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DNA methyltransferase inhibitor CDA-II inhibits myogenic differentiation

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ABSTRACT

CDA-II (cell differentiation agent II), isolated from healthy human urine, is a DNA methyltransferase inhibitor. Previous studies indicated that CDA-II played important roles in the regulation of cell growth and certain differentiation processes. However, it has not been determined whether CDA-II affects skeletal myogenesis. In this study, we investigated effects of CDA-II treatment on skeletal muscle progenitor cell differentiation, migration and proliferation. We found that CDA-II blocked differentiation of murine myoblasts C2C12 in a dose-dependent manner. CDA-II repressed expression of muscle transcription factors, such as Myogenin and Mef2c, and structural proteins, such as myosin heavy chain (Myh3), light chain (Mylpf) and MCK. Moreover, CDA-II inhibited C1C12 cell migration and proliferation. Thus, our data provide the first evidence that CDA-II inhibits growth and differentiation of muscle progenitor cells, suggesting that the use of CDA-II might affect skeletal muscle functions.

1. Introduction

Skeletal myogenesis is important for muscle growth and repair. In response to myogenic signals, quiescent satellite cells become activated and are induced to myoblasts. The myoblasts subsequently exit from proliferative stage, differentiate and fuse into multinucleated myotubes and finally form mature muscle fibers [1].

Myogenic differentiation is controlled by various myogenic regulatory factors (MRFs) and myocyte enhancer factors (MEFs) [2], such as MyoD [3], Myf5 [4] and Mef2c [5–8]. MRFs belong to the family of basic helix–loop–helix (bHLH) transcription factors including MyoD, Myf5, myogenin and MRF4 [9]. They form heterodimers to interact with a specific DNA module, the E-box, which locates in the regulatory regions of genes expressed in skeletal muscle [10]. MRFs initiate some gene regulatory processes of myogenic specification and expression of muscle differentiation markers. In this process, MRFs cooperate with MEF2 proteins. MEF2 proteins are a family of muscle-enriched nuclear factors that are essential for muscle differentiation and development. MEF2 proteins contain both MADS-box and MEF2 DNA-binding domain and have four members including MEF2A, B, C and D [11]. MEF2 factors alone do not possess myogenic activity, but bind as homodimers or heterodimers with the MRFs to the AT-rich consensus sequences within regions of muscle-specific genes, and then activate the myogenic differentiation program [12].

In the early stage of myogenesis, the paired box (Pax) transcription factors Pax3 and Pax7 mark myogenic precursor cells and regulate their entry into the process of skeletal muscle differentiation. In the determination stage, Pax3 directly activates Myf5 and upregulates MyoD expression and then induces precursor cells into myoblasts [13]. In the differentiation stage, MRFs and MEF2 induce muscle special genes expression and myoblasts induced differentiation and form multinucleated myotubes.

CDA-II (cell differentiation agent II), isolated from healthy human urine, is a DNA methyltransferase inhibitor [14–16]. Previous studies indicated that CDA-II had anti-cancer activity, capable of inhibiting cell proliferation, inducing apoptosis, activating tumor suppressor genes, and silencing oncogenes [14,15,17–20]. CDA-II was used for diverse cancer therapies and showed improved chemotherapeutic responses in treating breast cancer [21], non-small-cell lung cancer [22], hepatocellular carcinoma [23] and leukemia [17]. In 2004, the State Food and Drug Administration (SFDA) of China approved CDA-II as an anticancer drug for the treatment of patients with non-small-cell lung cancer and breast cancer [22]. Most studies to date have documented the mechanism of CDA-II in the cancer therapy is regulation of cell growth and certain differentiation processes [17,20–22]. However, it remains unknown whether CDA-II is involved in controlling myogenic differentiation and affects muscle functions.

The goal of this study is to investigate whether CDA-II has a role in regulating myogenesis. Using C2C12 myoblast differentiation

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model, we first observed that CDA-II treatment significantly inhibited C2C12 myoblast differentiation, reduced expression of muscle specific genes, and inhibited proliferation and migration of C2C12 myoblasts. Our findings demonstrate the DNA methyltransferase inhibitor CDA-II suppress the skeletal muscle growth and myogenesis.

