Eliciting the Low-Activity Aldehyde Dehydrogenase Asian Phenotype by an Antisense Mechanism Results in an Aversion to Ethanol

Eric Garver,[1] Guang-chou Tu,[1] Qing-Na Cao,[1] Maria Aini,[1] Feng Zhou,[1] and Yedy Israel[1,2]

[1]*Department of Pathology, Anatomy and Cell Biology, and the Alcohol Research Center, Thomas Jefferson University, Philadelphia PA 19107*
[2]*Laboratory of Gene Therapy and Millennium Institute, University of Chile, 11111 Santiago, Chile*

Abstract

A mutation in the gene encoding for the liver mitochondrial aldehyde dehydrogenase (*ALDH2–2*), present in some Asian populations, lowers or abolishes the activity of this enzyme and results in elevations in blood acetaldehyde upon ethanol consumption, a phenotype that greatly protects against alcohol abuse and alcoholism. We have determined whether the administration of antisense phosphorothioate oligonucleotides (ASOs) can mimic the low-activity ALDH2–2 Asian phenotype. Rat hepatoma cells incubated for 24 h with an antisense oligonucleotide (ASO-9) showed reductions in ALDH2 mRNA levels of 85% and ALDH2 (half-life of 22 h) activity of 55% equivalent to a >90% inhibition in ALDH2 synthesis. Glutamate dehydrogenase mRNA and activity remained unchanged. Base mismatches in the oligonucleotide rendered ASO-9 virtually inactive, confirming an antisense effect. Administration of ASO-9 (20 mg/kg/day for 4 d) to rats resulted in a 50% reduction in liver ALDH2 mRNA, a 40% inhibition in ALDH2 activity, and a fourfold ($P < 0.001$) increase in circulating plasma acetaldehyde levels after ethanol (1 g/kg) administration. Administration of ASO-9 to rats by osmotic pumps led to an aversion (−61%, $P < 0.02$) to ethanol. These studies provide a proof of principle that specific inhibition of gene expression can be used to mimic the protective effects afforded by the ALDH2–2 phenotype.

Key words: alcoholism • disulfiram • ALDH2-2 • acetaldehyde • treatment

Introduction

Research on the genetics of alcoholism indicates that there are (a) predisposing and (b) protective factors in the development of alcoholism. The heritability of predisposing factors has been estimated to be 40–60% (1), while the protective genetic influence can approach 100% (2). The protective genetic influence is associated with a low activity of liver mitochondrial aldehyde dehydrogenase (ALDH2),* an enzyme that plays a major role in the detoxification of acetaldehyde generated in the metabolism of ethanol. In some Asian populations, a dominant mutation in the gene that codes for this enzyme (*ALDH2–2*) renders the enzyme largely inactive (3, 4). Individuals carrying the *ALDH2–2* allele who consume small amounts of alcohol display elevated blood acetaldehyde levels and dysphoric effects that include dizziness, nausea, hypotension, and palpitations (5). It has been postulated that many of the intoxicating effects of ethanol are accentuated in subjects carrying the *ALDH2–2* allele (6, 7). Overall, the protective effect of the *ALDH2–2* allele against alcohol abuse and alcoholism ranges from 66 and 90% for heterozygotes to 100% for homozygotes (2, 8–12).

Disulfiram (Anatabuse®), a nonspecific drug that inhibits the activity of ALDH2 by reacting with sulfhydryl groups in the enzyme (13), has been shown to be effective in the treatment of alcoholism (14). However, a major problem with disulfiram is the lack of patient compliance which is mainly due to its side effects including sensory and motor neuropathies, optic neuritis, orthostatic hypotension, and hypersensitivity reactions (15–19). Hence, a new approach

Address correspondence to Eric Garver, Department of Pathology and Cell Biology, Thomas Jefferson University, 1020 Locust St., Rm. 275, Philadelphia, PA 19107. Phone: 215-503-5063; Fax: 215-923-9263; E-mail: emg002@jefferson.edu

**Abbreviations used in this paper:* ALDH2, aldehyde dehydrogenase; ASO, antisense phosphorothioate oligonucleotide; CHX, cycloheximide; GDH, glutamate dehydrogenase; RT, reverse transcriptase.

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to specifically inhibit ALDH2 without the undesirable side effects of disulfiram would be a welcomed development in the treatment of alcoholism.

With the advent of gene pharmacology, new approaches now exist by which the expression of a single gene can be inhibited. Antisense phosphorothioate oligonucleotides (ASOs) with a sequence complementary to the primary RNA transcripts or to mRNA can inhibit gene expression in a specific manner (20). The clinical applications of antisense are becoming accepted. An ASO (Vitravene™) designed to inhibit human cytomegalovirus replication was approved for commercialization by the FDA. In addition, over 20 other ASOs are undergoing clinical trials (www.recap.com).

In 1997, a consensus conference on the design of antisense concluded that a large number of ASOs (>30) had to be designed and tested empirically to find one effective antisense molecule (21). However, recent work in our laboratory led to the finding that about one half of the most active antisense deoxyoligonucleotides reported in the literature contain a 5′-TCCC-3′ motif in the molecule (22). Antisense deoxyoligonucleotides containing the TCCC motif and designed prospectively were shown to act by greatly reducing mRNA levels (22). This is in line with recent postulates that the RNA moiety of DNA-RNA hybrids is hydrolyzed by RNase H (23).

Here, we report studies on a phosphorothioate-modified deoxyoligonucleotide containing the 5′-TCCC-3′ motif directed against ALDH2 mRNA, which was shown to be most effective in: (a) reducing ALDH2 mRNA levels and mitochondrial ALDH2 activity in rat hepatoma cells in vitro, (b) reducing liver ALDH2 mRNA and mitochondrial ALDH2 activity in rats in vivo, (c) increasing fourfold the plasma acetaldehyde levels after an oral dose of ethanol, and (d) eliciting a marked reduction in ethanol consumption. Overall, we present studies in which an antisense molecule designed to anneal to ALDH2 mRNA in the rat results in a phenotype that mimics the elevated plasma acetaldehyde levels observed in Asians who have a low ALDH2 activity (ALDH2–2) and consume ethanol. Such an antisense molecule led to a marked reduction in voluntary ethanol consumption in animals.

