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PI3K/AKT and ERK regulate retinoic acid-induced neuroblastoma cellular differentiation

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ABSTRACT

Neuroblastoma, the most common extra-cranial solid tumor in infants and children, is characterized by a high rate of spontaneous remissions in infancy. Retinoic acid (RA) has been known to induce neuroblastoma differentiation; however, the molecular mechanisms and signaling pathways that are responsible for RA-mediated neuroblastoma cell differentiation remain unclear. Here, we sought to determine the cell signaling processes involved in RA-induced cellular differentiation. Upon RA administration, human neuroblastoma cell lines, SK-N-SH and BE(2)-C, demonstrated neurite extensions, which is an indicator of neuronal cell differentiation. Moreover, cell cycle arrest occurred in G1/G0 phase. The protein levels of cyclin-dependent kinase inhibitors, p21 and $p27^{Kip}$, which inhibit cell proliferation by blocking cell cycle progression at G1/S phase, increased after RA treatment. Interestingly, RA promoted cell survival during the differentiation process, hence suggesting a potential mechanism for neuroblastoma resistance to RA therapy. Importantly, we found that the PI3K/AKT pathway is required for RA-induced neuroblastoma cell differentiation. Our results elucidated the molecular mechanism of RA-induced neuroblastoma cellular differentiation, which may be important for developing novel therapeutic strategy against poorly differentiated neuroblastoma.

1. Introduction

Neuroblastoma is derived from embryonal neural crest cells and is the most common extra-cranial tumor in infants and children. It is biologically heterogeneous, hence lending to a wide spectrum of clinical presentations. Most notably, it is characterized by a propensity to differentiate and even spontaneously regress when diagnosed in infancy. Pharmacologic agents can also induce neuroblastoma regression. For example, retinoic acid (RA) is used clinically for its ability to induce cellular differentiation. RA, the biologically active form of vitamin A, is a natural morphogen and plays an important role in the early embryonic development and differentiation of the nervous system [1]. RA can also suppress malignant cell growth by induction of cell cycle arrest, differentiation, or apoptosis. However, in some cases, RA not only fails to inhibit tumor cell growth, but can also enhance cell proliferation and survival [1,2].

Biological effects of RA are mediated by binding all-trans-RA (ATRA) and retinoid X (9-cis-RA) receptors (RARs and RXRs), respectively. These nuclear receptors are activated by RA to form RXR-RAR heterodimers [1] resulting in induction of tumor suppressor gene expression [3] such as RARr, $p21^{Cip1}$, $p27^{Kip1}$, thioredoxin reductase and interferon-regulatory factor-1 [4]. RXR-RAR modulates expression of retinoid-responsive genes in two ways: (i) by binding to RA response element (RARE) in promoter regions of target genes or (ii) by antagonizing the enhancer action of transcription factors, such as AP1 or NF-IL6. RA can also act via nuclear receptor independent pathways, in which retinoid-related molecules that do not bind to classical retinoid receptors can induce apoptosis through caspase activation [5].

RA derivatives are used in therapy against neuroblastoma, and show promising clinical results [6,7]; however, the overall survival, unfortunately, has remained short of anticipated. Therefore, it is vital to elucidate the cellular mechanisms that are involved in RA-induced neuroblastoma differentiation. In particular, discerning the potential role of RA-mediated compensatory signaling pathways that lead to cell survival and evasion of apoptosis may shed some important information as to their perplexing clinical results. Activated by various growth factors, the PI3K/AKT signaling cascade

Abbreviations: PI3K, phosphatidylinositol 3-kinase; ERK, extracellular-signal-regulated kinase; RA, retinoic acid; RARs, all-trans-RA (ATRA) receptor; RXRs, retinoid X (9-cis-RA) receptor.

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is an important cell survival pathway, which can also regulate differentiation process of various cells [8]. Overexpression of constitutively active PI3K induces neurite outgrowth and expression of neuronal markers [9]. RA-induced activation of Rac1, a GTPase involved in cell growth and cytoskeleton remodeling, has been shown to be regulated by phosphorylation of p85 regulatory subunit by Src kinase in SH-SY5Y neuroblastoma cells [10]. The PI3K inhibitor, LY294002, blocks RA-induced neurite outgrowth and expression of neuronal markers, suggesting that activation of PI3K/Rac1 signaling is involved in the regulation of neuronal differentiation [11]. The ERK1/2 pathway is another signaling cascade that can be activated by mitogens, and cytokines to stimulate cell proliferation [12]. Aberrant activations of both PI3K and ERK1/2 pathways are observed in various cancers, hence, they are important potential targets for anti-cancer therapy [13,14]. Moreover, the PI3K/AKT and ERK1/2 pathways are critically involved in the regulation of apoptosis [15].

In this study, we investigated the effects of RA on neuroblastoma cell signaling processes. RA treatment increased expressions of cyclin-dependent kinase (CDK) inhibitor, p21 and $p27^{Kip}$ and halted cell cycle progression. Interestingly, we found upregulation of anti-apoptotic proteins, Bcl-2 and Bcl-xL, with RA treatment, implying potential mechanisms of RA-resistance and clinical treatment failures in patients with neuroblastoma. Moreover, the PI3K/AKT activity was critical in RA-induced neurite elongation and cellular differentiation.

