A Transgenic Model of Acetaldehyde Overproduction Accelerates Alcohol Cardiomyopathy

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ABSTRACT

Chronic alcohol consumption produces alcoholic heart muscle disease (AHMD), a prevalent form of congestive heart failure. Several hypotheses have been proposed to explain the damaging effects of alcohol on the heart, but neither the mechanism nor the ultimate toxin has been established. In this study, we use transgenic overexpression of alcohol dehydrogenase to elevate cardiac exposure to acetaldehyde, the major and most reactive metabolite of alcohol. Overexpression of alcohol dehydrogenase by 40-fold produced no detectable deleterious effects to the heart in the absence of alcohol. In the presence of alcohol, transgenic hearts contained 4-fold higher acetaldehyde than control hearts. Chronic alcohol exposure produced many changes similar to AHMD in transgenic hearts. Compared with control hearts, these pathological changes occurred more rapidly and to a greater extent: alcohol-exposed transgenic hearts were almost twice as large as control hearts. They demonstrated ultrastructural damage consistent with AHMD and had much lower contractility than alcohol-exposed control hearts. In addition, the transgenic hearts showed greater changes in mRNA expression for α-skeletal actin and atrial natriuretic factor than alcohol-exposed control hearts. Alterations in NAD+/NADH levels were insufficient to account for such severe damage in cardiomyopathic hearts. The increased damage produced in transgenic hearts suggests an important role for acetaldehyde in AHMD.

Almost one third of chronic alcoholics exhibit cardiac dysfunction (Urbano-Marquez et al., 1989), and a large proportion of these (Preedy and Richardson, 1994; Urbano-Marquez et al., 1995) develop alcoholic heart muscle disease (AHMD). Individuals with AHMD do not usually have vitamin deficiencies (Alexander et al., 1977a) or impaired nutritional status (Alexander et al., 1977b). Therefore, the development of AHMD is a function of alcohol intake rather than poor nutrition. The diagnosis of AHMD is made on the basis of deteriorating cardiac function, increased heart size, and a long history of alcohol abuse (Urbano-Marquez et al., 1989). Clinical characteristics of AHMD are reduced left ventricular ejection fraction, dilated cardiac chambers, and enlarged left ventricular mass. Evaluations of biopsy specimens at the light microscopic level demonstrate diffuse fibrosis, increased endocardial thickness, and cardiac myocyte hypertrophy (Preedy and Richardson, 1994; Urbano-Marquez et al., 1995). Electron microscopy reveals a loss or disruption of myofibrils and dilated sarcoplasmic reticulum. Swollen mitochondria with disorganized cristae are reported in most morphological studies. Apart from the history of alcoholism, these features are consistent with other dilated cardiomyopathies.

Neither the mechanism nor the specific toxic agent that produces AHMD has been established (Redetzki et al., 1983; Capasso et al., 1991, 1992; Zou et al., 1991; Mikami et al., 1993; Preedy and Richardson, 1994; Thomas et al., 1994). One potential toxin is the major metabolite of ethanol, acetaldehyde. Acetaldehyde has been found to concentrate in the heart (Espinet and Argiles, 1984) and has significant actions on cardiac function (Savage et al., 1995; Patel et al., 1997; Ren et al., 1997). Acute exposure to acetaldehyde produces a biphasic dose-dependent effect on cardiac contractility (Savage et al., 1995). At low doses, contractility is enhanced by a sympathetically mediated action, whereas at higher doses, contractility is decreased. The latter effect appears to be a direct action of acetaldehyde on the cardiac myocyte because inhibition is seen even in isolated myocytes (Ren et al., 1997). Acetaldehyde may contribute to the production of AHMD because it is highly reactive, producing measurable acetaldehyde-protein adducts even at very low concentrations (Hoerner et al., 1988). In addition, many patients with AHMD have circulating antibodies to cardiac acetaldehyde-protein adducts (Hoerner et al., 1988; Harcombe et al., 1995). However, a lack of suitable methods for chronically altering acetaldehyde concentration in vivo has prevented a definitive test of the acetaldehyde hypothesis. Confirmation or disapproval of the acetaldehyde hypothesis is currently the subject of the present study.