Materials and Methods

Chemicals

All chemicals used were purchased from Sigma-Aldrich, except for sucrose, sodium pyrophosphate, sodium phosphate, magnesium chloride, perchloric acid, hydrochloric acid, acetonitrile, and isooctane, which were purchased from Fisher Scientific.

ASOs

Phosphorothioate oligonucleotides with specific base sequences used for in vitro studies were manufactured by Genset Corp. Purified ASO-9 for in vivo work and for the last two in vitro studies was purchased from Hybridon. For the in vivo study, HPLC-purified ASO-9 was dissolved in PBS at a concentration of 20 mg/ml. A large stock solution was prepared a day ahead of the initiation of the in vivo study and aliquoted into separate tubes to prevent any freeze-thaw cycles, which were stored at −70°C until it was needed for dosing.

Cell Culture

Rat hepatoma cell line H4-II-E-C3 (H4) (American Type Culture Collection CRL-1600) was purchased from American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium containing 4.5 g/liter of L-glutamine (Cellgro®; Mediatech), 20% horse serum, and 5% fetal bovine serum (Life Technologies) in 10% CO_2 at 37°C. All delivery of oligonucleotides and incubations were conducted in the presence of 10% CO_2 at 37°C.

In Vitro Oligonucleotide Delivery

H4 cells were seeded 18–24 h before the delivery of phosphorothioate oligonucleotides at a density of 2.5×10^6 cells/100-mm culture dish. Oligonucleotides were prepared using cationic liposomes, Lipofectamine Plus™ (Life Technologies). For each 100-mm culture dish to be treated, varying amounts of oligo in 2.5 to 10 μl (800 μM stock) were added to 360 μl serum-free Dulbecco's modified Eagle's medium containing 4.5 g/liter of L-glutamine (DMEM) which were mixed with 27 μl PLUS reagent® in a microcentrifuge tube, while in another microcentrifuge tube, 40 μl of Lipofectamine (2 mg/ml) was added to 360 μl DMEM. The microcentrifuge tubes were incubated at room temperature for 15 min and then the contents of the microcentrifuge tube containing the oligo and PLUS reagent was added dropwise to the microcentrifuge tube containing lipofectamine and DMEM. The combined mixture was then incubated for an additional 15 min at room temperature to form the Lipofectamine Plus-oligo complex. The final Lipofectamine Plus-oligo complex (800 μl) was added to 7.2 ml of serum-free DMEM at 37°C. The 8.0-ml mixture constituted the complete culture medium in a 100-mm culture dish (final concentrations of ASO-9 ranged from 0.25 to 1.0 μM). Prior to the addition of the Lipofectamine Plus-oligo complex, the medium with serum was removed from the cells and the final 8 ml of prepared Lipofectamine Plus-oligo complex was added to the culture dish and incubated for 6 h in the absence of serum. 30 min before the end of the 6-h delivery, serum was added back to the cells for overnight culture. As the oligo concentration was reduced by addition of serum, a second addition of Lipofectamine Plus-oligo complex (280 μl) was prepared to maintain the desired concentration. A second addition was prepared using oligo, 9.45 μl PLUS reagent, and 14 μl lipofectamine. The final Lipofectamine Plus-oligo complex was added to the culture dish at 6 h with a mixture of 20% horse (2.2 ml) and 5% fetal bovine serum (0.6 ml) and incubated for an additional 18 h.

Mitochondria Isolation

Upon completion of incubations, H4 cells were collected by removing the culture medium containing the Lipofectamine Plus-oligo complex and washing once with 5 ml ice-cold mitochondrial isolation buffer comprised of 0.25 mM sucrose in 10 mM Tris-HCl, pH 7.4. Cells were collected and transferred to an ice-cold conical tube for centrifugation at 152 *g* for 5 min at 4°C to pellet the cells which were then resuspended in 200 μl of the isolation buffer. The concentrated cellular suspension was homogenized and fractionated as described previously (24). The final purified mitochondrial pellet was resuspended in 250 μl of 50 mM sodium pyrophosphate, pH 9.0, immediately frozen in liquid nitrogen, and stored at −70°C until the time of analysis.

Prior to analysis, the mitochondria were thawed on ice, solubilized by adding 1% Triton X-100, and incubated for 15 min on ice. In some studies (see study III, Table I and Table III), the Triton X–treated mitochondria were transferred to an ultracentrifuge tube and centrifuged at 4°C for 30 min at 69,500 *g* to remove insoluble particulates.

Mitochondria from rat livers collected from control (PBS) and ASO-9–treated animals were isolated in a similar fashion as described previously (24). Soluble mitochondrial protein content isolated from H4 cells or liver was determined by the Micro BCA Protein Assay Reagent Kit (Pierce Chemical Co.) as described by the manufacturer with bovine serum albumin as the standard.

Determination of ALDH and Glutamate Dehydrogenase Activity

The low-Km ALDH activity in the isolated mitochondria was assayed as described previously (25) with minor modifications. The assay was initiated by the addition of propionaldehyde to a final concentration of 14 μM, 80 μM NAD, and 2.5 mM $MgCl_2$ (26). The reaction was linear with time, and ALDH activity was expressed as nmoles NADH/min/mg protein. Glutamate dehydrogenase (GDH) was assayed as described with minor modifications (27). The assay was performed at 37°C in a 400-μl reaction mixture containing 25 μg of soluble mitochondrial protein. The reaction was linear with time, and GDH activity was expressed as the oxidation of NADH to NAD^+ in μmoles NAD/min/mg protein.

Determination of ALDH2 Half-Life by Cycloheximide

Initial studies were conducted to determine the optimal concentration of cycloheximide (CHX) required to maximally inhibit protein synthesis in the H4 cells. Cells were seeded at a density of 16×10^6 cells/T75 flask 24 h before the treatment with CHX to ensure that they were in a stationary phase. The incubation medium containing serum was removed from the cells and the cells were washed twice with 5 ml of methionine-free DMEM (L-glutamine was added; Life Technologies). Cells were then cultured for 1 h in 8 ml of methionine-free DMEM containing 1, 3, 5, 7, or 10 μg/ml CHX, after which a 15-min pulse of [^{35}S]methionine (1.5 μCi, SA = 1 CI/μM) was added to the flask. After the pulse, [^{35}S]methionine was removed from the flask and the cells were washed twice with 5 ml PBS. Cells were then lysed in 10 ml of 0.15 M KCl containing 1 mM EDTA (pH 8.0) and processed for radioactivity determination essentially as described earlier (28). The total cellular protein content of cells treated with or without CHX was determined as described above.

