International Immunopharmacology 21 (2014) 439–446

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Class I-specific histone deacetylase inhibitor MS-275 overrides TRAIL-resistance in melanoma cells by downregulating c-FLIP

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ARTICLE INFO

Article history:
Received 6 March 2014
Received in revised form 6 May 2014
Accepted 20 May 2014
Available online 2 June 2014

Keywords:
HDACis
Apoptosis
TRAIL
c-FLIP
Cutaneous melanoma
Uveal melanoma

ABSTRACT

Tumor-necrosis factor-related apoptosis-inducing ligand (TRAIL) has selective killing effect toward malignant cells; however some human melanomas are intrinsically resistant. In this study, we have shown that class I-specific histone deacetylase inhibitor (HDACi) MS-275 can synergize with TRAIL to induce apoptosis in TRAIL-resistant cell lines and to enhance susceptibility of sensitive cells. Conversely, class II-selective HDACi MC1575 has shown no effect on the resistance of melanoma cells and was able exclusively to increase TRAIL-induced cell death in responsive cells. Both the HDACis variably increased DR4, DR5, and procaspase 8 expression, regardless whether cells were TRAIL-sensitive or TRAIL-resistant. However, only MS-275 markedly decreased the expression levels of both the long and short c-FLIP isoforms. RNAi-mediated c-FLIP silencing resulted in caspase 8-dependent apoptosis in survivor cells which was comparable to that observed following MS-275 treatment. Accordingly, enforced expression of ectopic c-FLIP has abolished the cooperative induction of apoptosis by the combination of MS-275 and TRAIL. These data indicate that c-FLIP is a critical regulator of death ligand sensitivity in melanoma. Inhibition of class I HDAC isoenzymes 1, 2 and 3 has resulted to be functionally important for c-FLIP downregulation by MS-275. In contrast, knockdown of class II HDACs has had no effect on c-FLIP expression, thus explaining the dual incapacity of MC1575 to inhibit c-FLIP expression and sensitize cells resistant to TRAIL. The data reported here suggest that MS-275 represents a promising therapeutic approach in combination with TRAIL for treatment of cutaneous and uveal melanoma due to its ability to reduce c-FLIP expression.

1. Introduction

Melanoma is an aggressive form of cancer predominantly located in the basal layer of the epidermis and in the eyes [1]. Only when it is diagnosed at the very early stages, it can be cured by surgical resection with >95% success rate [2]. Unfortunately, melanoma lesions commonly go unnoticed or are asymptomatic for a long time [3] until they acquire a metastatic ability and disseminate to multiple organs [4]. Among the potential therapeutics in melanoma, TNF-related apoptosis-inducing ligand (TRAIL)-based strategies may yield positive responses. Permanent as well as inducible TRAIL-resistance seen in some melanomas may limit its applicability. The downregulation or malfunction of TRAIL receptors and initiator caspase 8 [5,6], and/or upregulation of antiapoptotic molecules such as Flice-inhibitory protein (c-FLIP) [7] were listed as major causes of TRAIL treatment failure. Therefore, efforts have been undertaken to induce or increase cellular sensitivity for TRAIL-induced apoptosis by modulating death pathway at several levels. These findings have spurred studies to characterize the role of epigenetics in the expression of regulatory proteins of apoptosis. Recently, we have shown that the downregulation of DcR1 and DcR2 due to promoter hypermethylation in cutaneous and uveal melanoma is responsible for the reduced levels of apoptosis, whereas the methylation status of DR4 and DR5 does not affect the gene expression and the sensitivity to apoptosis [8]. Previous works had already shown that certain histone deacetylase inhibitors (HDACis) can enhance programmed cell death inducing potential of TRAIL in TRAIL-sensitive cells and sensitize TRAIL-resistant cancer cells *in vitro* and thus can be considered as clinically viable candidates for combinatory therapy [9,10]. However, at present little is known about their effects on TRAIL-induced apoptosis and the underlying molecular mechanisms in melanoma [11–13]. In this study, we have investigated the impact of MS-275 (a class I-specific HDACi) and MC1575 (a class II-selective HDACi) on TRAIL-mediated apoptosis in cutaneous and uveal melanoma cells. Both the HDACis

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http://dx.doi.org/10.1016/j.intimp.2014.05.024
1567-5769/

increased in a variable manner DR4, DR5, and procaspase 8, but exclusively MS-275 significantly repressed c-FLIP expression. This latter effect has played a crucial role in altering TRAIL sensitivity, as only MS-275 is suitable to switch tumor cells from a TRAIL-resistant into a sensitive state.

