synthetic retinoids have shown great promise in the clinic when used as differentiation agents in neuroblastoma patients with minimal residual disease, while the mechanism of retinoid anticancer signalling in neuroblastoma is still not fully understood (Liu et al., 2005; Reynolds et al., 2003).

2. Neurotrophin and neurotrophin receptor-induced neuroblastoma cell differentiation

Among the best characterized factors involved in pre-cancerous neuroblast and neuroblastoma cell differentiation are the tyrosine kinase receptor (TRK), its ligands nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-5). Neuroblastoma cell lines generally lack functional neurotrophin receptors of the TRK gene family and therefore do not differentiate when stimulated with

neurotrophins including NGF, BDNF, NTF3 or NTF5, and absence of TRKA and TRKC gene expression in neuroblastoma tissues correlates with poor prognosis in neuroblastoma patients (Kogner et al., 1993; Suzuki et al., 1993; Yamashiro et al., 1996). When transfected with either TRKA or TRKC, neuroblastoma cell lines can differentiate into sympathetic neurons in response to treatment with their cognate ligands NGF and NT-3 (Edsjo et al., 2001; Lavenius et al., 1995). Moreover, treatment with growth inhibition agents such as aphidicolin and all-trans RA (atRA) results in NGF responsiveness through induction of TRKA gene expression, demonstrating that TRK-negative cells retain a capacity to respond to TRK-mediated cell differentiation (Hishiki et al., 1998; Poluha, Poluha, and Ross, 1995). Taken together, these data show that many neuroblastoma cell lines have the capacity to respond to physiological differentiation stimuli, although they have lost this capacity with regard to stimulation with NGF or with other neurotrophins unless the neuroblastoma cells are triggered to express the cognate receptors [reviewed in (Edsjö, Holmquist, and Påhlman, 2007)].

While TRKA- and TRKC-mediated signalling decreases neuroblastoma cell proliferation and aggressiveness, BDNF/TRKB signalling corresponds with poor outcome in neuroblastoma patients [reviewed in (Edsjö, Holmquist, and Påhlman, 2007)]. BDNF treatment of neuroblastoma cells transfected with a TRKB over-expressing construct does not induce differentiation (Ho et al., 2011), and BDNF stimulation of neuroblastoma cell lines expressing TRKB as a result of RA treatment does not affect proliferation, but increases survival and invasiveness (Matsumoto et al., 1995). In addition to these effects of TRKA and TRKB signalling, recent data also suggest a difference with respect to therapy resistance, invasiveness, angiogenesis, and possibly also genomic stability (Schramm et al., 2005).

atRA treatment of neuroblastoma cells can also induce the expression of the proto-oncogene RET, which codes for a tyrosine kinase growth factor receptor specifically binding glial cell line-derived neurotrophic factor (GDNF) (Airaksinen and Saarma, 2002). GDNF is required for the proper development of enteric and parasympathetic neuroblasts (Airaksinen and Saarma, 2002), and atRA-treated neuroblastoma cells differentiate further in response to GDNF (Hishiki et al., 1998). Additionally, activation of RET by GDNF leads to differentiation of neuroblasts and neuroblastoma cells into ganglia cells (D'Alessio et al., 1995), and GDNF synergizes with ciliary neurotrophic factor (CNTF) to enhance TRKA receptor expression, thereby strengthening NGF-mediated cell differentiation signal (Peterson and Bogenmann, 2004).

3. Fyn and Fyn kinase induce neuroblastoma cell differentiation

Other factors involved in neuroblastoma cell differentiation include the Fyn nonreceptor kinase. Expression of active Fyn induces differentiation and growth arrest in neuroblastoma cells (Berwanger et al., 2002). High expression of Fyn and high Fyn kinase activity are restricted to low-stage human neuroblastoma tissues, and expression of Fyn predicts long-term survival independently of MYCN gene amplification (Berwanger et al., 2002).