The half-life of ALDH2 (time to reduce the activity by 50%) in the H4 cells was determined in the presence of 5 μg/ml CHX. H4 cells (3 replicates/time per point) were treated for 1, 5, 7, 12, or 24 h with CHX in DMEM without serum present. The mitochondrial ALDH2 activity remaining at the different times of incubation with CHX was then determined.

Sequencing of H4-II-E-C3 ALDH2–1 cDNA by Reverse Transcriptase PCR

The published cDNA sequence of the mitochondrial ALDH2 in the Sprague-Dawley rat (29) was used to design PCR primers to amplify the cDNA obtained from the mRNA isolated from the H4 cell line. Total cellular RNA was isolated using Tri-Reagent® (Molecular Research Center, Inc.) and the first strand cDNA was made using a First Strand cDNA Synthesis Kit using random hexamer primers (Amersham Pharmacia Biotech) according to the manufacturer's protocols. Three sets of PCR primers were designed to amplify three fragments of 476, 753, and 598 bp which combined constituted 96% of the published Sprague-Dawley rat sequence. The PCR primers were as follows: (476 bp, forward 5′-CACgCCTgAgCCgCCTg-3′, reverse 5′-gCTgAAgAAgTCgCCATCg-3′), (753 bp, forward 5′-CgATggCgACTTCTTCAgC-3′, reverse 5′-CTTggCgATggT-CATgCCA-3′), (598 bp, forward 5′-TggCATgACCATCgC-CAAg-3′: reverse 5′-gAACCAgCATCCAgCACAg-3′). The primers were synthesized by the Nucleic Acid Facility at Thomas Jefferson University. All fragments were gel purified from 1% agarose gels using QIAGEN's gel purification kit (QIAGEN) and the forward primers of each fragment were used in the sequencing reactions performed by Thomas Jefferson University-Nucleic Acid Facility.

Determination of mRNA Expression by Competitive Reverse Transcriptase PCR

Competitive reverse transcriptase (RT)-PCR for ALDH2 mRNA was developed by first designing PCR primers to amplify a 584-bp fragment from the Sprague-Dawley rat cDNA, donated by Dr. Henry Weiner of Purdue University (West Lafayette, IN). The primers (specific to mitochondrial ALDH) used to generate the 584-bp fragment (referred to as a parent fragment) were as follows: forward (F) 5′-ACgTggTggTgATgAAAgTg-3′ and reverse (R1) 5′-gATgAAgTAACCACggTCTg-3′. The parent fragment was amplified from the cDNA template under the following conditions: denaturation at 95°C for 3 min followed by 30 cycles at 95°C for 1 min, 63°C for 30 s, 72°C for 1.2 min, and a final extension at 72°C for 10 min, and the resulting band was purified from a 1% agarose gel. An internal standard, which served as a competitor for primers of the parent fragment during reverse transcription (as recombinant RNA, rcRNA) and PCR (as first strand cDNA) reactions, was constructed using a similar technique to that described previously (30). The same forward primer mentioned above and a new reverse primer (NRP), comprised of the previous reverse primer sequence (R1, 5′-gAT-gAAgTAACCACggTCTg) and an added reverse primer sequence (R2, 5′-ACACTgCgTTCCACgAATTC-3′), was used to amplify a smaller internal standard fragment of 429 bp. The 40-mer NRP sequence (5′-gATgAAgTAACCACggTCTgA-CACTgCgTTCCACgAATTC-3′) allowed the parent fragment primers (F and R1) to also amplify the shorter artificial 429 bp internal standard that was spiked into experimental RNA samples at varying amounts for competition with the mRNA of the parent fragment.

The pGEM®-T Easy Vector (Promega) containing the 429-bp recombinant DNA fragment (rcDNA) was transformed into DH5α competent *Escherichia coli* cells according to the manufacturer's protocol (Life Technologies). The plasmid DNA was linearized using NotI restriction enzyme and then purified before use as template in the in vitro transcription reaction. The in vitro transcription reaction for generating RNA to be used as competitor in the RT-PCR reaction was performed using the Ribo-Probe® In Vitro Transcription System (Promega). The in vitro transcription and RNA purification was performed as recommended in the Promega technical manual.

A streamlined procedure similar to that reported by Vanden Heuvel et al. (31) for design of a recombinant RNA (rcRNA) was used to make the GDH internal standard which eliminated any subcloning and relied only on PCR to obtain the rcDNA template for in vitro transcription. The cDNA template obtained for GDH was obtained by reverse transcription using random hexamers (pd(N)6 20 ng/20 μl RT reaction; Amersham Pharma-

cia Biotech) and Omniscript RT (QIAGEN) from RNA isolated from H4 cells. Primers were designed to amplify a 782-bp fragment with a forward (F1) 5′-TCCTgggAggTCATCgAAg-3′ and a reverse (R) primer 5′-AACTgCTTCTCgCTggCTg-3′) by PCR using similar conditions to those described above for ALDH using 55°C for primer annealing. The PCR product was run on a 1% agarose gel and the 782-bp parent fragment was purified from the gel. The purified fragment was then amplified with a newly designed forward primer (NFP) and the same reverse primer (R) as mentioned above. The new forward primer was constructed using a T7 promoter sequence (5′-TgTAATACgACTCACTATAgggCgAAT) plus the previous forward primer sequence (F1) (5′-TCCTgggAggTCATCgAAg-3′), and a new forward primer sequence (F2) (5′-CgTgAgTgTggATgAggTg-3′) comprising a 65-mer: 5′-TgTAATACgACTCACTATAgggCgAATTCCTgggAggTCATCgAAgCgTgAgTgTggATgAggTg-3′, which amplified a shorter fragment (718 bp), which was subsequently used to prepare the GDH-mRNA internal standard. For this purpose, the smaller artificial fragment (718 bp) containing the T7 promoter sequence was then used as the DNA template for in vitro transcription to make RNA which served as the GDH internal standard for competitive RT-PCR reactions with sample RNA.

