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RNA interference against aldehyde dehydrogenase-2: development of tools for alcohol research

Gabriel Cortínez[a,*], Amalia Sapag[a], Yedy Israel[a,b,c]

[a]*Laboratory of Gene Therapy, Department of Pharmacological and Toxicological Chemistry, Faculty of Chemical and Pharmaceutical Sciences, and*
[b]*Department of Molecular and Clinical Pharmacology, Institute of Biomedical Sciences (ICBM), Universidad de Chile, Olivos 1007, Independencia, Santiago, 8380492, Chile*
[c]*Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, PA, USA*

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Abstract

Liver alcohol dehydrogenase oxidizes ethanol to acetaldehyde, which is further oxidized to acetate by aldehyde dehydrogenase-2 (ALDH2*1). Individuals who carry a low-activity ALDH2 (ALDH2*2) display high blood acetaldehyde levels after ethanol consumption, which leads to dysphoric effects, such as facial flushing, nausea, dizziness, and headache (''Asian alcohol phenotype''), which result in an aversion to alcohol and protection against alcohol abuse and alcoholism. Mimicking this phenotype may reduce alcohol consumption in alcoholics. RNA interference (RNAi) is a cell process in which a short interfering RNA (siRNA) of 21–25 bp guides the degradation of a complementary target mRNA. Thus, siRNAs may be useful in mimicking the Asian phenotype by inhibiting ALDH2 gene expression. We determined the inhibitory effect of three chemically synthesized siRNAs targeted against rat ALDH2 mRNA in human embryonic kidney cells (HEK-293 cell lines) transfected with a plasmid carrying the rat ALDH2 cDNA. Two of the three siRNAs were active, yielding a 65–75% reduction of ALDH2 activity. Based on the most promising siRNA sequence, three short hairpin RNA (shRNA) genes driven by the human U6 RNA promoter were designed and cloned in a plasmid. After transfection of HEK-293 cells, one of the genes was shown to be active, yielding a 50% reduction of ALDH2 activity. This effect is consistent with a 50% reduction in ALDH2 mRNA, whereas neither β-actin mRNA nor the interferon-inducible transmembrane protein-1 mRNA levels were affected. This study describes chemically synthesized siRNAs and an endogenously synthesized shRNA, which reduce ALDH2 activity and constitute tools that should be of value for further alcohol research. © 2009 Elsevier Inc. All rights reserved.

Keywords: Acetaldehyde; siRNA; shRNA; Interference; Gene therapy

Introduction

Liver alcohol dehydrogenase (ADH) oxidizes ethanol to acetaldehyde, which is further oxidized to acetate by aldehyde dehydrogenase-2 (ALDH2). Some individuals in the East Asian population carry a dominant negative ALDH2 allele (*ALDH2*2*), which codes for a less active ALDH2 (Yoshida and Davé, 1985). After alcohol consumption, these individuals display high blood acetaldehyde levels (Wall et al., 1997), which are responsible for a number of effects, such as facial flushing, nty 7ausea, dizziness and headache (''Asian alcohol phenotype''). Individuals carrying one or two copies of *ALDH2*2* drink moderately or are virtually abstemious respectively, and are protected against alcohol abuse and alcoholism by 60–99% (Chen et al., 1999; Harada et al., 1982b; Higuchi, 1994; Luczak et al., 2006; Thomasson et al., 1991; Tu and Israel, 1995; Zintzaras et al., 2006).

Disulfiram has been used in the treatment of alcoholism for many decades (Hald and Jacobsen, 1948). A disulfiram metabolite binds to the sulfhydryl groups of ALDH2 (Jin et al., 1994; Rossi et al., 2006), blocking the oxidation of acetaldehyde to acetate and increasing the levels of blood acetaldehyde after alcohol consumption (Jones and Teng, 1983). Patients treated with disulfiram experience the equivalent of the Asian phenotype (Harada et al., 1982a), developing an aversion to alcohol. However, disulfiram has several side effects (Bessero et al., 2006; Kalant and Khanna, 1998; Orakzai et al., 2007; Rossi et al., 2006) because of its nonspecific interaction with sulfhydryl groups of other proteins. An alternative to disulfiram to reduce the activity of ALDH2 would be to inhibit *ALDH2* gene expression by the process of RNA interference (RNAi).

* Corresponding author. Tel.: +56-2-978-2934; fax: +56-2-737-7291.
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E-mail address: gcortine@ciq.uchile.cl (G. Cortínez).

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RNAi occurs naturally in several species from plants to animals, including mammals (reviewed by McManus and Sharp, 2002; Fire, 1999; Hannon, 2002). This process requires double-stranded RNA molecules to direct the degradation of a complementary target mRNA, which results in the reduced synthesis of the encoded protein. In the process of RNAi, the antisense strand of the siRNA, usually ranging from 21 to 25 bp (Bernstein et al., 2001; Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000), is incorporated into the RNAi silencing complex (RISC), a cytosolic protein complex that guides the cleavage of the complementary mRNA. This degradation is highly specific, discriminating between messengers differing in a single nucleotide (Miller et al., 2004; Schwarz et al., 2006). Nevertheless, not all siRNAs are active. Although some rules have been established to guide their design (Elbashir et al., 2002; Reynolds et al., 2004), they do not take into account the fact that the secondary structure of the target mRNA may hinder the RNAi process (Ameres et al., 2007).

