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Histone deacetylase inhibition induces apoptosis and autophagy in human neuroblastoma cells

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ABSTRACT

Neuroblastoma (NB) is the most common solid extracranial tumor in children. Here we showed that trichostatin A, a histone deacetylase inhibitor (HDACi), decreases cell viability in three NB cell lines of different phenotypes. The treatment leads to G2/M-phase arrest, apoptosis and autophagy. Autophagy induction accompanies apoptosis in the most proliferative, N-Myc overexpressing cells. In contrast, autophagy precedes apoptosis and acts as a protective mechanism in the less proliferative, non-N-Myc overexpressing cells. Therefore, the autophagy induction is a relevant event in the NB response to HDACis, and it should be considered in the design of new treatments for this malignancy.

1. Introduction

The acetylation of histones regulates access of transcription factors to DNA and gene expression, contributing to the involvement of epigenetic processes in cancer genesis and progression [1]. The degree of acetylation is mediated by histone acetyltransferases and deacetylases. Hyperacetylated histones tend to result in transcriptional active genes, whereas hypoacetylation typically results in repressed transcription. Acetyltransferases, although initially described as histone acetylases, are also able to acetylate non-histone proteins. Similarly, histone deacetylases (HDACs) are a family of enzymes that reverts the protein acetylation of nuclear and cytoplasmic proteins, decreasing the expression of many genes and regulating the function of acetylable proteins in the cytoplasm. These HDAC substrates are directly or indirectly involved in numerous important cell pathways, including control of gene expression, regulation of cell proliferation, differentiation, migration, and death [2]. Therefore, HDAC inhibitors (HDACis) are able to induce cell growth arrest and cell death in several tumoral cell lines, especially in high-risk embryonic tumors [3], showing low toxicity towards normal cells [4]. HDACis have been extensively studied for cancer therapy, some of which are currently under clinical trials [2,5].

Neuroblastoma (NB), an embryonic tumor of the autonomic nervous system, is the most common extracranial form of solid tumor in childhood. The clinical presentation of NB is highly variable, and exhibits diverse and often dramatic clinical behaviors. Therapies are effective for patients with low- and intermediate-risk disease, but outcomes in high-risk NB remain a clinical challenge [6]. As in most cancers, the treatment against NB is mainly designed to promote the death of transformed cells, or to inhibit its cell-proliferation by inducing differentiation [6]. However, some NB cells can dedifferentiate, especially under hypoxic conditions [7], escaping the treatment and causing relapse.

Studies in NB have shown that some HDAC family members are aberrantly expressed in these tumors, correlating some of these alterations with the disease stage and prognosis. The knock-down of individual HDACs causes different phenotypes ranging from differentiation to apoptosis [8]. Experimental treatments of different human NB cell lines with HDACis have revealed that apoptosis is induced by gene expression modifications, and by cytosolic activation of proapoptotic protein Bax [9,10].

It has been recently described that tumor resistance to therapy could be related to cell survival through autophagic mechanisms. Autophagy is a process known to provide metabolic sources to the cell under nutrient depletion or other stresses, but it has also been linked to the death process itself. In the autophagy triggering, a number of factors (Atg proteins) are mobilized to induce *de novo*

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building of autophagy vacuoles that, after their fusion with lysosomes and conversion to autophagolysosomes, may feed the cell with the products of macromolecules degradation. However, the induction of autophagy may be a double-edged sword, since it is also a mechanism leading to cell death when destruction of cell cytoplasm components is activated in a long-lasting way [11]. In fact, autophagy has acquired a growing interest in cancer, offering new and suggestive targets for its treatment [12,13]. In solid tumors, not yet vascularized, hypoxic and nutrient limited regions develop autophagy to promote survival. At the same time, autophagy limits the extent of further genomic damage that could lead to more aggressive tumors and, depending on the type and stage of tumors, it can be a partner of apoptosis [11]. Interestingly, NB cell lines have the ability to develop autophagy in response to aggression, leading to cell death induction [14,15].