4. N-Myc blocks neuroblastoma cell differentiation

The most powerful endogenous blocker of neuroblastoma cell differentiation is the *MYCN* oncogene. N-Myc is well-known to repress neuroblastoma cell differentiation partly through repressing TRKA gene expression. A recent study demonstrates that N-Myc blocks TRKA gene expression by recruiting histone deacetylase 1 (HDAC1) to TRKA gene promoter and repressing its transcription (Iraci et al., 2011). Additionally, N-Myc represses the expression of growth arrest specific1 (GAS1), leading to increased RET tyrosine 1062 phosphorylation and decreased GDNF signaling (Lopez-Ramirez et al., 2008).

5. Histone deacetylase 1 and tissue transglutaminase in neuroblastoma cell differentiation

The transamidation activity of tissue transglutaminase (TG2) has been shown to be essential for the neuroblastoma and leukemia cell differentiation response to retinoid therapy (Balajthy et al., 2006; Tucholski, Lesort, and Johnson, 2001), and TG2 overexpression alone induces neuronal differentiation in neuroblastoma cells (Tucholski, Lesort, and Johnson, 2001). We have recently shown that repression of TG2 gene expression and reduction in transamidation activity are essential for N-Myc-induced neuroblastoma cell differentiation block (Liu et al., 2007). Importantly, dual step cross-linking chromatin immunoprecipitation and protein coimmunoprecipitation assays show that N-Myc acts as a transrepressor by recruiting HDAC1 protein to an Sp1-binding site in the TG2 gene core promoter in a manner distinct from it's action as a transactivator at E-Box binding sites (Liu et al., 2007). Histone deacetylase inhibitor treatment blocks the N-Myc-mediated HDAC1 recruitment and TG2 repression *in vitro*. In neuroblastoma-bearing N-Myc transgenic mice, histone deacetylase inhibitor treatment induces TG2 expression and demonstrates marked antitumor activity *in vivo*. Taken together, these data indicate the critical roles of HDAC1 and TG2 in N-Myc-induced oncogenesis and have significant implications for the use of histone deacetylase inhibitor therapy in N-Myc-driven neuroblastoma.

6. Opposing effects of two tissue transglutaminase protein isoforms in neuroblastoma cell differentiation

Uniquely, two isoforms of TG2 mRNA and protein, a short form (TG2-S) and a full length form (TG2-L), have been characterized (Begg et al., 2006). We have shown that TG2-L and TG2-S exert opposing effects on cell differentiation (Tee et al., 2010). Repression of TG2-L with small interfering RNA, which does not affect TG2-S expression, induces dramatic neuronal differentiation in neuroblastoma cells. In contrast, overexpression of TG2-S or a GTP-binding-deficient mutant of TG2-L (R580A), both of which lack the GTP-binding Arg-580 residue, induces neuroblastoma cell differentiation, which is blocked by an inhibitor of transamidase activity. Whereas N-Myc represses and retinoid activates both TG2 isoforms, repression of TG2-L, but not simultaneous repression of TG2-L and TG2-S, enhances neuroblastoma cell differentiation due to N-Myc small interfering RNA or retinoid. Moreover, suppression of vasoactive intestinal peptide (VIP) expression alone induces neuroblastoma cell differentiation, and VIP can be up-regulated by TG2-L, but not TG2-S. Taken together, these data indicate that TG2-L and TG2-S exert opposite effects on cell differentiation due to differences in GTP binding and modulation of VIP gene transcription (Tee et al., 2010).

7. Retinoid differentiation therapy

Conventional therapy of neuroblastoma patients now includes the differentiation agent retinoid. Unlike chemo-radiotherapy, retinoid differentiation therapy shows minimal side effects on normal cells, because normal non-malignant cells are already differentiated. The effects of retinoids are mediated by two classes of non-steroid nuclear hormone receptors, the retinoic acid (RAR α, β, γ) and the retinoic X (RXR α, β, γ) receptors (Reynolds et al., 2003). The naturally occurring all-trans-retinoic acid (atRA), 9-cis-retinoic acid and the synthetic 13-cis-retinoic acid (13-cis-RA) are examples of retinoids studied in neuroblastoma. It has long been established that atRA stimulation of neuroblastoma cells results in growth inhibition and neuronal differentiation as indicated by neurite outgrowth, increased NSE