Sample analysis for ALDH or GDH mRNA expression was performed using 2 μg of total RNA spiked with a range of internal standard RNA (ALDH 0–100 pg and GDH 0–32 ng) depending on the anticipated gene expression. Omniscript RT was used for all the reverse transcription reactions according to the QIAGEN protocol using random hexamers (Amersham Pharmacia Biotech) to prime first strand cDNA synthesis. The parent fragment and internal standard bands were analyzed on a Kodak Digital Science Image Station 440CF (Eastman Kodak Co.) by analyzing the net intensity of the UV/fluorescence emitted by the ethidium bromide in the bands. The actual amount of internal standard that was needed to compete for amplification with the parent fragment was determined by plotting the amount of internal standard against its corresponding net intensity ratio (internal standard band/parent fragment band). Using a linear relationship, the exact amount of internal standard needed to equally compete with the amount of sample mRNA present was determined from the linear regression equation when y = 1.

Animals and Treatment

Intravenous Administration of ASO-9. Male Lewis rats (Harlan) weighing 200–300 g were used. Prior to femoral vein catheterization, all rats were acclimated for at least 3 d to their cages. All animals were maintained on a 12 h light/dark cycle and had free access to laboratory rodent diet 5001 (PMI Feeds, Inc.) and tap water. Once animals were acclimated, surgery was performed to insert a femoral venous catheter comprised of a flexible Tygon plastic tubing (0.015 internal diameter [I.D.] × 0.03 outer diameter [O.D.]). Patency of catheters was maintained by using a lock solution containing heparin (444 U/ml), dextrose (22%), and streptokinase (16,667 U/ml), and a small piece of monofilament which was inserted at the exposed end of the cannula to retain the lock solution. Animals were treated for 4 d at 15 mg ASO-9/kg/day via intravenous catheters. An oral ethanol challenge (1 g/kg) was administered on day 5 and animals were killed 1 h later for determination of plasma acetaldehyde, ALDH, and GDH activity and mRNA expression.

Intraperitoneal Administration of ASO-9. Male Lewis rats as described above were surgically implanted with 2-ml osmotic pumps (Alzet Minipump 2 M1–1®) containing a solution of 25 mg/ml ASO-9 in PBS or PBS (controls) in the interperitoneal cavity. A priming dose of 20 mg/kg was administered intraperitoneally after implantation of the pumps. The pumps delivered ASO-9 at 24 mg/kg/day. 3 d after pump implantation, animals were deprived of water overnight while rat chow remained accessible. After the overnight fluid deprivation, on day 4 animals were offered 6% vol/vol ethanol as the only fluid and consumption was measured at hourly intervals for 5 h. Rehydration is a prime physiological drive and animals will initially consume the fluid offered. After the initial bout, rejection to continue consuming the fluid freely available indicates an aversion to the fluid offered. The general protocol follows that recently described (25). Animals were videotaped to capture their behavior after ethanol presentation (see Garver et al. in http://arc.tju.edu/egarver).

Acetaldehyde Determination by HPLC. Plasma acetaldehyde was determined as described previously (25).

Statistics

Statistical analysis was performed for the datasets in Microsoft Excel 1997. Significant inhibitions of treated versus control groups were calculated using a two-sample *t* test assuming unequal variances. Statistical significance was demonstrated at a 95% confidence level with P values < 0.05. Where applicable, mean values for groups were calculated, as well as the SEM.

Results

Low-Km Mitochondrial ALDH2 in Rat Hepatoma Cells and the Liver of the Lewis Rat

Lindahl and associates (32) have reported the existence of a mitochondrial ALDH2–1 in rat hepatoma H4-II-E-C3 cells (referred to as H4 cell line), with similar characteristics to ALDH2 in rat and human liver mitochondria. The ALDH2–1 cDNA sequence was reported for the Sprague-Dawley rat by Farres et al. (29). As the H4 cell line was derived from a tumor from an AxC-Buffalo rat cross, it was necessary to confirm that the ALDH2–1 cDNA sequence in the H4 cell line and in the inbred Lewis rat were similar to that reported for the Sprague-Dawley rat. We confirmed by RT-PCR and subsequent sequencing that the cDNA sequences of this isozyme in H4 cells and in the Lewis rat are >99% homologous to the cDNA sequence for which PCR primers were designed, which comprised 96% of ALDH cDNA. We further confirmed the studies by Huang and Lindahl (32) showing that ALDH2 activity is present in mitochondria of H4 cells and has a Km for acetaldehyde below 1 μM. Thus, the mitochondrial ALDH activity measured in the H4 cells has a similar high affinity for acetaldehyde as that in rat and human liver mitochondria (33).

Reduction in ALDH2–1 Activity of H4 Cells Incubated in the Presence of ASO-9

Half-Life. When using an antisense molecule to inhibit specific protein synthesis, the half-life of the preformed protein must be considered. To determine the half-life of the mature mitochondrial ALDH2 we used CHX to arrest the synthesis of all cellular proteins, which allowed us to measure the decay of the existing ALDH2 enzyme. We

first determined the minimal concentration of CHX required for maximal inhibition of protein synthesis in the H4 cell, measured by the rate of incorporation of [^{35}S]methionine into total proteins. CHX at a concentration of 5 μg/ml produced the maximal inhibition of ^{35}S incorporation. At this concentration of CHX methionine-derived ^{35}S incorporation was inhibited by 85% over 24 h. The residual incorporation could be due to posttranslational incorporation of [^{35}S]sulfate, and it was not further reduced by increasing the concentrations of CHX. Cell viability under these conditions exceeded 90%. Under these conditions, ALDH2 activity in the H4 cells was reduced by 56% from control values in 24 h. The calculated half-life of the mature ALDH2 in the mitochondria was 22 h. Such a half-life is in line with a half-life of 22 h also reported for Hela cells transduced with human ALDH2–1 (34).

Effect of ASO-9. We present here the results of antisense phosphorothioate deoxyoligonucleotide no. 9 (ASO-9), the most effective of nine antisense molecules containing the TCCC motif studied. Data in Table I show that after a 24-h incubation of H4 cells with ASO-9, mitochondrial ALDH2 activity was reduced by 54.9 ± 10.7% (mean ± SEM). Lipofectamine alone or lipofectamine plus control oligonucleotide(s) showed no reductions in ALDH2 activity. Given a half-life of 22 h, it was determined that the de novo synthesis of ALDH2–1 was inhibited by >95% after the 24 h of exposure to ASO-9 (Table I). Although ASO-9 proved to be very effective in inhibiting the synthesis of ALDH2, the exact mechanism by which this molecule elicited its effects was unknown. Thus, studies were conducted to determine whether ASO-9 reduced ALDH2–1 mRNA levels.

