Toxicology in Vitro 35 (2016) 86–92

Contents lists available at ScienceDirect

Toxicology in Vitro

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

Calmidazolium chloride inhibits growth of murine embryonal carcinoma cells, a model of cancer stem-like cells

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ARTICLE INFO

Article history:
Received 6 November 2015
Received in revised form 7 April 2016
Accepted 27 May 2016
Available online 28 May 2016

Keywords:
Cancer stem-like cells
Calmidazolium chloride
Embryonal carcinoma cells
Growth inhibition

ABSTRACT

Calmidazolium chloride (CMZ) is widely used as a calmodulin (CaM) antagonist, but is also known to induce apoptosis in certain cancer cell lines. However, in spite of the importance of cancer stem cells (CSCs) in cancer therapy, the effects of CMZ on CSCs are not yet well understood. We investigated the effects of CMZ on the F9 embryonal carcinoma cell (ECC) line as a surrogate model of CSCs. To avoid bias due to culture conditions, F9 ECCs and E14 embryonic stem cells (ESCs) were grown in the same culture medium. Results obtained using a cell-counting kit showed that CMZ significantly inhibited growth in F9 ECCs compared with growth in E14 ESCs. CMZ also induced apoptosis of F9 ECCs, but not of E14 ESCs, which was associated with caspase-3 activation and an increased fraction of the sub-G1 cell population. In addition, our data revealed that the expression of stemness-related genes including c-Myc was selectively down regulated in CMZ-treated F9 ECCs. Our results suggest that CMZ can inhibit the growth of ECCs by inducing apoptosis and down regulating stemness-related genes, without causing any harm to normal stem cells. These findings indicate a potential application of CMZ in the development of anti-CSC therapeutics.

1. Introduction

Cancer stem cells (CSCs) are considered to be tumor-initiating subpopulations in many cancers, including leukemia and solid tumors (Reya et al., 2001; Sagar et al., 2007; Bonnet and Dick, 1997; Pardal et al., 2003; Hill, 2006). The self-renewal and tumor-initiation capabilities of CSCs are thought to be the basis of their role in cancer outbreak, progression, metastasis, and especially, relapse and drug resistance (Gao et al., 2010; Chen et al., 2013; Nguyen et al., 2012; Visvader and Lindeman, 2008, 2012). Therefore, the development of novel compounds and therapeutic strategies that selectively target CSCs is a very important challenge.

Embryonal carcinoma cells (ECCs) comprise a highly malignant subset of stem cells in germ cell tumors, such as teratocarcinomas. As stem cells, ECCs have the abilities of self-renewal, pluripotency, and differentiation into diverse cell types (Silván et al., 2009; Andrews, 2002; Blelloch et al., 2004; Andrews et al., 2005). Because CSCs and ECCs share common features, ECCs can be used as a surrogate model for therapeutic studies of CSCs.

The level of differentiation in human tumors is an important factor that is commonly evaluated in a clinical setting, and poorly differentiated tumors are generally correlated with the worst patient prognosis (Hermann et al., 2007; Dean et al., 2005; Guzman et al., 2002; Glinsky et al., 2005; Ben-Porath et al., 2008). Preferential overexpression of genes, which is generally abundant in undifferentiated embryonic stem cells (ESCs), is also frequently observed in histologically poorly differentiated tumors. Overexpressed genes that are crucial for the function of ESCs include sex-determining region Y-box 2 (Sox2), octamer-binding transcription factor 4 (Oct4), Nanog, and c-Myc (Gidekel et al., 2003; Li et al., 2004; Rodriguez-Pinilla et al., 2007; Santagata et al., 2007; Vita and Henriksson, 2006). Thus, a key issue for curing cancers is determining how to induce differentiation and apoptosis in CSCs.