activity and accumulation of norepinephrine (Pahlman et al., 1984; Sidell, 1982) . Even though atRA induces neuronal differentiation, the outcome differs depending on the neuroblastoma cell line studied. While some cell lines develop a sympathetic noradrenergic phenotype, a cholinergic switch has been suggested in other cell lines (Handler et al., 2000; Hill and Robertson, 1997). Mechanism studies have revealed that the down-regulation of the proto-oncogenes N-Myc, MYB and HRAS precedes the morphological differentiation, with changes in the expression of other proto-oncogenes following thereafter (Thiele, Deutsch, and Israel, 1988; Thiele, Reynolds, and Israel, 1985). As discussed in the previous sections, atRA treatment can also result in neuroblastoma cell differentiation by up-regulating the expression of the neurotrophin receptors TRKA, TRKB and RET, leading to responsiveness to their ligands NGF, BDNF and GDNF (Cerchia et al., 2006; Hishiki et al., 1998).
Phase I clinical trials show that higher and more sustained drug levels can be obtained with 13-cis-RA relative to atRA, and a phase III randomized clinical trial shows that high-dose, pulse therapy with 13-cis-RA given after completion of intensive chemoradiotherapy (with or without autologous bone marrow transplantation) significantly improved event-free survival in high-risk neuroblastoma patients (Reynolds et al., 2003) (Matthay et al., 1999). Moreove, *In vitro* as well as clinical data indicate that synthetic retinoids such as Fenretinide might be effective against neuroblastoma cells resistant to 13-cis-RA therapy. This effect seems to be independent of retinoid receptors, does not involve differentiation but is involved in the induction of programmed cell death (Reynolds et al., 2003).

8. Combination differentiation therapy with retinoids and histone deacetylase inhibitors

Although 13-cis-RA significantly improves event-free survival in high-risk neuroblastoma patients (Matthay et al., 1999), resistance to retinoid therapy eventually develops in more than 50% of neuroblastoma patients. Combination therapies with retinoids and other anti-cancer agents have therefore been studied extensively. Interestingly, during screening a small-molecule library for compounds enhancing histone deacetylase inhibitor-induced neuroblastoma cell differentiation, retinoids were found to be the top hit compounds (Hahn et al., 2008). Secondary assays confirmed greater neuroblastoma differentiation with the combination therapy of histone deacetylase inhibitors and retinoids. In a xenograft model of neuroblastoma, animals treated with the combination therapy with the histone deacetylase inhibitor SAHA and atRA had the longest survival. This work suggests that combination therapy with histone deacetylase inhibitors and retinoids could be a promising new strategy for differentiation therapy of children with neuroblastoma (Hahn et al., 2008).

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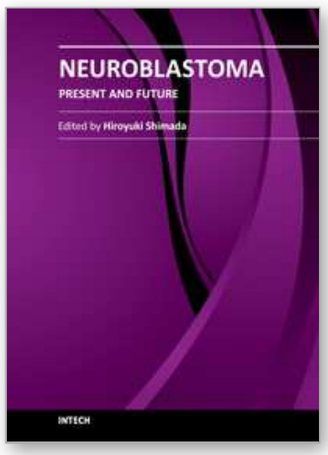

Neuroblastoma - Present and Future

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Neuroblastoma, once called "enigmatic", due to "unpredictable" clinical behaviors, is composed of biologically diverse tumors. Molecular/genomic properties unique to the individual tumors closely link to the clinical outcomes of patients. Establishing risk stratification models after analyzing biologic characteristics of each case has made a great success in patient management. However, the trend of improving survival rates in neuroblastoma over the last 30 years has started to level off, and currently available treatment modalities have almost reached to their maximized intensity. Furthermore, aggressive treatment causes significant long-term morbidities to the survivors. We really need to make the next step to the level of personalized medicine with more precise understanding of neuroblastoma biology. This book includes useful data and insights from the world's experts in this field. I believe this book can make an excellent contribution to all the investigators working hard and fighting for the children stricken by this disease.

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