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Biochemical and Biophysical Research Communications 328 (2005) 206–212
BBRC
www.elsevier.com/locate/ybbrc

Stable siRNA-mediated silencing of antizyme inhibitor: regulation of ornithine decarboxylase activity

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Received 24 November 2004
Available online 6 January 2005

Abstract

Ornithine decarboxylase (ODC) is the rate-limiting enzyme involved in the biosynthesis of polyamines essential for cell growth and differentiation. Aberrant upregulation of ODC, however, is widely believed to be a contributing factor in tumorigenesis. Antizyme is a major regulator of ODC, inhibiting ODC activity through the formation of complexes and facilitating degradation of ODC by the 26S proteasome. Moreover, the antizyme inhibitor (AZI) serves as another factor in regulating ODC, by binding to antizyme and releasing ODC from ODC-antizyme complexes. In our previous report, we observed elevated AZI expression in tumor specimens. Therefore, to evaluate the role of AZI in regulating ODC activity in tumors, we successfully down-regulated AZI expression using RNA interference technology in A549 lung cancer cells expressing high levels of AZI. Two AZI siRNAs, which were capable to generate a hairpin dsRNA loop targeting AZI, could successively decrease the expression of AZI. Using biological assays, antizyme activity increased in AZI-siRNA-transfected cells, and ODC levels and activity were reduced as well. Moreover, silencing AZI expression decreased intracellular polyamine levels, reduced cell proliferation, and prolonged population doubling time. Our results directly demonstrate that downregulation of AZI regulates ODC activity, intracellular polyamine levels, and cell growth through regulating antizyme activity. This study also suggests that highly expressed AZI may be partly responsible for increased ODC activity and cellular transformation.

Keywords: Antizyme inhibitor; Ornithine decarboxylase; Antizyme; siRNA

Ornithine decarboxylase (ODC) is a key enzyme in polyamine biosynthesis. Polyamines such as putrescine, spermine, and spermidine are essential for cell growth and differentiation [1]. Therefore, mechanisms of synthesis, catabolism, and transport are highly regulated. Although polyamines are essential for normal cell growth, increased activity of ODC beyond an undefined minimum threshold can induce cell transformation and tumor formation [2]. Elevated ODC activity has been reported in transformed cell lines [3], in virtually all animal tumors including the stomach, skin, colon, esophagus, colorectal [4–7], and in certain tissues predisposed to carcinogenesis [8,9]. Several studies have also demonstrated that upregulation of ODC and polyamine accumulation are necessary for the development of tumor model. Experimentally, induced ODC activity has been observed in transformation caused by oncogenic ras [10], v-Src [11], activated RhoA [12], and overexpression of eukaryotic initiation factor 4E [13], and the c-myc protooncogene can also upregulate ODC transcription [14].

Given the absolute requirement of polyamines for cell growth and the potentially oncogenic consequences of their overproduction, ODC activity and polyamine levels are subjected to tight regulation. Control of mammalian ODC is extremely complex, in part because ODC regulation is both cell-type and stimulus-specific [15–17]. Activities associated with ODC are not only regulated by

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doi:10.1016/j.bbrc.2004.11.172

various growth stimuli, but also by polyamines through gene transcription [18], mRNA degradation [19], mRNA translation [20], and enzymatic degradation [21].

Antizyme is a specific inhibitor of ODC, whose synthesis is stimulated by translational frameshifting in response to increased cellular polyamine content [22,23]. Functionally, antizyme binds to the free ODC monomer and prevents the formation of the enzymatically active homodimer. The binding of antizyme to the ODC monomer stimulates the degradation of ODC by the 26S proteasome, thereby reducing ODC level and activity [24]. In addition to inhibiting polyamine biosynthesis, antizyme has also been recently reported to suppress the polyamine transporter [25]. Thus, in a negative feedback system, antizyme plays a pivotal role in the regulation of ODC to prevent excess accumulation of cellular polyamines.

The antizyme inhibitor (AZI) is also involved in the regulation of ODC. AZI stabilizes the ODC level by trapping antizyme. AZI binds to the antizyme with higher affinity than ODC and releases it from the ODC-antizyme complex, preventing ODC from being degraded [26]. Rat and human cDNAs encoding AZI have been cloned and sequenced [26,27], and results show that AZI closely resembles ODC, except that AZI homodimers lack ODC activity. The AZI and ODC monomers do not form heterodimers.

Previously, we found that AZI is highly expressed in tumor tissues compared with counterpart normal tissues [28]. Therefore, it is proposed that enhanced expression of AZI may be responsible for the upregulation in ODC activity in cancer cells. To elucidate the mechanism responsible for AZI regulation of ODC in cancerous cells, we successfully used a recent technological breakthrough that allows delivery of short dsRNA, specifically small interfering RNA, via a plasmid into eucaryotic cells to modulate AZI expression. Using this system, antizyme activity, ODC level and activity, polyamine content, and cell growth in AZI-siRNA-transfected cells were determined. Our study results demonstrate that downregulation of AZI increases antizyme activity, resulting in reduced ODC level and activity, reduced intracellular polyamine content, and eventually leading to decreased cell proliferation.