Reduction in ALDH2 mRNA Levels Induced by ASO-9 on H4 Cells

The steady-state level of ALDH2 mRNA was determined by quantitative competitive RT-PCR as described in Materials and Methods. Table II shows that after a 24-h treatment with ASO-9, ALDH2 mRNA in the H4 cells was reduced by 84.8 ± 4.7%. This result suggests that the mechanism of action for ASO-9 is likely mediated by RNA hydrolysis by RNase H.

Specificity

Sequence. The effect of minor sequence changes on the ASO-9 molecule was tested by incubating cells with phosphorothioate oligonucleotides containing 2-, 3-, or 4-base mismatches compared with ASO-9. One of the mismatches was always located in the "TCCC" motif. The studies showed that mismatches of 2-, 3-, or 4 bases in ASO-9 blunted the oligonucleotide's ability to reduce the levels of mRNA. The greater the number of mismatches incorporated into the sequence of ASO-9, the less effective the modified ASO-9 became (Table II; experiments 4 and 6).

mRNA Levels. The specificity of ASO-9 was also demonstrated by determining mRNA levels of ALDH2 and of GDH, another mitochondrial enzyme, in the same cells. Fig. 1 shows the reduction in mRNA ALDH2 afforded by ASO-9 (see Fig. 1, A1) and its ineffectiveness on GDH mRNA levels (Fig. 1, B1).

In Vivo Inhibition of ALDH2 Activity and mRNA by Antisense Oligonucleotide ASO-9

As ASO-9 proved to be most effective in reducing ALDH2 activity in cell culture and was both sequence and gene specific for *ALDH2*, we determined whether this molecule could also reduce the activity of hepatic ALDH2 in vivo. The activity of GDH was determined as a control mitochondrial enzyme. ALDH2 and GDH gene expression (mRNA) were also determined in vivo. Antisense ASO-9 was administered for 4 d via an indwelling femoral vein catheter with either ASO-9 (15 mg/kg) or PBS. 24 h after the last dose of ASO-9 or PBS, animals received an oral dose of 1 g/kg ethanol (from a solution of 7% wt/vol in water) and were killed 60 min later for the determination of mRNA levels, enzymatic activities, and plasma acetaldehyde levels. Treatment with ASO-9 led to a 50% reduction

Table I. *Effect of ASO-9 on the Activity of the Low Km ALDH2 in Mitochondria of H4-II-E-C3 Rat Hepatoma Cells*

		ALDH2 activity (nmoles/min/mg protein)				
	Control (no lipofectamine)	Lipofectamine (no oligo)	Lipofectamine plus control oligo[a]	Lipofectamine plus ASO-9[b]	Inhibition[c] (%)	*P* value
Study I[d]	18.4 ± 0.8 (4)	16.4 ± 3.1 (4)	15.9 ± 3.4 (4)	3.9 ± 1.4 (4)	76.2	<0.02
Study II[d]		14.0 ± 2.2 (4)		7.5 ± 1.3 (4)	46.5	<0.02
Study III[e]		37.1 ± 3.4 (4)		21.5 ± 4.0 (4)	42.1	<0.02

Where applicable, all oligonucleotides were kept at 1 μM throughout the 24-h incubation.
[a]Sequence 5′-CAgATgACCTCCCCCgTggAA-3′.
[b]Sequence 5′-TCCTCCTTgTTCCCTTCggCT-3′.
[c]Inhibition of ASO-9 with respect to lipofectamine alone. The calculated inhibition of ASO-9 based on the half-life of 22 h of ALDH2-1 was 98.1 ± 19%. Values reported as mean ± SEM were applicable.
[d]Studies I and II were conducted with mitochondrial fractions resuspended in 1% Triton X-100.
[e]Study III was conducted with mitochondrial fractions resuspended in 1% Triton X-100 and centrifuged to remove nonsoluble debris.

Table II. *Reduction of ALDH2 mRNA Levels by ASO-9*

	ALDH2 mRNA	Inhibition
	pg mRNA/ μg total RNA	*%*
Experiment no. 1		
Control oligo (1.0 μM)	12.5	NA[a]
ASO-9 (1.0 μM)	2.5	80
ASO-9 (0.5 μM)	2.0	84
ASO-9 (0.25 μM)	1.5	88
Experiment no. 2		
Control oligo (1.0 μM)	100	NA
ASO-9 (1.0 μM)	3	97
Experiment no. 3		
Control oligo (0.5 μM)	25	NA
ASO-9 (0.5 μM)	5	80
Experiment no. 4		
2 bp mismatch of ASO-9 (1.0 μM)	7.5	NA
ASO-9 (1.0 μM)	2.5	67[b]
Experiment no. 5		
Lipofectamine Plus (no ASO)	23.4	NA
ASO-9 (1.0 μM)	4.7	80
Experiment no. 6		
3 bp mismatch of ASO-9 (1.0 μM)	12.95	NA
4 bp mismatch of ASO-9 (1.0 μM)	21.8 ± 8.3	NA
ASO-9 (1.0 μM)	5.6 ± 0.5	74[b]

Control oligonucleotide in experiments no. 1–3: 5′-CgTCT-TCACTTCCgTgTAggC-3′; 2 base mismatch of ASO-9 in experiment no. 4: 5′-TCCTCg TTgTTCgCTTCg gCT-3′; 3 base mismatch of ASO-9 in experiment no. 5: 5′-TCCTCgTTgTTCgCATCg gCT-3′; 4 base mismatch of ASO-9 in experiment no. 6: 5′-TCCACg TTg-TACgCATCg gCT-3′.

[a]NA, not applicable.

[b]Inhibition versus the 2-bp (Experiment no. 4) or 4-bp (Experiment no. 6) mismatch. Mean inhibition by ASO-9: 84.8 ± 4.7% was calculated from the six experiments shown above.