2. Materials and methods

2.1. Plasmids and reagents

The following luciferase reporter plasmids and recombinant DNA constructs were used: the 3 × MEF2-luc reporter containing three copies of MEF2-responsive sites in pGL2 vector was from Dr. Eric Olson (University of Texas Southwestern Medical Center, Dallas, TX) [24]; the Myogenin promoter reporter (Myog-luc) was kindly provided by Dr. Kiyoshi Kawakami (Jichi Medical School, Japan) [25]; MCK-luc reporter containing muscle creatine kinase promoter and enhancer was attained from Dr. Andrew Lassar (Harvard Medical School, Boston, MA) [26].

The following antibodies were used in the Western blotting: Myosin heavy chain (1:5000; RD Systems, MF20), Myogenin (1:1000; BD Pharmingen, 556358) and α-tubulin (1:5000; Santa Cruz, sc-5286).

CDA-II was purchased from Hefei Everlife Pharmaceutical Co., Ltd. (Anhui, China).

2.2. Cell culture, differentiation assay, reporter activity assay

C2C12 cells (ATCC) were cultured in DMEM with 20% inactivated fetal calf serum (FCS). For the myogenesis assay, C2C12 cells were grown to 80–90% confluence, and induced for differentiation by switching from growth medium to differentiation medium (DM: DMEM containing 2% horse serum).

Transient transfection of cultured C2C12 cells was achieved using SuperFect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol. 24 h after transfection, cells were cultured in differentiation medium with or without CDA-II (1 mg/ml) for another 48 h. Luciferase-based reporter activity assays were performed as described previously [27].

2.3. Real-time RT-PCR

C2C12 total RNA was prepared with Qiagen RNeasy kit (Qiagen, Hilden, Germany) and cDNA was generated using GeneAmp RNA PCR kit (Applied Biosystems, Carlsbad, CA). Real-time PCR was performed using the StepOneplus Real-time PCR system(Applied Biosystems, Carlsbad, CA) with the SYBR Green PCR Core Reagents kit (Applied Biosystems, Carlsbad, CA). The primers used in these analyses are listed in the Supplementary Table 1. The following genes were analyzed: Myh3 (myosin heavy chain 3, skeletal muscle, embryonic), Mylpf (myosin light chain, phosphorylatable, fast skeletal muscle), Myog (Myogenin), MCK(muscle creatine kinase), Mef2c, and the control Gapdh.

2.4. Immunofluorescence, proliferation assay, and scratch assay

For immunofluorescence, C2C12 myoblasts were cultured in 6-well plate. When cells were 80–90% confluent, the grown medium was replaced by differentiation medium with the following CDA-II treatments: 0, 0.1, 0.25, 0.5, 1.0, 2.0 mg/ml for 72 h. The cells were incubated with primary antibody against MHC (1:200, RD Systems, MF20).

For proliferation assay, C2C12 myoblasts (5×10^5) were cultured in 100 mm plates with grown medium overnight. CDA-II was added to the medium the next day with the following concentrations: 0, 0.1, 0.25, 0.5, 1.0, 2.0 mg/ml. Cells were then trypsinized, and cell number was determined by Trypan blue assays at Day 2 and Day 4 after treatment.

For scratch assay, C2C12 myoblasts were seeded in a 6-well plate in grown medium to reach a confluence. A scratch in the monolayer was made using a yellow tip and the cells were washed and cultured in grown medium with CDA-II with different concentrations for 24 h. Migration of the cells into the scratch area was observed by Leica DM3000 microscope (Leica, Buffalo Grove, IL) and quantified by TScratch software [28].

