Original Paper

The Combined Effect of Retinoic Acid and LSD1 siRNA Inhibition on Cell Death in the Human Neuroblastoma Cell Line SH-SY5Y

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Key Words

Neuroblastoma • Retinoic acid • Lysine-specific demethylase 1 • Apoptosis • SH-SY5Y cell line

Abstract

Aims: Retinoic acid (RA) is used pharmacologically to treat neuroblastoma (NB), but its mechanism of action is unclear and it has limited use against refractory disease. This study investigated the expression of LSD1 (also known as KDM1A) in tumors, and assessed the efficacy of combining RA treatment with the inhibition of LSD1 expression. **Methods:** LSD1 protein expression levels were assessed semi-quantitatively in specimens of NB and ganglioneuroblastoma (GNB), along with the apoptosis markers, Bcl-2 and Bax. The combined effect of RA and LSD1 siRNA inhibition on cell death was then assessed in the human neuroblastoma cell line, SH-SY5Y. **Results:** LSD1 expression was higher in NB compared to GNB, and LSD1 overexpression directly correlated with Bcl-2 expression and inversely correlated with Bax expression. RA treatment or LSD1 siRNA inhibition alone inhibited the growth of SH-SY5Y cells, but did not cause significant apoptosis or cell death. Combined treatment led to higher rates of SH-SY5Y cell death, as reflected by an increased Bax/Bcl-2 ratio. **Conclusions:** The combined effect of RA and LSD1 siRNA inhibition had a synergistic effect on promoting the apoptosis of NB cells. This novel approach may improve the clinical treatment of NB.

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Introduction

Neuroblastoma (NB) is the most predominant tumors occurring during early childhood. For example, it is the most common extracranial solid neoplasm in children, is responsible for 7% of malignancies in patients younger than 15 years, and represents ~15% of deaths due to childhood cancer [1]. NB has a favorable prognosis when diagnosed prenatally or in the newborn period; however, tumors that are diagnosed after the first year are often more

aggressive with extensive metastatic disease and an unfavorable prognosis [2]. The clinical course of NB is heterogeneous, and has been classified as low, intermediate or high risk. Low risk disease has a favorable prognosis, whereas high risk NB is difficult to treat, even with intensive chemotherapy, surgery, radiotherapy and stem cell transplantation. NB has been linked to distinct genetic variants, including *N-myc* amplification [3], 1p36 and 11q deletion [4], and 1q21.1 deletion or duplication [5]. About 20% to 50% of patients with high risk NB are progressive or refractory [6], and therapies are still very poor for refractory disease.

Retinoic acid (RA) is a natural and synthetic derivative of vitamin A [7] which is known to exert profound effects on cell proliferation, differentiation and morphogenesis, by acting through nuclear RA receptors and retinoid X receptors [8, 9]. Previous studies have reported that RA has antitumor effects on NB-derived cell lines, accompanied by a marked decrease in the expression level of *N-myc* [10]. It has also been reported that exposure to all-trans-RA can induce NB-derived cells to undergo neuronal differentiation, cell cycle arrest [11, 12]. RA is used pharmacologically to treat NB. However, the mechanism of action of RA remains unclear and its effects against refractory disease are limited [13]. Clinical resistance to this agent is a significant problem for ongoing NB treatment [14].

Lysine-specific demethylase 1 (LSD1), also known as KDM1A, was the first histone demethylase to be identified [15]. LSD1 belongs to the flavin adenine dinucleotide-dependent amine oxidase superfamily, and is known to specifically demethylate mono- or dimethylated histone H3 lysine 4 and H3 lysine 9 via a redox process [16-18]. Since LSD1 was discovered, increasing evidence has shown that its deregulation has a significant impact on human carcinogenesis, for example, LSD1 has been implicated in the etiology of various cancers including breast cancer [19], prostate cancer [20], and Ewing's sarcoma [21]. In this study we observed that LSD1 was strongly expressed in NB, and that its expression strongly correlated with the expression of Bcl-2. While RA treatment or LSD1 inhibition alone slightly affected the growth of SH-SY5Y cells, combined treatment caused significant apoptosis, which was associated with an inhibition of Bcl-2 expression and increased Bax expression.