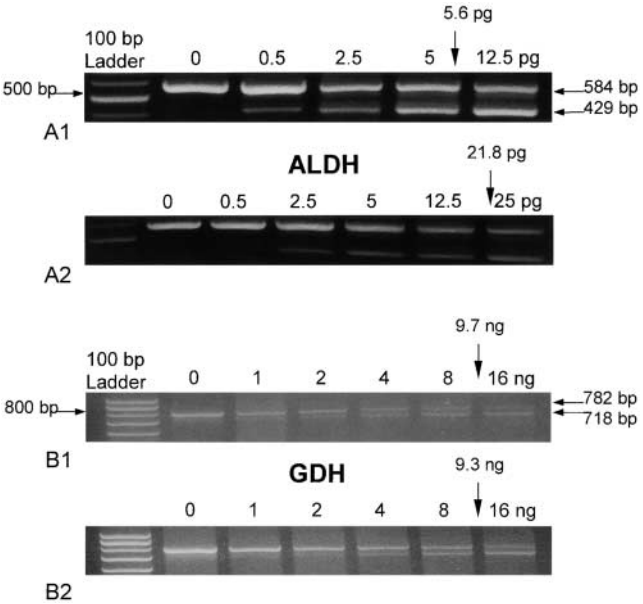

Figure 1. Quantitation of ALDH2–1 and GDH steady-state mRNA by competitive RT-PCR. A1 and A2 show ALDH2 mRNA expression and B1 and B2 show GDH mRNA expression. H4-II-E-C3 hepatoma cells were incubated with Lipofectamine Plus™ in the presence of 1 μM ASO-9 (A1 and B1) or the 4-base mismatch of ASO-9 (A2 and B2, nucleotide sequence in Table II). The concentration shown represents the pg of ALDH2 mRNA competitor added/μg total RNA (A1 and A2) and ng GDH mRNA competitor added/μg total RNA (B1 and B2). As can be observed, lesser competitor was necessary to compete with ALDH2 mRNA in ASO-9–treated cells than in cells treated with the 4-base mismatch of ASO-9 (control). In three replicates, the relative concentration of ALDH mRNA for 4-base mismatch control cells was (indicated by arrows) 21.8 ± 8.3 pg/mg total RNA; that for the ASO-9–treated cells was 5.6 ± 0.5 pg/mg total RNA. ASO-9 did not affect the GDH mRNA levels when compared with the 4-base mismatch oligonucleotide. In three replicates, the relative concentration of GDH mRNA for 4-base mismatch control cells was (indicated by arrows) 9.65 ± 0.19 ng/mg total RNA; that for the ASO-9–treated cells was 9.26 ± 0.18 ng/mg total RNA.

in ALDH2 mRNA compared with PBS control animals (Table III). Administration of ASO-9 reduced ALDH2 activity by 38–45% and led to a fourfold increase in plasma acetaldehyde levels after ethanol administration, compared with acetaldehyde levels in control animals that received the same dose of ethanol (Table IV). GDH activity and GDH mRNA levels were not affected by ASO-9, thus also demonstrating in vivo the specificity of ASO-9 on ALDH2 gene expression (Tables III and IV).

Inhibition of Ethanol Consumption after Treatment with ASO-9

Studies were performed to determine if ASO-9 could establish an aversion to ethanol and reduce ethanol consumption. The animal model used was essentially as reported previously (25) to test a drug-induced aversion to ethanol. Rats were surgically implanted with osmotic pumps which delivered ASO-9 at 24 mg/kg/day intraperitoneally. 3 d after pump implantation, animals were deprived of water overnight. Thereafter, animals were offered 6% vol/vol ethanol as the only fluid, and consumption was measured at hourly intervals for 5 h. A reduction in ethanol consumption after the first bout indicates an aversion to the fluid offered. Cumulative ethanol consumption (mean ± SEM) at each hourly interval is shown in Fig. 2. Initial ethanol consumption was similar in both the ASO-9 and control (PBS) groups during the first hour of ethanol presentation, amounting to 1.12 ± 0.09 g ethanol/kg (ASO-9) and 1.70 ± 0.68 g ethanol/kg (controls). After the first hour of ethanol presentation, consumption in the 1–5 h interval was reduced significantly ($P < 0.015$) in the ASO-9 group (0.48 ± 0.23 g ethanol/kg) compared with the control group (1.22 ± 0.16 g ethanol/kg) (Fig. 2). This equates to a 61% reduction in ethanol consumption in ASO-9–treated animals after experiencing the drug (ASO-9) ethanol reaction. Cumulative ethanol consumption at 5 h (which includes the amount consumed in the first hour of presentation) was

Table III. *Reduction of the Steady-State Levels of ALDH2-1 and GDH mRNA after Treatment with PBS or ASO-9*

	ALDH2-1	GDH	Inhibition[a]
	pg of mRNA/ μg total RNA	*ng of mRNA/ μg total RNA*	*%*
PBS			
1	13.0	2.6	NA[b]
2	15.3	2.6	NA
3	16.1	2.3	NA
4	19.6	–	NA
	16 ± 1.4	2.5 ± 0.1	
ASO-9			
1	7.4	2.3	53.8
2	9.6	2.7	40.0
3	6.4	2.6	60.0
4	8.4	–	47.5
	8.0 ± 0.7	2.5 ± 0.1	50.3 ± 4.2

t test of PBS versus ASO-9, $P < 0.007$.
[a]Inhibition for each animal in ASO-9 group calculated from mean ALDH2-1 mRNA expression of PBS group.
[b]NA, not applicable.

Table IV. *In Vivo Effect of ASO-9 Administration on the Activity of Liver ALDH2-1, GDH, and Plasma Acetaldehyde Levels after Administration of Ethanol (1 g/kg)*

Treatment group	ALDH specific activity	GDH specific activity	ALDH/GDH	Plasma acetaldehyde
	nmol/min/ mg protein	*μmol/min/ mg protein*	*Ratio*	*μM*
PBS				
1	58.9	6.4	9.2	1.5
2	48.2	6.4	7.5	1.9
3	42.9	8.4	7.0	3.6
4	58.9	8.4	7.0	3.6
	52.2 ± 4.0	7.1 ± 0.5	7.4 ± 0.7	2.2 ± 1.0
ASO-9				
1	37.5	7.7	4.9	7.5
2	37.5	7.7	4.9	6.1
3	32.2	8.4	3.8	7.5
4	42.9	8.4	5.1	8.1
5	37.5	7.7	4.9	9.4
6	32.2	7.7	4.2	11.4
	36.6 ± 1.6[a]	7.9 ± 0.2[b]	4.6 ± 0.2[c]	8.3 ± 1.9[d]

ASO-9 (15 mg/kg/day) was administered intravenously to rats for 4 d. 24 h after the last dose of the oligonucleotide, animals received 1 g/kg ethanol orally. Group values are means ± SEM.
[a]Effect of ASO-9 on mitochondrial ALDH activity: $P < 0.01$.
[b]Effect of ASO-9 on mitochondrial GDH activity: not significant.
[c]Effect of ASO-9 on ALDH/GDH ratio: < 0.015.
[d]Effect of ASO-9 on plasma acetaldehyde levels: < 0.002.

