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A novel apoptosis-inducing mechanism of 5-aza-2′-deoxycitidine in melanoma cells: Demethylation of TNF-α and activation of FOXO1

Shunsuke Noguchi [a,b,*], Takashi Mori [c], Masaya Igase [d], Takuya Mizuno [a,b]

[a] Laboratory of Molecular Diagnostics and Therapeutics, Joint Faculty of Veterinary Medicine, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan
[b] Biomedical Science Center for Translational Research, The United Graduate School of Veterinary Science, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan
[c] Laboratory of Veterinary Clinical Oncology, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[d] The United Graduate School of Veterinary Science, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan

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ABSTRACT

Melanoma is a poor-prognosis cancer in both humans and dogs, and so the anti-tumor effects of 5-aza-2′-deoxycitidine (5-aza) on solid tumors such as melanoma have gained much attention. However, its anti-tumor mechanism remains entirely unclear. This present study revealed a part of the anti-tumor effects of 5-aza, focusing on apoptosis induction, on human and canine melanoma cells. Treatment with 5-aza markedly induced obvious apoptosis in melanoma cells. 5-Aza-induced apoptosis was possibly due to induced expression of cytotoxic cytokines such as *TNF-α*. We revealed hypermethylation of the promoter region of *TNF-α* as a consequence of treatment with 5-aza. Concurrently, we evaluated the effect of 5-aza on the Akt/FOXO1 signaling cascade, which plays a pivotal role in the transcription of cytokine genes. As a result, 5-aza inactivated Akt and inversely activated FOXO1, which contributed to the up-regulation of *TNF-α*. Furthermore, up-regulation of *TNF-α* by 5-aza administration was found in *in vivo* experiments. These current data suggest a novel apoptosis-inducing mechanism of 5-aza and indicate that 5-aza could be a promising therapeutic agent for the treatment of human and canine melanomas.

Introduction

Melanoma is one of the most common cancers in humans as well as in dogs. This cancer can easily metastasize to other organs in both species. Human melanoma occurs in the skin, whereas canine melanoma often develops in the oral cavity. However, canine melanoma is considered to be a good preclinical model for studies on the human one because it has an expression pattern of several genes, i.e., those for MAPK and Akt, similar to that of human melanoma, as well as similar clinical and histopathological features [1,2].

The B-Raf proto-oncogene, a serine/threonine kinase (BRAF), mitogen-activated protein kinase kinase 7 (MEK), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and programmed cell death 1 (PD-1) have been focused on as therapeutic targets in studies on human melanoma, and clinical trials targeting such molecules have been performed around the world [3]. Although the therapeutic modalities targeting these factors are considered to be promising, melanoma still remains a problem to be overcome. Therefore, additional therapeutic approaches are desired.

Decitabine, which is 5-aza-2′-dexycitidine (5-aza), inhibits DNA methylation through the inhibition of DNA methyltransferase 1 (DNMT1) and has been applied to the treatment of human myelodysplastic syndrome in the USA [4]. Also, 5-aza experimentally exhibits anti-tumor effects such as cytotoxicity, rescue from chemoresistance, and activation of antitumor immunity, and many clinical trials have focused on utilizing 5-aza as the sole agent or as part of a combination chemotherapy approach for the treatment of several kinds of human solid tumors [5]. The induction of apoptosis as an anti-tumor effect of 5-aza has especially been a matter of focus. 5-Aza induces apoptotic cell death by up-regulating Fas-signaling or the expression of TNF-related apoptosis-inducing ligand (TRAIL) in human leukemic cells [6,7]. Also, in melanoma, inhibitors of DNA methylation are considered to be promising therapeutic agents because DNA methylation contributes to the silencing of many kinds of tumor-suppressive genes [8–11]. However, the reason for the anti-tumor effects of, and mechanisms of inhibition of DNA methylation by, these inhibitors have mostly remained unclear.

Although DNA methylation, especially methylation of promoter regions, plays a pivotal role for gene expression, histone

Abbreviations: αTNF-α, mAb against human TNF-alpha; Bi-seq, bisulfite sequencing; ChIP, chromatin immunoprecipitation; FasL, Fas ligand; FOXO, Forkhead box O; mAb, monoclonal antibody; MSP, methylation-specific PCR; TNF-α, tumor necrosis factor-α; TRAIL, TNF-related apoptosis-inducing ligand; 5-aza, 5-aza-2′-dexycitidine.

* Corresponding author. Tel.: +81 83 933 5897; fax: +81 83 933 5930.
E-mail address: snoguchi@yamaguchi-u.ac.jp (S. Noguchi).