Materials and Methods

Tissue microarrays and immunohistochemistry

Tissue microarrays (TMAs) were constructed according to a previously described method [22]. TMA blocks were cut into 5µm sections and processed for immunohistochemistry in accordance with a previously described protocol [23]. In brief, TMA sections were first dewaxed and rehydrated, endogenous peroxidase activity was then blocked using 0.3% H₂O₂. Sections were incubated with 10% goat serum for 30 mins and incubated with the following primary antibodies overnight at 4°C: anti-LSD1 (1:100 dilution; Cell Signal Technology, USA) anti-Bcl-2 (1:200 dilution; Santa Cruz Biotechnology, USA) or anti-Bax (1:200 dilution; Santa Cruz Biotechnology, USA). Subsequently, sections were incubated with a secondary antibody (1:2000 dilution; Thermo Scientific, USA) or 1 h at room temperature. Visualization of immunostaining was achieved using the Envision System with diaminobenzidine (Dako, Glostrup, Denmark).

Evaluation of immunostaining

LSD1, Bcl-2, and Bax immunostaining were scored using a semi-quantitative method to evaluate the percentage of positive tumor cells (0–100%). Scores were categorized in 25% increments (i.e. 0%, 25%, 50%, 100%), as described previously [24]. Protein expression was scored by three independent pathologists, who were blinded to the clinical data. A score was accepted if at least two of the pathologists were in agreement.

Cell culture

SH-SY5Y cells were obtained from the American Type Culture Collection. The cell line was chosen because SH-SY5Y cells are aggressive and poorly differentiated. SH-SY5Y is a “N” type NB cell line with small rounded adherent clumps and short spiny neuritic processed form. Cells were cultured in 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum at 37°C in a humidified 5% CO₂ atmosphere.

Cell proliferation assay

Cell proliferation was measured using a cell counting kit-8 (CCK-8, Dojindo Molecular Technologies, Japan), in accordance with the manufacturer's protocol. Briefly, the cell counting reagent was added to SH-SY5Y cells growing in 96-well plates, which were then incubated at 37°C for 2 h in a humidified 5% CO₂ atmosphere. Metabolically-active cells convert the water-soluble tetrazolium salt to a yellow-colored formazan dye product. The formazan dye is soluble in tissue culture medium and the quantity of formazan product, measured by absorbance at 450 nm, is directly proportional to the number of living cells in culture.

Apoptosis and Cell cycle analysis

To detect the changes of apoptosis, SH-SY5Y cells were analyzed with Annexin V-EGFP/PI Apoptosis Detection Kit (R&S Biotech, Shanghai) according to the protocol of manufacture. To monitor changes in the cell cycle distribution, cells were harvested 48 hr after treatment, washed with PBS, fixed in 70% ethanol and stained with 10 mg/ml propidium iodide.

LSD1 siRNA transfection

Cells were seeded in a six-well cell culture plate (2 × 10⁵ cells/well) in 2 ml antibiotic-free normal growth medium supplemented with 10% FBS. The cells were incubated at 37°C in a CO₂ incubator until the cells were 60-80% confluent. This usually took 18-24 hours. Then, SH-SY5Y cells were transiently transfected with a small interfering RNA (siRNA) targeted against *LSD1*, or with a scrambled control siRNA (Santa Cruz Biotechnology, USA) according to the manufacturer's protocol.

Protein extraction and Western blot analysis

Western blotting was used to determine *LSD1*, PARP, Bcl-2 and Bax expression levels in extracts from SH-SY5Y cells grown in a 6-well plate. The cells were washed twice with cold PBS before adding radio-immunoprecipitation assay lysis buffer containing a protease inhibitor cocktail and a phosphatase inhibitor cocktail (Thermo Scientific). Extracts were centrifuged at 12,000 g for 15 mins at 4°C and the protein concentration was determined using a BCA protein assay (Thermo Scientific). Forty microgram-amounts of extracted protein were run on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were then incubated with primary antibodies, anti-full length PARP and anti-cleaved PARP from Abcam (USA), and others as indicated above, at 4°C overnight; the membranes were then incubated with secondary antibody coupled to horseradish peroxidase for 2 h. Immunoreactivity was visualized using enhanced chemiluminescence reagents (Thermo Scientific) and protein bands were quantified with a densitometer (Chemidoc MP; Bio Rad).