Calmidazolium chloride (CMZ) is a specific and promising inhibitor of calmodulin (CaM) (Belle, 1981), a calcium-binding messenger protein that performs various important cell functions by activating CaM-dependent enzymes (e.g., CaM-dependent kinase II, nitric oxide synthase, and calcineurin) (Takano et al., 2003). Additional actions of CMZ include inhibition of Ca^{2+}-ATPase activity in skeletal muscle sarcoplasmic reticulum and regulation of Ca^{2+} concentration in kidney cells, myocardial cells, and osteosarcoma cells (Jan and Tseng, 2000; Khan et al., 2000; Kumar et al., 2009; Tseng et al., 2004). Importantly, several lines of evidence directly support the idea that CMZ may be a potential

Abbreviations: CMZ, calmidazolium chloride; CaM, calmodulin; CSCs, cancer stem cells; DMSO, dimethyl sulfoxide; ECCs, embryonal carcinoma cells; ESCs, embryonic stem cells; FACS, fluorescent-activated cell sorting; ICM, inner cell mass; LIF, leukemia inhibitory factor; Oct4, octamer-binding transcription factor 4; PI, propidium iodide; qRT-qPCR, quantitative real-time PCR; RIPA, radioimmunoprecipitation assay; SDS, sodium dodecyl sulfonate; Sox2, Sex determining region Y-box 2.

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http://dx.doi.org/10.1016/j.tiv.2016.05.015
0887-2333/

candidate for development as an anti-cancer therapeutic. For example, CMZ has been shown to induce apoptosis in breast cancer and hepatoma cells, and to enhance differentiation of colon cancer cells (Newton et al., 2000; Liao et al., 2009; Rochette-Egly et al., 1988). To date, however, the effects of CMZ on CSCs have not been elucidated.

Here, we present the results of our investigation into the effects of CMZ on the F9 ECC line, which was used as a surrogate model of CSCs, compared with those on the E14 ESC, used as a normal counterpart of ECCs. Our results shed light on the potential therapeutic impact of CMZ on CSCs.

2. Materials and methods

2.1. Chemicals

Calmidazolium chloride (CMZ) was purchased from Tocris Bioscience, Bristol, UK (Cat. #2561), dissolved in dimethyl sulfoxide (DMSO), and diluted before use.

2.2. Cell culture

The F9 ECCs and E14 ESCs were maintained in a humidified incubator at 5% CO_2 and 37 °C. Both cell types were cultured in Knockout Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA), supplemented with 15% (v/v) of fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO), 2 mM L-glutamine, 1× MEM Non-essential Amino Acid (NEAA); Welgene Inc., Gyeongsangbuk-do, Republic of Korea), 1× penicillin-streptomycin solution (Cat. #30–002-cl; Corning, Mediatech Inc., Manassas, VA), 20 μg/mL ciprofloxacin (Sigma-Aldrich), 55 μM 2-mercaptoethanol (Gibco, Thermo Fisher Scientific, Waltham, MA), and 1000 U/mL of leukemia inhibitory factor (LIF; Cat. #GSR-7001; Global Stem, Gaithersburg, MD). The F9 ECC and E14 ESC lines were grown on 0.1% gelatinized tissue culture dishes.

2.3. Cell growth assay

Cell proliferation was measured using a cell-counting kit-8 (CCK-8 assay kit; Dojindo Corporation, Kumamoto, Japan). Cell viability is easily determined by CCK-8 assay using WST-8. In live cells, WST-8 gives rise to an orange-color formazan dye by cellular dehydrogenases activity. The number of living cells can be estimated by measuring the amount of the dye. Twenty-four hours prior to experiments, cells were plated onto each well of a 96-well plate and exposed to either DMSO as positive control or different concentrations of CMZ for an additional 24 h. The CCK-8 solution was added to 100 μL of culture medium, and the cells were incubated at 37 °C for 2 h. The optical density was measured at 450 nm using a microplate reader.

The inhibition concentration of 50% cell growth (IC_{50}) was calculated by the linear equation of sample concentration and cell survival rate %.