3. Results

3.1. CDA-II inhibits myogenic differentiation

We first examined whether CDA-II affects myogenic differentiation. C2C12 myoblasts were induced to undergo differentiation in the presence of various concentrations of CDA-II, ranging from 0.1 mg/ml to 2 mg/ml. Three days later, immunofluorescence staining was performed using an antibody against myosin heavy chain(MHC) to observe the effect of CDA-II treatment on myotube formation (Fig. 1A). We found that C2C12 myoblasts differentiated properly in the absence of CDA-II, giving rise to extensive formation of myotubes. In contrast, CDA-II treatment reduced myotube formation in a dose-dependent manner. The highest concentration of CDA-II (2 mg/ml) used generated barely detectable myotubes. To quantify the degree of cell fusion, we calculated the fusion index (Fig. 1B). The fusion index was 44.7 ± 5.1 in non-treated cells, but only 3.4 ± 1.1 in CDA-II treated (1 mg/ml) cells. The decreased fusion index of the myotubes suggested that the CDA-II might specifically repress the myoblasts fusion. Therefore, these results strongly indicated that CDA-II repressed myogenic differentiation.

3.2. CDA-II represses the expression of myogenic genes

The myogenic differentiation consists of a series of events including the induction of expression of myogenic transcription factors such as Myogenin and Mef2c, and muscle structural genes such as Myh3, Mylpf, and MCK. Therefore, we performed Western blotting analysis to determine whether CDA-II repressed expression of muscle differentiation markers including Myogenin and MHC (Fig. 2A). We found that expression of Myogenin and MHC was markedly decreased in a concentration-dependent manner three days after C2C12 myoblast differentiation.

We also performed real-time RT-PCR to investigate whether CDA-II affects the expression of muscle-specific genes at the transcriptional level. C2C12 myoblasts were treated with or without CDA-II (1 mg/ml) for 72 h in differentiation medium. 1 mg/ml CDA-II is sufficient to significantly inhibit myogenic differentiation (Fig. 1). Real-time RT-PCR data were presented in Fig. 2B. The relative levels of genes transcribes in CDA-II-treated cells as compared to non-treated cells were significantly decreased for Myogenin (0.63 ± 0.04), Mef2c (0.03 ± 0.01), Myh3 (0.02 ± 0.01), Mylpf (0.21 ± 0.02) and MCK (0.11 ± 0.03).

Furthermore, we performed reporter assays to determine whether CDA-II affected the promoter activity of MCK, Myogenin and Mef2. We found that CDA-II treatment decreased activities of the MCK reporter (0.26 ± 0.05) (Fig. 2C), the Myogenin reporter (0.24 ± 0.12) (Fig. 2D) and the artificial Mef2 reporter (0.14 ± 0.05) (Fig. 2E). The results suggested that CDA-II repressed the activity of muscle-specific transcription factors as well as muscle structural proteins, which contributed to CDA-II inhibition of myogenesis.