1.599 ± 0.23 g ethanol/kg (ASO-9) and 2.928 ± 0.59 g ethanol/kg (controls) corresponding to a 45% reduction ($P < 0.035$) in the total ethanol consumption in the ASO-9–treated animals when compared with controls (Fig. 3 A). This reduction is identical to the reduction (46%) in ethanol consumption experienced in earlier studies in rats treated with disulfiram, a nonspecific drug used to induce an aversion to ethanol (25; Fig. 3 B).

Discussion

The studies presented show that an antisense phosphorothioate deoxyoligonucleotide (ASO-9) was specific and effective in reducing the de novo synthesis of mitochondrial ALDH2 in rat hepatoma cells. Given a 22-h half-life for the mature enzyme, an inhibition of 95% in ALDH2 synthesis was calculated to occur after a 24-h incubation with ASO-9. Actual remaining activity at 24 h was 55%.

Present knowledge indicates that there are two major mechanisms of antisense action: (a) occupancy of mRNA and (b) occupancy-activated RNase H hydrolysis of the RNA targets (20). Our earlier studies on the mechanisms of action of ASOs which incorporate the TCCC motif in the molecule led to the finding that steady-state mRNA levels are significantly reduced by these ASOs. As RNase H only exists in the nucleus and the most effective ASOs had targets mainly in intron and 3′-untranslated regions, it was postulated that the TCCC-mediated mechanism of antisense action was localized in the nucleus (22).

In the studies presented here, nine ASOs were designed which incorporated the TCCC motif (22) targeting exons (a message also present in the nucleus), as only the cDNA sequence of the Sprague-Dawley rat *ALDH2* was known at the time of starting these studies (29). We found that two of the nine antisense molecules (two ASO's in 3′-untranslated region [UTR], and seven ASOs in open reading frame including ASO-9) had inhibitory effects on the ALDH2 enzyme activity, with ASO-9 being the most effective. As ASO-9 targeted exon 2, it was possible that the antisense mechanism of ASO-9 might have been due to occupancy-mediated inhibition of translation rather than to RNase H–mediated hydrolysis. To determine if the mechanism of action followed translation arrest or a reduction in mRNA transcript levels, we determined the steady-state levels of mRNA and whether any reduction in mRNA levels correlated with the loss of ALDH2 activity. Analysis of ALDH2 steady-state mRNA by competitive RT-PCR demonstrated that ASO-9 indeed reduced the levels of mRNA by 85%, which paralleled the calculated 95% inhibition of enzyme synthesis. These data suggest that the mechanism of action of ASO-9 is likely one involving mRNA hydrolysis by RNase H, although some additional transcriptional arrest by occupation cannot be ruled out.

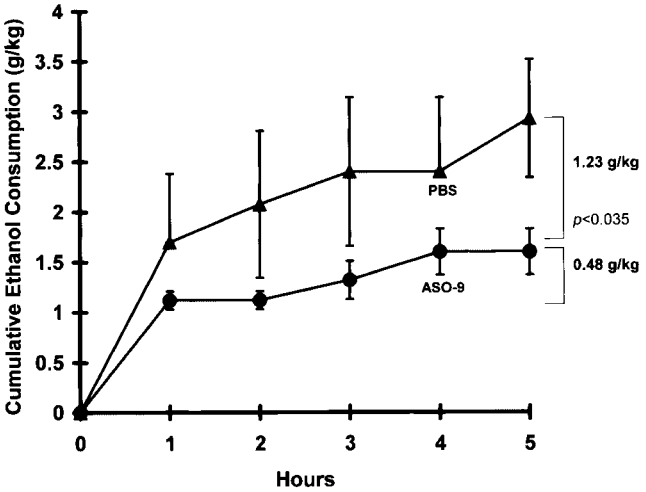

Figure 2. Effect of ASO-9 on ethanol consumption after an 18-h water deprivation period. Data show cumulative ethanol consumption (g ethanol/kg) by animals during the first 5 h of access to a 6% ethanol (vol/vol) solution after an 18-h water deprivation. Prior to the study, rats received ASO-9 (24 mg/kg/hr) or PBS for 4 d from implanted osmotic pumps. Values represent mean ± SEM for five animals. Ethanol consumption was not significantly different in the two groups in the first hour of fluid reaccess. However, after the primal rehydration bout (and after experiencing a combined ethanol-ASO-9 effect), rats that had received ASO-9 consumed 61% less ethanol than control animals in the following 4 h (0.48 vs. 1.23 g ethanol/kg; $P < 0.015$).