Abbreviations: AHMD, alcoholic heart muscle disease; KH, Krebs-Henseleit buffer; SkActin, α-skeletal actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ANF, atrial natriuretic factor; ADH, alcohol dehydrogenase.
of this hypothesis is essential to understanding the basis of the disease. Unfortunately, the current evidence was largely indirect and correlative. Until now, more direct tests have had serious technical problems. Earlier experiments used metabolic inhibitors to alter the concentration of acetaldehyde (Weishaar et al., 1978; Hillbom et al., 1983; Preedy and Richardson, 1994). However, these inhibitors are only partially effective and nonspecific, greatly inhibit alcohol intake, and are potentially toxic and difficult to maintain chronically in experimental animals. The direct administration of acetaldehyde is also unsuitable for chronic studies. In this study, we increased cardiac exposure to acetaldehyde by genetically increasing the activity of enzymes that produce it, specifically in the heart. Our results indicate that a 4-fold increase in cardiac acetaldehyde levels greatly increases the rate of onset of AHMD. These results are consistent with the hypothesis that acetaldehyde plays an important role in alcohol-induced cardiomyopathy.

Materials and Methods

Development of Transgenic Mice. The MyADH transgene was produced by replacement of the catalase coding sequences in the transgene MyCAT (Kang et al., 1996) with the coding sequences for rat ADH I (Crabb and Edenberg, 1987). FVB mice obtained from the University of North Dakota Biomedical Research Center were used to produce transgenic lines containing the MyADH transgene. Standard procedures were used for producing transgenic animals. A second transgene containing a cDNA for the enzyme tyrosinase was coinjected with MyADH. The enzyme tyrosinase produces coat color pigmentation in albinoid mice (Overbeek et al., 1991) and was used to conveniently identify transgenic animals. All animal procedures were approved by the U.S. Department of Agriculture-certified institutional animal care committee.

Analysis of Transgene Expression. The activity and tissue specificity of the transgene were determined by measuring RNA on Northern blots and enzyme activity in tissue homogenates. RNA for Northern blots was prepared with RINazol as we previously described (Voss-McCowen et al., 1994; Kang et al., 1997) from heart, liver, skeletal muscle, brain, kidney, and lung of transgenic and normal mice. A 150-nucleotide fragment of the 3' untranslated sequence of the transgene was labeled with 32P and used as probe. This sequence had little detectable cross-reactivity with any other mouse mRNA. The strength of the hybridization signal was determined on a PhosphorImager (model 445 SI; Molecular Dynamics, Sunnyvale, CA), and quantification was facilitated with image analysis software.

Alcohol dehydrogenase (ADH) enzyme assays were performed as previously described (Agarwal and Goedde, 1990). Activity was calculated from the conversion of NAD+ to NADH in the presence of tissue extract and ethanol. Extracts were prepared by homogenization in 9 volumes of 0.1 M sodium phosphate, pH 7.0. Activity was measured in 0.5 ml of 33 mM sodium pyrophosphate, pH 8.8, containing 3 mM NAD+, 16 mM ethanol, and 10 μl of tissue extract. Reactions were run at 25°C, and the production of NADH was determined by the change in absorbance at 340 nm. Absorbance was measured continuously on a Beckman recording spectrophotometer (model DU-62) equipped with a kinetics software module. Only the linear portion of the activity curve was used for calculation. Absorbance from heat-inactivated tissue samples was subtracted as blank. Enzyme activity from normal and transgenic samples was expressed per microgram of extracted protein. At least three normal and transgenic values were determined for each tissue.

Chronic Treatment of Mice with Ethanol. Age- and sex-matched transgenic and normal animals between 9 and 12 weeks of age were placed on a nutritionally complete, all-liquid ethanol-containing diet (Lieber and DeCarli, 1982). Diets were purchased from Dyets Inc. (Bethlehem, PA). The standard diet contains 6% alcohol, which we found to produce essentially 100% mortality rates in FVB mice. Therefore, we used 4% alcohol diets containing maltose dextrin to compensate for the reduced calories. Normal FVB mice were found to have a higher intake than control mice and lost weight when exactly pair fed with transgenic animals. To compensate for this, control animals were given access to 25% higher intake than that consumed by transgenic mice. Over the course of the study, control animals consumed 17% more diet per day than transgenic animals. Despite this, transgenic animals gained 4 g b.wt. over the course of the study, whereas control animals gained less than 1 g b.wt.