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methylation is also important. Histone H3K4 tri-methylation (H3K4me3) is positively associated with gene expression, whereas H3K27 tri-methylation (H3K27me3) correlates with gene silencing [12].

It is well established that Fas ligand (FasL) and TRAIL signaling induce apoptosis by binding to their respective receptors, Fas and TRAILR1 [13]. Notably, TRAIL elicits tumor cell-specific apoptotic cell death. Therefore, a therapeutic modality utilizing TRAIL signaling is considered promising for cancer therapy. On the other hand, apoptosis induced by another cytokine, tumor necrosis factor-α (TNF-α), is more complicated; i.e., the binding of TNF-α to TNFR1, but not that to TNFR2, triggers opposing biological responses such as cell survival and apoptosis [14,15]. Nuclear factor-κB (NF-κB) activation is associated with cell survival, whereas caspase-8 activation contributes to apoptosis via TNF-α signaling [13].

The transcription of several cytokines such as FasL and TRAIL are promoted by Forkhead box O (FOXO) transcription factors, especially FOXO1 and FOXO3 [16]. FOXOs are converted to their inactive phosphorylated form by activated phosphoinositide 3-kinase (PI3K)/Akt signaling. Altogether, FOXOs act as a tumor-suppressor. Thus, the PI3K/Akt/FOXOs signaling cascade plays a crucial role in the expression of these cytokines and apoptosis induced by them.

In this present study, we examined an apoptosis-inducing mechanism of 5-aza by focusing on the up-regulation of *TNF-α* in human and canine melanoma cells. Also, we partly revealed the mechanism of silencing of *TNF-α*. Our results suggest that canine melanoma is an important preclinical model that is useful for evaluating the potential beneficial effects of inhibitors of DNA methyltransferase such as 5-aza on human melanoma.

Materials and methods

Cell culture and cell viability

Human melanoma cell lines A2058 and Mewo were purchased from Health Science Research Resources Bank (Osaka, Japan). Human melanoma cell line colo679 was obtained from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University. Canine melanoma cell lines KMeC, LMeC, CMeC-1, and CMM1 were gifts from The University of Tokyo. Human normal epidermal melanocytes HEMa-LP and NHEM were obtained from Invitrogen (Carlsbad, CA, USA) and ScienCell Research Laboratories (Carlsbad, CA, USA), respectively. The cells were maintained according to the manufacturers' instructions. The number of viable cells was determined by performing the trypan blue dye exclusion test.

Clinical specimens

Twenty canine melanoma tissues were collected from the oral cavity of dogs with malignant melanoma at the animal hospital of Gifu University. All dogs had melanoma histopathologically confirmed by several veterinary pathologists at Gifu University. All dog owners had consented to protocols approved by the each institutional ethical committee at Gifu University. All tumor specimens were obtained at the time of diagnosis or surgical excision before chemotherapy or radiation therapy.

Assessment of apoptosis

For assessment of the morphological characteristics of apoptosis, the cells were collected at 120 hr after the treatment. The cells were stained with Hoechst33342 (5 μg/ml; Dojindo, Kumamoto, Japan) at 37 °C for 30 min, washed once with phosphate-buffered saline (PBS), resuspended, pipetted dropwise onto a glass slide, and examined by fluorescence microscopy using an Olympus microscope (Tokyo, Japan) equipped with an epiilluminator and appropriate filters. The cells with condensed and/or fragmented nuclei stained with Hoechst33342 were taken to be apoptotic.

To assess early apoptosis, we stained the cells with annexin V (AV) and propidium iodide (PI) by using Annexin V-FITC early Apoptosis Detection Kit (Cell Signaling Technology, Inc., Danvers, MA, USA) at 48 hr after treatment of the cells with 5-aza (10 μM). The assessment was performed by flow cytometric analysis using a BD Accuri™ C6 flow cytometer (BD Biosciences, San Jose, CA, USA).

Cell transfection with siRNA and treatment with 5-aza

Cells were seeded into 6-well plates at the concentration of 0.5×10^5 (each melanoma cell line) or 1.0×10^5 (HEMa-LP and NHEM) cells per well the day before treatment. 5-Aza (Sigma, St. Louis, MO, USA) was diluted in DMSO at a dose of 10 mM and used at 1, 5 or 10 μM concentration. 0 μM 5-aza indicated the treatment with DMSO alone. Short-interfering RNA (siRNA) for *foxo1* (5′-GCUGCAUCCAUGGACAACAACAGUA-3′; siR-*foxo1*, Invitrogen), which targets both human and canine *foxo1*, was used for transfection of the cells. Transfection was achieved by using cationic liposomes, Lipofectamine RNAiMAX (Invitrogen), at a concentration of 10 nM (siR-*foxo1*) according to the manufacturer's Lipofection protocol. Pre-miR miRNA Precursor Molecules-Negative Control #2 (Applied Biosystems) was used as a non-specific control siRNA.