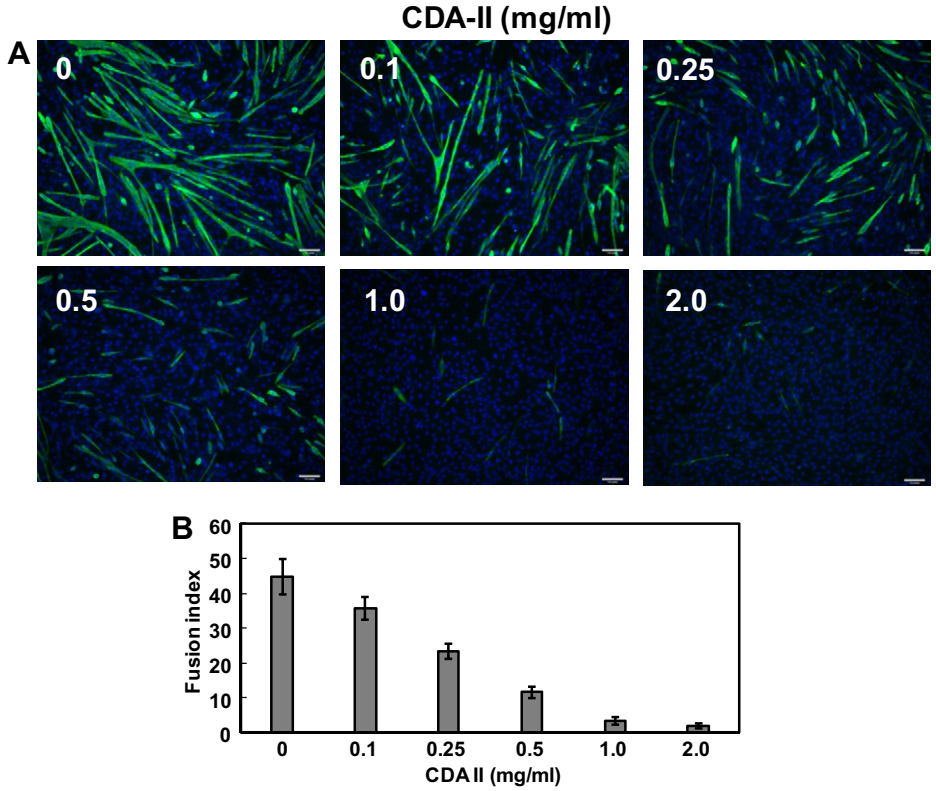

Fig. 1. CDA-II inhibits myogenic differentiation of C2C12 myoblasts. (A) C2C12 myoblasts were treated with different concentrations of CDA-II and induced to differentiate. Myogenic differentiation was scored by immunostaining with myosin heavy chain (MHC, green) antibodies after cells were cultured in differentiation medium for 72 h. DAPI staining (blue) marked the nuclei. (B) Fusion index, calculated as the ratio of the number of nuclei within myotubes (>2 nuclei) to the total number of nuclei in the field, was quantified based on (A). The average values of three independent experiments ± SD were presented. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

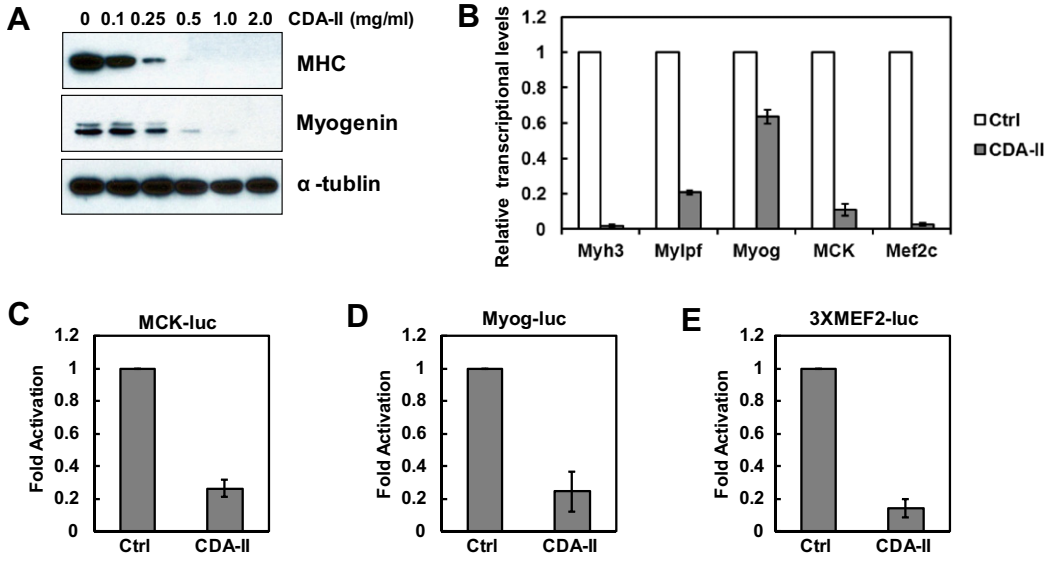

Fig. 2. CDA-II represses expression of muscle-specific genes. (A) C2C12 myoblasts were induced to undergo myogenic differentiation in the presence of various concentrations of CDA-II. Three days later, expression levels of myosin and myogenin were subsequently analyzed by Western blotting. (B) C2C12 cells were induced to differentiation with or without CDA-II (1 mg/ml) treatment. RNA sample were collected 72 h after differentiation, and real time RT-PCR was performed to analyze the transcript levels of muscle-specific genes including Myog, MHC, Mylpf, MCK and Mef2C. (C) Effects of CDA-II treatment on a luciferase reporter driven by the MCK promoter and enhancer. C2C12 cells were cotransfected with the MCK-luc and pEF-RL reporters. Transfected cells were cultured with or without CDA-II (1 mg/ml) treatment in differentiation medium for 48 h before measuring luciferase activities. (D and E) Similar transcriptional assay were performed using the myogenin promoter luciferase reporter (Myog-luc) and an artificial reporter containing 3 copies of MEF2 binding site (3XMEF2-luc).