2.4. Colony formation assay

The colony formation assay was performed as described in a previous report (Park et al., 2014). Briefly, E14 cells were seeded at 1×10^2 cells per well and F9 cells were seeded at 2×10^2 per well in 24-well plates. Twenty-four hours later, the cells were exposed to either DMSO as positive control or different concentrations of CMZ for 9 h and then maintained with fresh medium for 7 d. Cells were fixed with fixation solution and stained with 0.02% crystal violet. The colonies were scanned with flat-bed color scanner and counted using Image J software.

2.5. DNA fragmentation assay

Degradation of DNA into a specific fragmentation pattern is a characteristic of apoptosis (Collins et al., 1997). Cells were harvested after treatment for 24 h with either DMSO as positive control or different concentrations (0–10 μM) of CMZ. The cells were then resuspended in 500 μL of phosphate-buffered saline (PBS), lysed by treatment with 55 μL of lysis buffer [containing 50 mM Tris-HCl (pH 8.0), 200 mM EDTA, and 5% Triton X-100] for 20 min at 4 °C, and centrifuged at 12,000 rpm for 30 min. The supernatant was incubated with 0.1 volume of Proteinase K (20 mg/mL) for 10 min at 56 °C. Fragmented DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and then precipitated with 0.1 volume of sodium acetate and 2 volumes of ethanol. DNA fragments were dissolved in Tris-EDTA (TE) buffer [containing 0.02 volume of RNaseA (100 mg/mL)] and electrophoresed on a 1.5% agarose gel.

2.6. Cell-cycle analysis

After cells were incubated for 24 h in medium in the presence of vehicle (DMSO), 7, and 10 μM CMZ, they were trypsinized, resuspended, and fixed overnight with cold 70% ethanol at -20 °C. Fixed cells were resuspended in PBS containing 50 μg/mL propidium iodide (PI) and 20 μg/mL RNase A, and incubated for 30 min at 37 °C. Cellular DNA content was then assessed by flow cytometry with fluorescent-activated cell sorting (FACS).

2.7. Western blotting

Western blot analysis was carried out as previously described (Kim et al., 2015). Briefly, cells were treated with either vehicle (DMSO) or CMZ at different concentrations for 24 h, harvested, and centrifuged. The pellets were resuspended in radioimmunoprecipitation assay (RIPA) buffer [150 mM NaCl, 50 mM Tris (pH 8.0), 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and protease inhibitors] and incubated on ice. The supernatants containing the proteins were collected after centrifugation. Protein concentration was determined by the Bradford assay. For western blot analysis, proteins were separated on 12% SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA). Immunoblotting was then performed using a rabbit polyclonal anti-caspase-3 antibody (Cell Signaling 9665S).

2.8. Isolation of RNA and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis was performed as described in a previous report (Kim et al., 2015). Total RNA was isolated with TRI-Reagent (Cat. #TR118; Molecular Research Center Inc., Cincinnati, OH). Expression levels were analyzed by qRT-PCR with SYBR Premix Ex Taq II (Takara, Japan) and an Applied Biosystems 7300 Real-Time PCR system, using primer sets for target genes. The qRT-PCR analysis was performed in quadruplicate using the following primers: for mouse Oct4, (sense) 5′-AGAAGTGGGTGGAGGAAG-3′ and (antisense) 5′-GGCACTTCAGAAACATGG-3′; for mouse Sox2, (sense) 5′-ACAACTCGGAGATCAGCA-3′ and (antisense) 5′-TCATGAGCGTCTTGGTTT-3′; for mouse Nanog, (sense) 5′-TGCACTCAAGGACAGGTT-3′ and (antisense) 5′-TGCACTTCATCCTTTGGT-3′; for mouse c-Myc, (sense) 5′-GAGGAGCTGGAATCTCTC-3′ and (antisense) 5′-AAGGTTGTGAGGTTAGGC-3′.

2.9. Statistical analysis

Data are presented as mean plus or minus standard error of the mean (S.E.M.) and were analyzed by a two-tailed student *t* test. Results with $P < 0.05$ were considered significantly different.