Herein we describe that trichostatin A (TSA), a non-specific inhibitor of HDACs, induces G2/M-cycle phase arrest, activation of the pRB protein, and induction of apoptosis and autophagy in different human NB cell lines. However, the significance of the autophagy induction differs from one cell line to the other, ranging from the activation of survival pathways to the initiation of cell death programs. Therefore, we consider that this effect is worth taking into account, to improve the therapy-outcome of NBs, as well as to develop new treatment strategies for this malignancy.

2. Materials and methods

2.1. Cell culture and reagents

Three human NB cell lines were used in this study supplied by the Children's Hospital Sant Joan de Déu of Barcelona (Spain): the N-type NB cells LA1-55N, with small cell bodies and neurite-like processes; the I-type SK-N-JD, with and intermediated phenotype between N- and S-type NB cells, resembling that of stem cells; the S-type SK-N-AS, characterized by bigger cell bodies and the absence of neurite-like processes, is considered the least tumorogenic type [16]. A characteristic of LA1-55N and SK-N-JD, but not of SK-N-AS cells, is *N-MYC* gene amplification [17]. Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Biological Industries, Kibbutz Beit Haemek, Israel). Cells were maintained in a humidified 10% CO_2 atmosphere at 37°. The experiments were performed in 6–12- or 96-well culture plates at 70% confluence. Cells were treated with the pan-HDAC inhibitor trichostatin A (TSA) (Sigma–Aldrich, Saint Louis, MO), the broad spectrum caspases inhibitor N-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD.fmk, 100 μM) (Bachem, Bubendorf, Switzerland), or the autophagy inhibitor 3-methyladenine (3-MA, 10 mM) (Sigma–Aldrich, Saint Louis, MO), recently described as a PI3 K-III inhibitor [18]. TSA and zVAD were both dissolved in DMSO (Sigma–Aldrich, Steinheim, Germany); 3-MA was dissolved in RPMI 1640 medium.

2.2. Viability

Cell survival was evaluated using the MTT colorimetric assay (Sigma, Steinheim, Germany). 10^4 cells were incubated in 96 well microtiter cell culture plates, in the absence (control cells) or presence of TSA, in a final volume of 100 μl. After the indicated treatment, cells were incubated for 3 h at 37 °C in RPMI containing 10 μM MTT (diluted in PBS). The blue MTT formazan precipitate was then dissolved in 100 μl of isopropanol and the absorbance was measured at 570 nm on a multiwell plate reader. The absorbance measured was related to the protein content (BCA (bicinchoninic acid) Protein Assays, Pierce, Rockford, IL) and considered proportional to the number of viable cells. Cell viability was expressed as a percentage of these values in treated cells in comparison with the non-treated control cells. Data are shown as the mean ± standard error media of triplicate cultures.

Cell viability was also assessed by trypan blue assay. In that case 2×10^5 cells were cultured in six well plates, after the indicated treatment cells were washed twice in phosphate buffer solution (PBS) and dyed with 0.2% trypan blue (Sigma–Aldrich, Steinheim, Germany) for 2 min. The percentage of dyed (death) cells and not dyed (alive) cells was counted with a Neubauer cell counting chamber.

2.3. Apoptosis and cell cycle analysis by the propidium iodide staining method

Cells (2×10^6/plate) were seeded and collected 24 h after the indicated treatment. Cells were then washed twice with ice-cold PBS complemented with 1% FBS, resuspended in 500 μl of the same solution, and fixed with 5 ml of 70% ice-cold ethanol O/N at −20 °C. For staining with propidium iodide (PI, Bender MedSystems, Vienna, Austria), cells were washed twice in ice-cold PBS with 1% FBS, resuspended in 300 μl citrate–phosphate tampon, and incubated for 30 min at room temperature. Cells were centrifuged and the pellet was resuspended in 400 μl of PBS complemented with 1% FBS containing 0.5 mg/ml PI and 10 mg/ml RNaseA and incubated for 30 min at room temperature. The stained cells were analyzed using a FACS-Calibur flow cytometer and Cellquest Software (Becton Dickinson, Mountain View, CA) [19].