3.3. CDA-II inhibits migration and proliferation of C2C12 cells

To determine the effects of CDA-II on C2C12 cell migration, we monitored the migratory capacities of C2C12 cells using the *in vitro* scratch assay. Non-treated cells grown in growth medium displayed high migratory ability as the scratch wound was almost recovered by the migratory cells after 24 h incubation. However, migration of CDAII-treated cells was inhibited in a dose-dependent manner (Fig. 3A).

In addition, we investigated the effects of CDA-II on C2C12 cell proliferation. A reduction of C2C12 myoblasts proliferation was detected after CDA-II treatment in a dose-dependent manner (Fig. 3B). Specifically, significant decrease of cell growth was observed after treatment with 1 or 2 mg/ml CDA-II as compared to the untreated control. Therefore, CDA-II appeared to inhibit both cell migration and proliferation.

4. Discussion

Although CDA-II is well studied in cancer therapies, its function in muscle differentiation is largely unknown. In this report, we first demonstrate that CDA-II inhibits myogenic differentiation by attenuating the expression of muscle specific genes. As a result of this effect, once undifferentiated myoblasts are exposed to CDA-II and immediately incubated in the differentiation medium, the myotube formation is blocked completely. As CDA-II inhibits the migration and proliferation of C2C12 myoblasts, it is possible that CDA-II represses the myogenic differentiation by reducing the cell migration capacity to prevent myoblasts alignment and fusion into multinucleated myotubes. A small number of short myotubes were detected when C2C12 myoblasts were cultured in differentiation medium in the presence of CDA-II.

Myogenesis is strictly regulated by both genetic and epigenetic mechanisms including modifications of histone and DNA. DNA methylation has been postulated as one of the major repressive systems acting on muscle gene loci [29]. Demethylation of muscle regulatory regions at the beginning of the differentiation program seems necessary for the differentiation program to proceed [30]. It has been demonstrated that DNA methyltransferase inhibitor, 5-Azacytidine, enhances myogenic differentiation by up-regulation of muscle genes at the myoblast stage [31]. Previous studies found that MyoD and Myog promoters became demethylated at the onset of C2C12 muscle cells differentiation [30,32]. These evidences suggest that DNA demethylation may contribute to myogenesis. However, other studies suggest that overexpression of DNA methyltransferase (DNMT) in C2C12 myoblast cells promote not block myogenic differentiation [33]. In accordance with this evidence, our result implied that the inhibition of methyltransferase by CDA-II can block myogenesis. Thus, DNA methylation/demethylation can not only promote but also inhibit myogenic differentiation, potential reason of which may be the different regions of methylation/demethylation. The precise mechanism regulating methylation/demethylation during myogenesis are still largely unknown, further studies are needed to investigate the mechanism of specific DNA methyltransferases and demethylases acting during myogenesis [29].