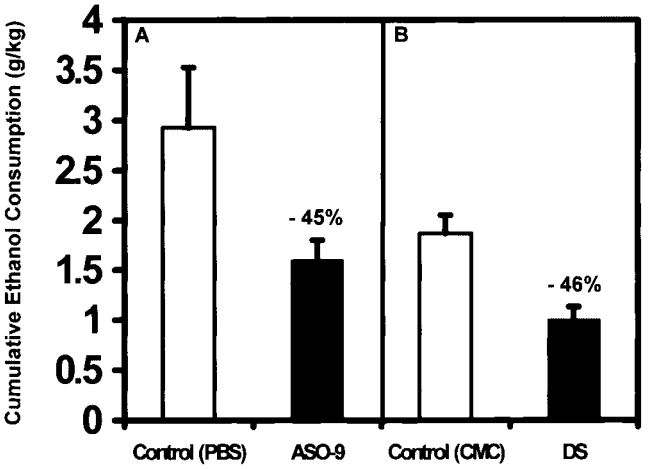

Figure 3. Comparison of the inhibitory effect on ethanol consumption of ASO-9 (24 mg/kg/day) for 4 d (data from this study) and of disulfiram administration (100 mg/kg/day) for 4 d (data from reference 25). The effect on ethanol consumption of ASO-9 and of disulfiram (a nonspecific inhibitor of ALDHs), was determined after an 18-h water deprivation period of inbred Lewis rats. Data show total ethanol consumption (g ethanol/kg) by animals during 5 h (zero to 5 h) of access to a 6% ethanol (vol/vol) solution after the period of water deprivation. Control for disulfiram was carboxymethyl-cellulose (CMC) in saline administered orally 2 h before fluid reaccess. Control for ASO-9 was PBS delivered continuously from the osmotic pumps, which may account for the differences in fluid consumption (6% ethanol) in the two control groups. The inhibitory effect on ethanol consumption of the two drugs (ASO-9 and disulfiram) was identical (45 vs. 46%, not significant). DS, disulfiram.

A true antisense mechanism was demonstrated by the fact that as the number of mismatches from the original ASO-9 molecule were increased from 2- to 3- and 4 bases, the oligonucleotide's ability to inhibit ALDH mRNA levels was reduced. An oligonucleotide with 4 base mismatches from ASO-9 was essentially inactive. Furthermore, ASO-9 was specific for its *ALDH2* target, as ALDH2 mRNA levels were reduced whereas the mRNA levels for GDH, another mitochondrial enzyme, remained constant.

Despite the variation in basal mRNA levels in the H4 cells, the inhibition afforded by ASO-9 over six experiments was quite constant and averaged 85%. During in vitro studies it was noted that basal ALDH2 mRNA levels varied between experiments. In each of these experiments, serum was added at 6 h and the serum lots varied from one experiment to another. Previously, it has been shown that hormones such as insulin, progesterone, and dihydrotestosterone, found at different levels in serum lots, can increase or reduce the expression of *ALDH2* by as much as 50% (35). In addition, H4 cells at various passages were used over the course of the experiments and we found that the ALDH2 activity could be reduced by as much as 50% after many (>5) passages.

When "naked" (without liposomal conjugation) ASO-9 was administered intravenously for 4 d and animals were killed 24 h after the last dose of the oligonucleotide, hepatic ALDH2 mRNA was reduced by 50% and ALDH2 activity was reduced by 40%. Administration of ethanol to animals pretreated with ASO-9 elicited a fourfold increase in plasma acetaldehyde levels versus that in control (PBS) animals. In the same animals, ASO-9 did not modify the activity of mitochondrial GDH, thus again indicating the specificity of action of this antisense oligonucleotide seen in cell cultures.

We further determined if treatment of ASO-9 would establish an aversion to ethanol consumption in rats. We have previously reported the development of a rat model to measure a drug-induced aversion to ethanol using disulfiram as the aversive test drug (25). Animals treated with ASO-9 showed a 61% reduction in alcohol consumption after experiencing the ASO-ethanol reaction compared with control animals. Overall, ASO-9 showed an effectiveness in the same order as that reported previously for large doses of disulfiram.

Theoretically, an ASO of 17–21 bases with a sequence complementary to the primary RNA transcripts or the mRNA sequence can inhibit gene expression through Watson-Crick DNA-RNA hybridization in a specific manner because a sequence of this length is unlikely to repeat more than once in the human genome. Here, we demonstrated that ASO-9 did not affect GDH activity or mRNA levels, which strongly suggested that its effects were not due to nonantisense effects on another protein. In addition to specificity of antisense oligonucleotides for their target, antisense oligonucleotides have been shown to have minimal side effects over a wide range of doses (36). Both the specificity and lack of serious side effects of an antisense oligonucleotide would be advantageous when compared with disulfiram.

Disulfiram displays a large number of side effects, is nonspecific for its target, and requires daily administration, which has markedly reduced its clinical use (15, 16). The half-life of phosphorothioate oligonucleotides is 48 h in rodents (37–39) while in humans, antisense molecules have been reported to have a half-life of 6 to 14 d (40). The protracted half-life in humans would make it possible to reduce the frequency of administration, maintaining the desired efficacy and improving compliance. Disulfiram is the only drug that inhibits ALDH2 which is approved in the U.S. for the treatment of alcoholism. Controlled studies show that daily administration of disulfiram is indeed effective in the treatment of alcoholism (41, 42), although disulfiram has been criticized for not lengthening the time elapsed until full abstinence is broken (43). However, as patients must experience the effects of the disulfiram-ethanol reaction before the aversive effects take place, the time to break the abstinence is not expected to be affected by the drug. The main problem with disulfiram relates to the lack of compliance by patients with medication taking, mainly due to sensory and motor neuropathies, optic neuritis, orthostatic hypotension, and hypersensitivity reactions (15–19). In a number of studies in which disulfiram was administered under supervision its effectiveness in reducing alcohol consumption was clearly seen (14). However, due to its side effects, doses that are fully therapeutic are rarely achieved. At the doses normally employed (250–300 mg daily), less than 50% of patients display the disulfiram-ethanol reaction (44, 45). Thus, there is the obvious need to develop alternatives to disulfiram to mimic the strong protective effects of a low ALDH2 activity seen in Asians carrying the *ALDH2–2* allele.

Studies presented in this communication provide a strong proof of principle that inhibition of *ALDH2* gene expression can mimic the ALDH2–2 Asian phenotype, resulting in (a) low liver mitochondrial ALDH2–1 activity, (b) elevated plasma acetaldehyde levels after the administration of ethanol, and (c) an aversion to ethanol, shown as a reduction in alcohol consumption.

Our appreciation to Dr. Henry Weiner for the gift of the rat ALDH2 cDNA and to Drs. Ronald Lindahl and Eric Wickstrom for helpful discussions. Dr. Eric Garver would like to add this dedication to Dr. Guang-chou Tu, who passed away on July 20th, 2001 as a result of liver cancer. George served as my right hand man for my Ph.D. thesis from which this paper was generated. Despite the repeated chemotherapy sessions, George maintained his input and assistance right up to the revision stage of this manuscript. He was a very accomplished scientist and a wonderful mentor. We will greatly miss his collaborations and friendship.

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