Materials and methods

Cell culture. The A549 human lung cancer cell line obtained from ATCC was cultured in DMEM (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (Life Technologies, Gaithersburg, MD) and 1% penicillin/streptomycin (Life Technologies) in a humidified atmosphere containing 5% CO_2 at 37 °C.

SiRNA construction and transfection. To design specific siRNAs targeting AZI, DNA sequences of the type AA(N19) were selected using siRNA Target Finder (www.ambion.com). Four DNA sequences were selected for candidate siRNAs targeting AZI. Scrambled siRNA, 5′-AAACTCTTACGGTCACCAA-3′, was also used as negative control; it does not match any mammalian sequences currently available on online databases. Sense and antisense oligonucleotides were annealed and inserted into the *Bam*HI and *Hin*dIII sites of the pSILENCER 2.1-U6-neo plasmid vector (Ambion, St. Louis, MO), which is a U6 promoter plasmids coding for RNAs composed of two identical 19-nucleotide sequence motifs in an inverted orientation, separated by a 9 base pair spacer to generate a hairpin dsRNA loop capable of mediating AZI inhibition (Fig. 1). A549 cells were transfected using the lipofectamine plus transfection reagent (Invitrogen, Carlsbad, CA) with AZI-siRNAs containing plasmids, scrambled siRNA containing plasmid, and pSILENCER vector control containing no siRNA. Stably expressed clones were selected based on G418 antibiotic resistance (700 μg/mL).

RT-PCR. Total RNA was isolated from transfected A549 cells using Trizol reagent (Life Technologies, Grand Island, NY) and then treated with RNase-free DNase I (Promega, Madison, WI). To detect the presence of AZI in transfected cells, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using primers specific for AZI, forward: 5′-TATGACTTTCGGCTTTGT-3′, reverse: 5′-TCTGGTCC CAAATAGCTA-3′. For detecting expression of β-actin, antizyme, and ODC, the following specific primers were used: β-actin, forward: 5′-GT GGGGCGCCCCAGGCACCA-3′, reverse: 5′-CTCCTTAATGTCA CGCACGATTTC-3; antizyme: forward: 5′-CCTCCACTGCTGTAG TAACCCG-3′, reverse: 5′-CCAAAAAGCTGAAGGTTCGGA-3′; and ODC, forward: 5′-AAAGCAAAGTTGGTTTTGCGG-3′, reverse: 5′-CCTCTGGAAGCCATTGAACGT-3′.

Western blot analysis. Transfected cells were harvested, pelleted by centrifugation, washed with ice-cold phosphate-buffered saline (PBS),

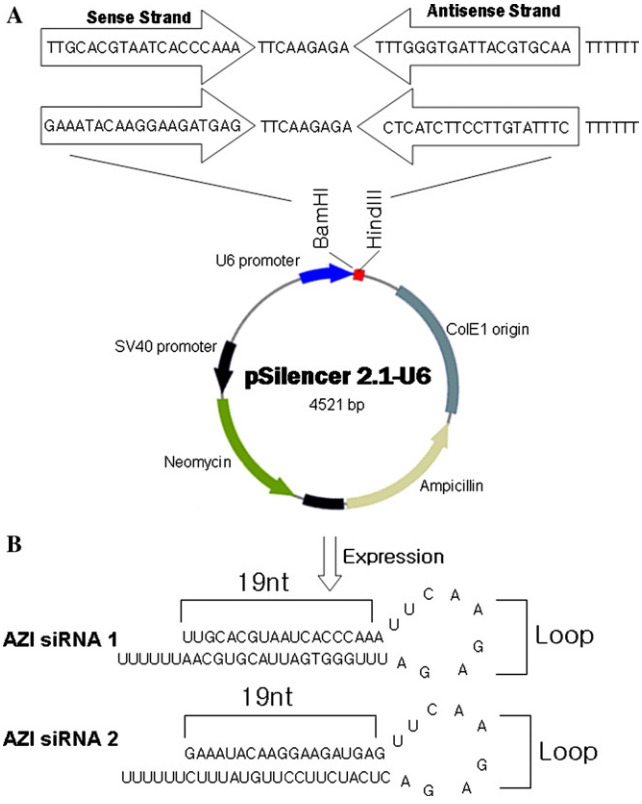

Fig. 1. (A) Schematic representation of the U6 RNA polymerase III promoter-based siRNA expression vector. Sequences encoding siRNA are inserted immediately downstream of the U6 promoter. The 6 thymidine residues serve as the termination signal for polymerase III. (B) Predicted second structure of the AZI-siRNA transcripts from the expression vector.