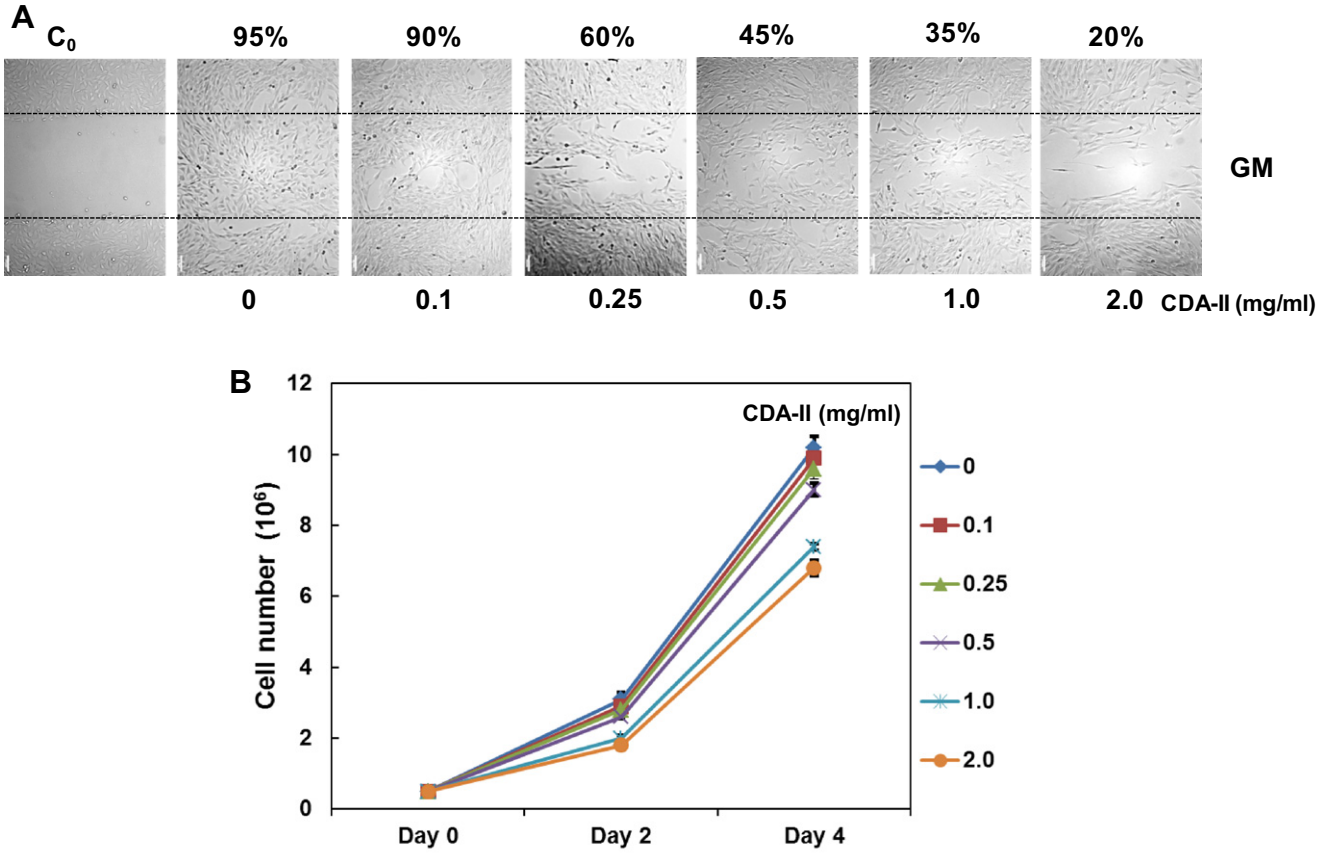

Fig. 3. CDA-II inhibits migration and proliferation of C2C12 cells. (A) Migratory ability of C2C12 cells was analyzed by scratch assay. A scratch wound was made in the confluent monolayer of C2C12 cells, and then cells were cultured in the presence of 0, 0.1, 0.25, 0.5, 1 and 2 mg/ml of CDA-II in growth medium for 24 h. Cells were then photographed to observe the degree of the closure of the scratches. C_0 shows the scratch made in the monolayer at time zero. The percentages of the scratch area covered with migrating cells are shown. (B) C2C12 cell proliferation was determined by counting cells after cells were cultured in growth medium with various concentrations of CDA-II for 48 h and 96 h. Data were presented as average values ± SD.

In summary, our results indicate that DNA methyltransferase inhibitor CDA-II can repress myogenesis by down-regulation of the myogenic genes. This would bring an therapeutic questions: the use of CDA-II in clinical treatment might affect skeletal muscle functions. Further studies are needed to better understand the mechanism of CDA-II in skeletal muscle development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.068.

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