Statistical analysis

Statistical analysis was performed using SPSS Version 19.0 (SPSS; IBM, USA). The association between *LSD1* expression and other variables was analyzed using the Spearman rank test. Other statistical analyses were carried out using the Student's t-test for unpaired data. A difference was considered significant if the *p*-value from a two-tailed test was less than 0.05.

Results

LSD1 is expressed in neuroblastoma and correlates significantly with Bcl-2 expression

In this study, we initially analyzed expression of *LSD1* using a TMA containing 48 specimens, including 32 NBs and 16 GNBs. The clinical data for each specimen, as well as the score for *LSD1*, Bcl-2 and Bax expression is presented in Table 1. Our data shows that *LSD1* was expressed at a relatively higher level in NB compared to GNB (Fig. 1). Similarly, Bcl-2 was also overexpressed in NB, and at relatively higher levels than in GNB (Fig. 1). In contrast, the expression of Bax was much lower in NB and GNB (Fig. 1). Correlations amongst *LSD1*, Bcl-2 and Bax expression were analyzed by the Spearman's rank test (Table 1). The results indicated that *LSD1* expression positively correlated with the expression of Bcl-2 (*LSD1* vs. Bcl-2: $\rho = 0.537$; $p = 0.001$), and was inversely correlated with the expression of Bax (*LSD1* vs. Bax; $\rho = 0.434$, $p = 0.03$).

Samples NO	Gender (M/F)	Years (Y/M)	Diagnosis	Clinical Stages	Lymph node metastasis (Y/N)	LSD1	Bcl-2	Bax
1	M	8M	NB	IV	N	+++	++	—
2	F	18M	NB	III	Y	++	++	—
3	F	2Y	NB	I-II	N	++	++	—
4	M	22M	NB	IV	N	++	++	—
5	M	1Y	NB	IV	Y	+++	+++	—
6	M	3Y	NB	IV	N	+	++	—
7	M	2Y	NB	IV	Y	+++	+++	++
8	M	2Y	NB	III-IV	Y	++++	++++	—
9	F	14M	NB	IV	Y	++++	++	—
10	M	11M	NB	III	Y	++++	++++	+
11	M	6M	NB	II	Y	++++	++++	++
12	F	5Y	NB	IV	Y	++++	++++	+
13	M	8M	NB	I	Y	+++	+++	—
14	M	3Y	NB	IV	N	++++	++	++
15	F	1M	NB	I	N	++++	++++	+
16	M	1M	NB	IV	Y	+++	++++	+
17	M	7M	NB	III	Y	++++	++++	+
18	M	2Y	NB	I	Y	++++	++++	++
19	M	13M	NB	I-II	Y	+++	++++	++
20	M	2Y	NB	I	Y	++++	++++	++
21	F	3Y	NB	I	N	+++	++	+
22	M	20M	NB	IV	N	++++	++++	++++
23	M	2Y	NB	III	Y	+++	+++	—
24	M	9M	NB	III	Y	++++	++++	+++
25	F	13M	NB	III	Y	++++	++++	++
26	M	2Y	NB	IV	Y	+++	++++	+
27	M	7Y	NB	I	Y	++++	++	—
28	F	15Y	NB	I	N	++	++	+
29	F	3Y	NB	III	Y	++++	+++	+
30	M	17M	NB	IV	Y	+++	++++	+
31	F	6Y	NB	I	Y	+++	+++	—
32	F	8Y	NB	I	Y	++++	++	—
33	M	3Y	GNB	IV	Y	++	+++	—
34	M	3Y	GNB	I	Y	+++	++	—
35	M	4Y	GNB	IV	Y	++++	+++	—
36	F	3Y	GNB	IV	N	+	+	+
37	F	3Y	GNB	IV	Y	+++	++++	+
38	M	5Y	GNB	IV	Y	++++	++++	++
39	M	5Y	GNB	IV	Y	++++	++++	+
40	F	3Y	GNB	III-IV	N	++++	++++	+++
41	F	4Y	GNB	I	N	++++	+++	++
42	M	2Y	GNB	III-IV	N	++++	++	++
43	M	2Y	GNB	I	Y	++++	++++	++
44	M	3Y	GNB	IV	Y	+++	+++	++
45	F	2Y	GNB	II	N	+++	+++	+
46	M	4Y	GNB	II	N	+++	+++	++
47	M	7M	GNB	III	Y	+++	++++	+
48	M	5Y	GNB	I	Y	++++	++++	++