2. Materials and methods

2.1. Cell culture and treatment

The cutaneous melanoma cell line IGR-37 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) was grown in 85% DMEM with 4 mmol/l L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, and 15% fetal bovine serum. The uveal melanoma cell lines OCM-1 (provided by J. Mellon, Department of Ophthalmology, UT Southwestern Medical Center, Dallas, TX), OCM-3, Mel270 and 92.1 (provided by Martine J. Jager, Leiden University Medical Center, Leiden, The Netherlands) were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin, and 10% FBS. G361 cutaneous melanoma cells (ECACC) were grown in McCoy's 5a medium modified with 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin. To exclude that our observations were restricted to cell lines maintained in long-term culture, we also examined primary tumor cells derived from two patients with cutaneous melanoma (CMel-1, derived from a 72-year-old white male with nodular melanoma of the trunk; CMel-2, derived from a 72-year-old white female with superficial spreading melanoma on lower leg) and two patients with uveal melanoma (UMel-1, derived from a 52-year-old white male with choroidal melanoma; UMel-2, derived from a 59-year-old white male with ciliary body melanoma). Collection of biopsies and preparation of primary melanoma cell cultures were conducted following Institutional Review Board guidelines and approval. Low passage primary melanoma cells (CMel-1, CMel-2, UMel-1, and UMel-2) were cultured in DMEM-F12 medium supplemented with 10% FCS. Cells were incubated for 24 h in the presence of TRAIL (50 ng/ml) and, where indicated, pretreated with MS-275 (Sigma-Aldrich, Milano, Italy) or MC1575 (provided by Dr. Mai, University of Rome "La Sapienza", Italy) at concentrations ranging from 0.5 to 5 μM and for different times (12, 24, and 48 h) as previously reported [14]. The concentration resulting in 50% of cell growth inhibition (IC50) was found to be 1 μM for MS-275 and MC1575 and 50 ng/ml for TRAIL (data not shown).

2.2. Transient transfections

Small interfering RNAs (siRNAs) were designed to downregulate either both c-FLIP splice variants (referred to as FT) or to specifically target the long form (FL) or the short form (FS) (Dharmacon, Lafayette, USA), as described previously [15]. Specific siRNAs targeting HDACs 1, 2, 3, 4, 5, 6, 7, 9, and 10 were purchased from Santa Cruz Biotechnology.

c-$FLIP_L$ and c-$FLIP_S$ coding regions were PCR amplified and ligated into the pcDNA/V5-His TOPO vector according to the manufacturer's instructions (Life Technologies Inc.).

Cells were transiently transfected with specific siRNAs or expression vectors using METAFECTENE® PRO transfection reagent (Biontex Laboratories GmbH, Germany).

2.3. Total RNA and DNA extraction

Total RNA and DNA extraction was performed by Recover All Total Nucleic Acid Isolation kit (Ambion Inc) from FFPE samples, and by TRIzol Reagent (Invitrogen) from cells.

2.4. Reverse transcription and quantitative real-time PCR

Total RNA was reverse-transcribed with IMProm-II™ reverse transcriptase kit (Promega). Quantitative Real-Time PCR was performed by ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Milan, Italy). Primers and probes are listed in Table 1. The mRNA levels of specific genes were normalized to endogenous β-actin (Applied Biosystems).

2.5. Western blot analysis

Total cell extracts (50 μg) were resolved by SDS-PAGE and blotted onto nitrocellulose membranes with specific antibodies against procaspase 8, c-$FLIP_L$, and c-$FLIP_S$ (Abcam, Cambridge, MA), as well as against HDACs 1, 2, 3, 4, 5, 6, 7, 9, and 10 (Santa Cruz Biotechnology).

2.6. Apoptosis assay

Apoptosis was quantified using the Biocolor APOPercentage™ assay (Biocolor Ltd. Newtonabbey, UK).

2.7. Statistical analysis

Comparisons between groups were made with Student's *t* test. Differences were considered significant when the *p* value was <0.05.

3. Results

3.1. Effect of HDACis on the apoptosis-inducing potential of TRAIL

Because HDACis have been combined with TRAIL in a variety of cancers to overcome resistance to apoptosis [16–18], we investigated the effects of pretreatment with the class I-specific HDACi MS-275 and the class II-selective HDACi MC1575 on TRAIL-induced apoptosis in human cutaneous and uveal melanoma cell lines. As shown in Fig. 1, at 24 h treatment and 1 μM concentration, MS-275 induced apoptosis in all the cell lines examined, including those resistant to TRAIL. Interestingly, the sequential treatment of cells with MS-275 followed by TRAIL very dramatically sensitized survivor cells (IGR-37, OCM-1, OCM-3, 92.1, CMel-2, and UMel-2) to undergo apoptosis and was more effective in inducing cell death in TRAIL-sensitive lines (G361,

Table 1
Primers, probe sequences of the genes analyzed by quantitative real-time PCR, and size of amplicons generated.

Gene	Forward primer[a]	Reverse primer[a]	Probe[b]	Product size (bp)
DR4	AGTGCATGGACAGGGTGTGTT	CGTTGCTCAGAATCTCGTTGTG	TTGGGTCTCCTACGAGGG	82
DR5	CAAGACCCTTGTGCTCGTTGT	TTGGGTGATCAGAGCAGACTCA	CCGCGGTCCTGCTGTTGGTCTC	70
Procaspase 8	GGATTTATCATCACCTCAAACGAGAT	GGCCATCCCCAGCAGAA	TCCCGGATGAGGCTG	79
c-$FLIP_L$	GCTGGCAGCTGATTAGATGGT	TTTGAGTCAGTGGACTGGGAAA	CCACCCAGATTGAGG	70
c-$FLIP_S$	GAGTGCCGGCTATTGGACTTT	CCTCCTCCCGTGGTCCTT	TCCAGTGACAGCTGAGAC	62

Sequences for the primers and probes are shown in 5′ to 3′ orientation.
[a] Designed with Primer Express Software V3.0 (Applied Biosystems).
[b] Probes contain a FAM fluorescent as a reporter at the 5′ end and a MGBNFQ as a quencher at the 3′ end.