2.4. Transmission electron microscopy (TEM)

After treatments, 2×10^6 cells were centrifuged and the pellets were fixed in a solution containing 2% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate tampon for 1 h at 4 °C. Afterwards they were postfixed in 2% osmium tetroxide for 4 h at 4 °C, dehydrated in ethanol and propylenoxide and embedded in Spurr. Finally, ultrathin sections were stained with uranyl acetate and lead citrate and examined in a JEOL 1010 transmission electron microscope. Digital images were obtained using a Bioscan Image Digitalization System (Gatan). Autophagy was quantified in three different experiments by counting the number of cells with at least five autophagic vacuoles (double membrane and internal cytoplasmic content) in 25 different fields containing near 50 cells each one, and expressed as percentage ±SEM.

2.5. RT-multiplex ligation-dependent probe amplification (RT-MLPA)

RNA was analyzed by reverse transcriptase multiplex ligation-dependent probe amplification (RT-MLPA) using SALSA MLPA KIT R011-B1 Apoptosis mRNA from MRC-Holland (Amsterdam, The Netherlands) for the simultaneous detection of 30 messenger RNA molecules. In brief, RNA samples (200 ng total RNA) were first reverse transcribed using a gene-specific probe mix. The resulting cDNA was annealed overnight at 60 °C to the MLPA probe mix. Annealed oligonucleotides were ligated by adding Ligase-65 (MRC-Holland, Amsterdam, The Netherlands) and incubated at 54 °C for 15 min. Ligation products were amplified by PCR (33 cycles, 30 s at 95 °C; 30 s at 60 °C, and 1 min at 72 °C) with one unlabeled and one FAM labeled primer. The final amplified PCR fragments were separated by capillary electrophoresis on a 48-capillary ABI-Prism 3730 Genetic Analyzer (Applied Biosystems/Hitachi, Foster City, CA). Peak area and height were measured using GeneScan analysis software (Applied Biosystems). mRNA levels for each gene were expressed as normalized ratios of the peak area divided by the peak area of a control gene, this result expresses the relative abundance of mRNA of the gene of interest. Areas were normalized to β-glucuronidase (*GUSB*) [20].

2.6. Western blot

Experiments were performed with whole cell extracts. Protein concentration was determined with the BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein were loaded on each lane, and electrophoresed on SDS–polyacrylamide gels with tris–glycine running buffer, and transferred to nitro-cellulose membranes. Blots were saturated with 5% skim milk, 0.1% Tween in TBS and incubated with antibodies against: beclin-1 (1:500), caspase-3 (1:200) (BD-Pharmingen, San Diego, LC3 (1:2000) (kindly provided by Dr. T. Yoshimori, N.I.G. Shizuoka-ken, Japan), ATG-5L (1:500), ATG-12 (1:500) (Abgent, San Diego, CA), acetylated histone-3 (1:1000), acetylated histone-4 (1:2000) (Upstate Biotechnology, Lake Placid, NY), N-Myc (1:500) (Calbiochem, San Diego, CA), pRB (1:5000) (New England Biolabs, Beverly, MA), E2F1 (1:5000), PARP (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA), survivin (1:250) (Novus Biological, Littleton, CO) and β-actin (1:5000) (Abcam, Cambridge Science Park, Cambridge) which was used as a normalizing protein. After being washed, the membranes were incubated with biotinylated secondary antibody labeled with horseradish peroxidase (1:1000) (Amersham, Buckinghamshire, United Kingdom), for 1 h at room temperature, washed again and developed with the chemiluminescence ECL Western blotting system followed by apposition of the membranes to autoradiographic films (Hyperfilm ECL, Amersham, Buckinghamshire, United Kingdom). The results shown are indicative for two–three different experiments in each case.

3. Results

3.1. TSA decreases viability and induces cycle arrest at G2/M phase in NB cells

Histone deacetylase inhibition by 0.5 μM TSA in human NB cell lines in culture was assessed by Western blotting after 24 h treatment. At this low micromolar concentration TSA notably increased the level of acetylated histone H3 and H4 in the three cells lines (Fig. 1A). The effect of TSA on cell viability was assessed after exposition to different concentrations of TSA for 24 and 48 h. Although TSA treatment decreased the number of viable cells in a time- and dose-dependent manner in all the cell lines (Fig. 1B), this effect was