Marked inhibition of the expression of several target genes has been achieved by transfecting cells in culture with siRNAs (see Akhtar and Benter, 2007). For in vivo studies, genes encoding short hairpin RNAs (shRNAs), which are endogenously generated in the cell and further hydrolyzed to siRNA, allow longer acting effects than siRNA (McAnuff et al., 2007). The shRNA genes can be transcribed by RNA polymerase III, which typically synthesizes small RNAs lacking both a Cap structure and a poly A tail (Geiduschek and Tocchini-Valentini, 1988). An RNA pol III promoter that has been widely used to drive the synthesis of therapeutic shRNAs is that of the U6 RNA gene (Brummelkamp et al., 2002; Paul et al., 2002). Once formed, shRNA molecules are processed by Dicer, a type III RNAse (Bernstein et al., 2001), releasing a small double-stranded RNA, which may be recognized and loaded onto RISC. The aim of this study was to design and test synthetic siRNAs and shRNA-encoding gene constructs to reduce ALDH2 activity.

Materials and methods

Cell culture

The HEK-293 cells were obtained from the American Type Culture Collection (ATCC catalog no. CRL-1573) and were grown in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/L of glucose (Gibco-Invitrogen, Grand Island, NY) and 1.5 g/L of sodium bicarbonate, supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) in 5% CO_2 at 37°C.

Short interfering RNA formation

Short RNA hybrids (siRNAs) of 19 bp were formed by annealing two 21-mer oligoribonucleotides (Eurogentec, Belgium), each having two thymidines at their 3′ end (Fig. 1). The sense and antisense oligonucleotides were incubated together (1.5 nmol each) in 75 μL of 50 mM Tris (pH 7.5) and 100 mM NaCl for 2 min at 94°C, 5 min at 78°C and 5 min at 65°C. Finally, the annealed siRNAs were cooled down to 20°C, aliquoted, and stored at −80°C. All the cooling down transitions were carried out at a rate of 2°C/min.

Cell type model

It is well known that primary hepatocytes cannot be readily transfected with lipofectamine, the standard reagent in the field (Holmen et al., 1995). Moreover, the half-life of ALDH2 in primary hepatocytes (Garver et al., 2001) does not allow a rapid estimation of the inhibitory effects of siRNA or shRNA. To circumvent these problems, we lipofected HEK-293 cells simultaneously with both a plasmid coding for the rat ALDH2 enzyme and with the siRNA molecules or a siRNA precursor (shRNA gene). The HEK-293 cells are readily lipofected, and, in addition, do not express ALDH2 activity (0.16 ± 0.14 nmol of NADH/mg of protein/min; not significantly different from zero). The ALDH2 activity of HEK-293 cells transfected with the plasmid encoding the rat ALDH2 ranges from 5 to 10 nmol NADH/mg of protein/min. In vivo, liver ALDH2 activity is reported as 12 nmol NADH/mg of protein/min (Ocaranza et al., 2008), whereas that in rat hepatoma cells (H4-II-E-C3) is 3 nmol NADH/mg of protein/min (Karahanian et al., 2005). H4-II-E-C3 cells are not readily lipofected (only 4% in our hands; data not shown).

Lipofection of HEK-293 cells with short interfering RNA and plasmid

The HEK-293 cells were plated in 1 mL of medium at 1×10^6 to 1.2×10^6 cells per 35-mm culture plate. The

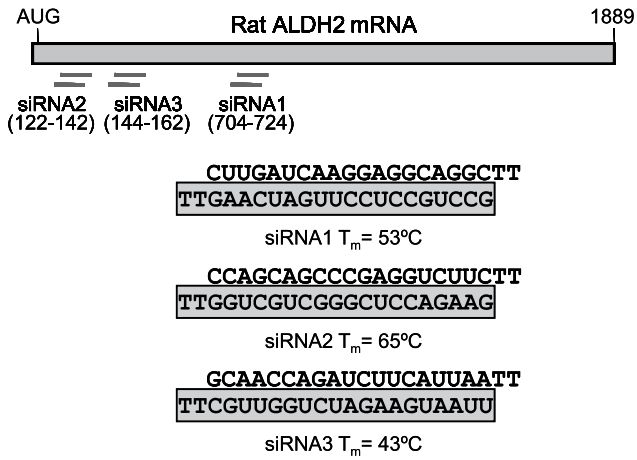

Fig. 1. Targets for short interfering RNA (siRNA) on rat aldehyde dehydrogenase-2 (ALDH2) mRNA. The oligoribonucleotides have two thymidines at the 3′ end that improve resistance to nuclease degradation. The antisense oligoribonucleotides are highlighted in *gray boxes*. The antisense strand of siRNA2 is also complementary to the human ALDH2 mRNA. The T_m was calculated according to Xia et al. (1998).

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