2. Materials and methods

2.1. Materials

All-trans-RA and β-actin antibodies were purchased from Sigma–Aldrich (St. Louis, MO). DNA fragmentation and cell cycle analysis kits were obtained from Roche Applied Science (Indianapolis, IN). The inhibitors, LY294002 and U0126, and antibodies against phospho-AKT, AKT, PTEN, Bad, Bcl-2, Bcl-xL, p21, $p27^{Kip}$, phospho-ERK1/2, ERK1/2 were purchased from Cell Signaling (Beverly, MA). Anti-neuron specific enolase (NSE) was obtained from Abcam (Cambridge, MA). All secondary antibodies against mouse and rabbit IgG were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The TransLucent RXR Reporter Vector was purchased from Panomics (Redwood City, CA).

2.2. Cell culture and transfection assays

Human neuroblastoma cell lines, SK-N-SH and BE(2)-C, were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 media (Cellgro Mediatech Inc., Herndon, VA) supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich) in a humidified atmosphere of 5% CO_2 at 37 °C. For transfections, 5×10^4 cells were seeded per well in a 24-well plate and transfected the next day with Lipofectamine™ 2000 (Invitrogen, Rockville, MD). Cells were harvested at indicated time points, and luciferase activity assay was performed with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Luciferase activity was measured by Luminometer Monolight 2010 (Analytical Luminescence Laboratory, San Diego, CA) and normalized to Renilla luciferase activity using the same samples. Cells were seeded on culture plates, serum-starved for 24 h and then treated with RA. RA concentration of 10 μM was used based on phase I clinical trial data in patients with neuroblastoma [7]. Morphologic neurite formation was assessed daily with a Nikon inverted phase-contrast microscope (Nikon Eclipse TS100).

2.3. Cell cycle analysis

Cell cycle distribution was analyzed by flow cytometry. Briefly, 1×10^6 cells were trypsinized, washed once with PBS, and fixed in 70% ethanol. Fixed cells were again washed with PBS, incubated with 100 μg/ml RNase for 30 min at 37 °C, stained with propidium iodide (50 μg/ml), and analyzed on a FACScan flow cytometer. The percentage of cells in different cell cycle phases was analyzed using Cell-FIT software (Becton–Dickinson Instruments, San Jose, CA).

2.4. Cell survival and cell death assays

Cells were seeded in 96-well plates at $5–10 \times 10^3$ cells/well in RPMI 1640 culture media with 10% FBS and incubated overnight. They were then cultured in serum-free RPMI media overnight and treated with RA (10 μM). Cell survival was assessed daily using the Cell-Counting Kit-8 (Dojindo Molecular Technologies, Inc., Gaithersburg, MD). The values were read at OD450 with the EL808 Ultra Microplate Reader (Bio Tek Instrument, Inc., Winooski, VT). DNA fragmentation was measured as previously described [16]. Cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) were detected using a Cell Death Detection ELISAplus kit (Roche Applied Science) according to the manufacturer's protocol. Each assay was performed in triplicate.

2.5. Western blot analysis

Protein concentrations were quantified using the Bio-Rad Protein Assay kit (Hercules, CA). Equal amounts of protein were fractionated by electrophoresis on 4–12% NuPAGE Novex Bis-Tris gels (Invitrogen), transferred to PVDF membranes, and probed with antibodies. The bands were visualized by an enhanced chemiluminescent detection system (ECL Plus) according to the manufacturer's instructions (Amersham Inc., Piscataway, NJ).

3. Results

3.1. RA induced neuroblastoma cellular differentiation by G0/G1 cell arrest

The basis for therapeutic application of RA is its ability to induce tumor cell differentiation followed by cell death. Thus, we first examined the effects of RA on neuroblastoma cellular differentiation. In both SK-N-SH and BE(2)-C human neuroblastoma cell lines grown in serum-free RPMI media, a very little evidence of cell differentiation was observed at baseline (Fig. 1A, left). However, with administration of RA (10 μM), cells exhibited distinct morphological changes consistent with differentiation as evidenced by development of long, out-branched neurites (Fig. 1A, right). Correlative to morphologic evidence, the expression of NSE, a marker of neuroendocrine cellular differentiation, also increased significantly at 5 days after treatment with RA (Fig. 1B).

RA induction of cellular differentiation is preceded by cell cycle arrest [17]. To investigate the effects of RA on cell cycle progression, we next measured the distribution of cells in cell cycle phases using flow cytometry. After serum starvation for 24 h, SK-N-SH cells demonstrated cell cycle arrest with 84.64% of cells in G1 phase. When treated with RA, the percentage of SK-N-SH cells in G1 phase increased to 86.82%. The percentage of cells in S phase decreased from 13.18% to 10.68% (Fig. 1C). RA also increased cells in G1 phase from 76.23% to 77.60% when assessed under without serum starvation (data not shown). Thus, RA treatment consistently induced neuroblastoma cell cycle arrest. Expressions of CDK inhibitors p21 and $p27^{Kip}$, which are thought to be important in regulating the G1 phase checkpoint [18], were measured by