Table 1. Clinicopathological features and results of LSD1, Bcl-2 and Bax immunostaining in neuroblastic tumor samples. LSD1, Bcl-2, and Bax immunostaining were scored using a semi-quantitative method to evaluate the percentage of positive tumor cells (0–100%). Scores were categorized in 25% increments (–, negative; +, 25% positive; ++, 50%; +++, 75%; +++, 100%). Protein expression was scored by three independent pathologists, who were blinded to the clinical data. A score was accepted if at least two of the pathologists were in agreement. (LSD1 vs. Bcl-2: $\rho = 0.537$; $p = 0.001$) (LSD1 vs. Bax: $\rho = 0.434$; $p = 0.03$).

Combined effect of retinoic acid and LSD1 inhibition on SH-SY5Y cells

Taking into consideration that LSD1 was overexpressed in neuroblastic tumors, we further analyzed its functional relevance in NB. SH-SY5Y cells were transiently transfected with a small interfering RNA (siRNA) directed against *LSD1*, or with a scrambled control siRNA. A significant knockdown of LSD1 protein expression was detected 48 h after transfection (Fig. 4). Upon siRNA-induced knockdown of *LSD1*, a decrease in cell viability

Fig. 1. LSD1, Bcl-2 and Bax expression in NB and GNB tissue microarrays. LSD1 expression was relatively higher in NB specimens (A, B) compared to GNB (C, D). Bcl-2 was also overexpressed in NB (E, F), and present at slightly lower levels in GNB (G, H). The expression of Bax was much lower in NB (I, J) and GNB (K, L).

Fig. 2. The effect of RA and LSD1 inhibition alone or in combination on the growth of SH-SY5Y cells. SH-SY5Y cells were transfected with siRNA against LSD1 (10 µM) or RA treatment (10 µM) slightly inhibited the proliferation of SH-SY5Y cells. SH-SY5Y cells treated with combined RA and LSD1 siRNA inhibition showed a significant reduction in cell numbers after a 48 hr incubation.

was detected using cell proliferation assays (Fig. 2). Decreased viability was accompanied by the appearance of morphologic features indicating differentiation or apoptosis, such as outgrowth of neurite-like structures and fragmentation of the nucleus. Moreover, flow cytometric analysis showed that apoptosis and subG1 fraction was increased significantly when treatment with LSD1-siRNA and RA (Fig. 3).

To examine the possible effects of RA on growth and viability of SH-SY5Y cells, the cells were cultured with 10 µM RA and the numbers of viable cells were counted after 48 h. As shown in Fig. 2, 10 µM RA didn't significantly inhibited the proliferation of SH-SY5Y cells. To analyze the combined effect of LSD1 siRNA inhibition and RA on the death of SH-SY5Y cells, the cells were transfected with siRNA targeting LSD1 and exposed to 10 µM RA for 48 h. As shown in Fig. 2, we observed an increase in cell death following the combined treatment. Furthermore, morphological changes in the form of neurite-like structures and fragmentation of the nucleus became more significant (Fig. 3).

Fig. 3. Induced changes in cell morphology of SH-SY5Y cells by RA treatment and/or LSD1 inhibition using phase-contrast microscopy. (A,D,E) Arrows indicate cells with a neurite phenotype. Combined RA treatment and LSD1 siRNA inhibition resulted in a significant increase in the number and length of neurite-like structures (E), magnified by 200-fold (A-E). Nuclear fragmentation into oligonucleosomes and chromatin condensation served as an indicator of apoptosis (J). Complete nuclear body fragmentation increased significantly after combined treatment with RA and LSD1 siRNA. Arrows indicate apoptotic cells, magnification 400-fold (F-J). Flow cytometric analysis of SH-SY5Y cells after siRNA-mediated LSD1 knockdown, RA treatment or combination of them. The apoptosis and the subG1 fraction was increased significantly when treatment with LSD1-siRNA and RA, indicating increase apoptotic cell death (K,L).