Quantitative RT-PCR (qRT-PCR) using real-time PCR

Total RNA was isolated from cells and tissues by the phenol/guanidium thiocyanate method with DNase I treatment. For determination of mRNA expression levels, total RNA was reverse transcribed with a PrimeScript® RT Reagent Kit (Takara, Otsu, Japan). Real-time PCR was then performed by using THUNDERBIRD® SYBR qPCR Mix (TOYOBO, Osaka, Japan) and a CFX96 Real-Time PCR Detection System under the following cycling conditions: 95 °C for 1 min, 40 cycles of 95 °C for 15 s, and 60 °C for 50 s. Primer sequences used for each mRNA amplification are shown in Table 1. Primer design was performed by using Primer3 software (http://bioinfo.ut.ee/primer3/). The relative expression level of mRNA was calculated by the $\Delta\Delta Ct$ method, and *GAPDH* was used as an internal control.

Western blotting

Total protein was extracted from whole cells, and SDS-PAGE and electroblotting were performed according to the procedure described previously [17]. The antibodies used in this study were the following: anti-Akt (pan) rabbit mAb (#4691), anti-phosphorylated-Akt (Ser473; p-Akt) rabbit mAb (#4060), anti-cleaved caspase-3 rabbit mAb (#9664), anti-caspase-8 rabbit mAb (#4790), anti-TNF-α rabbit mAb (#6945), anti-FOXO1 rabbit mAb (#2880), anti-phosphorylated-FOXO1 (Ser256; p-FOXO1[S256]) rabbit mAb (#9461), anti-FOXO3a rabbit mAb (#2497), and anti-phosphorylated-FOXO1 (Thr24)/FOXO3a (Thr32; p-FOXO1[T24]/FOXO3a[T32]) rabbit mAb (#9464), all of which were from Cell Signaling Technology, Inc. and properly diluted with TBS-T containing 2% bovine serum albumin and 0.01% sodium azide. The loading control was prepared by re-incubating the same membrane with anti-β-actin antibody (Sigma, St. Louis, MO, USA).

DNA methylation assay

Genomic DNA (gDNA) was extracted from cells and tissues by using NucleoSpin® Tissue (Takara) according to the manufacturer's protocol. Bisulfite treatment of gDNA was achieved with a MethylEasy Xceed Rapid DNA Bisulphite Modification Kit (Human Genetic Signatures, Randwick, Australia) used according to the manufacturer's instructions. Then, for validation of the DNA methylation status, methylation-specific PCR (MSP) for human *TNF-α* (*hTNF-α*) and canine *TNF-α* (*cTNF-α*) was performed with an Episcope® MSP Kit (Takara). Primers used for the methylated MSP (M-MSP) and unmethylated MSP (U-MSP) are shown in Table 2. MSP was performed according to the procedure previously described [18]. For semi-quantitative MSP (semi-qMSP), the amount of methylated DNA was calculated by real-time PCR and the subsequent $\Delta\Delta Ct$ method according to the manufacturer's protocol.

Table 1
Primer sequences for qRT-PCR.

Gene	Forward primer	Reverse primer
Human *TRAIL*	GTCAAGTGGCAACTCCGTCA	ACTCCTTGATGATTCCCAGG
Canine *TRAIL*	ACAAGTACTCCCAAAGTGGC	GGTCGCTTACTACGTAAGGA
Human *FasL*	GCACTTTGGGATTCTTTCCA	CCTCCATTTGTCTGGCTCAT
Canine *FasL*	CAAGATCCATCCCTCTGGAA	GCTTGTTGTTGCAGGACTGA
Human *TNF-α*	TCCTTCAGACACCCTCAACC	AGGCCCCAGTTTGAATTCTT
Canine *TNF-α*	ACCACACTCTTCTGCCTGCT	CTGGTTGTCTGTCAGCTCCA

Table 2
Primer sequences for MSP.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
hTNF-α		
M-MSP	TAGAAGGTGTAGGGTTTATT ATCGT	TACCTTTATATATCCCTAAAACGAA
U-MSP	TAGAAGGTGTAGGGTTTATT ATTGT	TACCTTTATATATCCCTAAAACAAA
cTNF-α		
M-MSP	TTTGGAAATTAGAGGAAAAT AGGTC	AAACATCAAAAATACTTCCTACTCG
U-MSP	TTTGGAAATTAGAGGAAAAT AGGTT	AACATCAAAAATACTTCCTACTCACC