To elucidate the molecular mechanisms underlying the combined effect of RA and LSD1 inhibition on apoptotic cell death in SH-SY5Y cells, we examined whether poly-ADP-ribose polymerase (PARP), one of the physiological substrates of activated caspase-3, could be proteolytically activated in response to RA and LSD1 inhibition. As shown in Fig. 4, cleavage of PARP was observed in cells with LSD1 inhibition and RA treatment alone, as well as cells treated in combination. It was apparent that the cleavage of PARP in cells with the combined treatment was higher than the single treatment groups. The Bcl-2 family includes anti-apoptotic members such as Bcl-2, and pro-apoptotic members such as Bax. The ratio of Bax to Bcl-2 has been reported to be correlated with apoptosis. Our results show that treatment of cells with 10 μ M RA and LSD1 siRNA inhibition induced an increase in the protein level of Bax and a decrease in the protein level of Bcl-2 (Fig. 4). Furthermore, there was an approximate 3-fold increase in the ratio of Bax/Bcl-2 expression in these cells, compared with cells treated with 10 μ M RA or LSD1 siRNA inhibition alone.

Fig. 4. The effect of LSD1 inhibition and RA treatment on LSD1, PARP cleavage, Bcl-2, and Bax expression in SH-SY5Y cells. Western blot analysis of LSD1, PARP, Bcl-2, and Bax in SH-SY5Y cells after exposure to 10 μ M RA and/or LSD1 siRNA inhibition for 48 h (left). Bcl-2 and Bax protein levels in SH-SY5Y cells were assessed using Western blotting and densitometric analysis. There was an approximate 3-fold increase in the ratio of Bax/Bcl-2 expression compared with cells treated with 10 μ M RA or LSD1 siRNA inhibition alone (right).

Discussion

Present therapy for NB includes surgery, chemotherapy, radiation therapy and biological approaches [25]. RA has been shown to induce neuronal differentiation and/or apoptosis, and is widely used as a chemotherapeutic agent for treating patients with NB. Despite its efficacy, the effects of RA against refractory disease are limited. Moreover, resistance to RA represents a significant drawback to its clinical application. In this study, we demonstrated that LSD1 inhibition could enhance the ability of RA to promote the death of NB cells. LSD1, the first identified histone demethylase, is overexpressed in breast cancer, small cell lung cancer, colorectal, bladder and prostate cancers and neuroblastoma [19, 26, 27].

We also found that LSD1 is strongly expressed in NB, and at much higher levels than in benign GNB. This result was consistent with reports that LSD1 expression was higher in undifferentiated neuroblastic tumors than in the differentiated tumors [27]. To further analyze the role of LSD1 in the pathogenesis of NB, we found that transient knockdown of *LSD1* using siRNA increased the death of SH-SY5Y cells. Furthermore, we found that the effect of RA on the death of SH-SY5Y cells was significantly enhanced after LSD1 siRNA inhibition.

The pathogenesis of a wide variety of human diseases is frequently related to impaired control of apoptosis [28]. Indeed, we observed that Bcl-2 expression was high in neuroblastic tumors, but Bax expression was relatively low or undetectable. Moreover, expression of these proteins was correlated with LSD1 expression. This correlation suggested that the effect of RA and LSD1 inhibition on human SH-SY5Y cells may be mediated through the mitochondrial apoptotic pathway. Bcl-2 and Bax are important endogenous regulators of apoptosis and cellular activity in response to a variety of physiological and pathological insults [29, 30]. It is known that an increase in the Bax:Bcl-2 ratio can trigger the release of mitochondrial pro-apoptotic factors such as cytochrome C, Smac and AIF into the cytosol, which mediates apoptosis [29, 31]. Interestingly, Anna et al. indicated that overexpression of Bcl-2 after RA-induced differentiation of NB cells represented a loss of the apoptotic response or resistance

to other chemotherapeutic drugs [32]. Treatment with RA can be led to an increase in H3K4me2 level [33], and loss of RA receptor target genes expression is associated with a reduction in H3K4me2 [34]. Here, we found that inhibition of LSD1 enhanced the effect of RA on the death of SH-SY5Y cells, through reducing the expression of Bcl-2 and increasing the levels of Bax (Fig. 4). Our results showed that there was an approximate 3-fold increase in the ratio of Bax/Bcl-2 expression in these cells, compared with cells treated with 10 μ M RA or LSD1 siRNA inhibition alone. In conclusion, our results suggest that a combination of LSD1 inhibition and RA treatment could be a new therapeutic option for the management of NB.

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