Alcohol-Induced Modifications in Cardiac Gene Expression. To assess changes in gene expression in AHMD and to determine how they were affected by overexpression of ADH, cardiac RNA was isolated and probed on Northern blots. Mice were sacrificed by cervical dislocation. Hearts were weighed, and RNA was isolated as described above. Northern blots were probed with labeled oligonucleotides for atrial natriuretic factor (ANF), α-skeletal actin (SkAc-tin), and glyceroldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Probes were labeled using polynucleotide kinase and [γ-32P]ATP. Sequences of the probes were obtained from Jones et al. (1996). Hybridization for each probe was quantified on Molecular Dynamics PhosphorImager and normalized to the signal for GAPDH mRNA.

Measurement of Contractility. Mice were anesthetized by i.p. injection of 100 mg/kg ketamine and 32 mg/kg xylazine coadministered with 100 IU of heparin. When deep anesthesia was achieved, the left side of the chest was opened. A small incision was made in the descending aorta to allow insertion of a cannula. Immediately on insertion of the cannula into the aorta, the inferior vena cava was cut, and flow was initiated at a rate of 1.5 ml/min. The innominate artery, common carotid artery, and subclavian artery were then ligated and cut. The heart was removed from the chest and transferred to the perfusion apparatus. From the point of cannulation and throughout the entire procedure, the heart was retrogradely perfused with Krebs-Henseleit buffer (KH) consisting of 120 mM NaCl, 20 mM NaHCO3, 4.6 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgCl2, 1.25 mM CaCl2, and 11 mM glucose. KH buffer was prefiltered through a 0.22-μm filter. Throughout the perfusion, KH buffer was continuously equilibrated with 95% O2/5% CO2, which maintained pH 7.4.

The temperature of the heart and KH buffer was maintained at 37°C. Perfusion pressure was continuously monitored on a Gould/Statham p23Db physiological pressure transducer. Preload tension on the heart was set to 3g. The heart was paced throughout the procedure at 5 Hz (6 V, 3 ms). Contractile force and heart rate were measured by means of a Grass FT03 force transducer hooked to the apex of the heart and connected to an ETH 400 bridge amplifier that feeds into a Grass FT03 force transducer hooked to the apex of the heart and connected to an ETH 400 bridge amplifier that feeds into a Powerlab/400 amplifier. Data were continuously analyzed on using AD Instruments Chart for Windows version 3.3.5. Perfusion pressure, heart rate, and contractile force were continuously recorded. Cardiac work was analyzed as the product of force, and heart rate was divided by the weight of the heart.

Electron Microscopy. Anesthesia of the mice was achieved through i.p. injection of sodium pentobarbital (70 mg/kg). The abdomen and thorax were opened, and the right atrium was cut to allow the release of blood. Perfusion fixation was immediately initiated at a flow rate of 5 ml/min for 1 to 3 min with washout solution made up as 6.6 g of paraformaldehyde, 1.15 ml of 70% glacial acetic acid, 0.5 g of CaCl2, 0.25 g of AlCl3, and 1 g of procaine hydrochloride in 360 ml of 0.2 M sodium cacodylate and distilled water to make 1000 ml. A small amount (1–3 drops) of 1 N NaOH was required to get the paraformaldehyde into solution. The entire solution is finally brought to pH 7.4. Washout solution is followed by perfusion with chilled (4°C) Karnovsky's fixative (pH 7.4) at a rate of approximately 3 ml/min for 10 min. The heart is removed with surgical scissors and immersed immediately in chilled Karnovsky's fixative (Karnovsky, 1965). The heart is cut to form small tissue blocks (1 mm3) while immersed in fixative. Tissue fixation continues in the cold (4°C) for 2
alcoholic KOH and NAD
mice and pulverized in liquid nitrogen. NADH was extracted with
genberg, 1985). Briefly, the heart was excised from anesthetized
were measured according to spectrophotometric procedures (Klin-
was then centrifuged at 10,000
l loop injection system on a Varian 3700 gas chro-
and other components were separated on a 15-m VOCOL capillary
tissue was dehydrated through graded ethanols and pro-
lysine oxide, embedded in Epon/Araldite, and cured for 48 h at 60°C. Thin sections (silver-gray interference color) are cut on an MT-2B
knife. Sections are collected on naked copper (300-mesh) grids and
DuPont-Sorvall ultramicrotome equipped with a Diatome diamond
Acid (60 min in dark) and 0.5% aqueous uranyl acetate (4°C, 90 min). These are observed with either a JEOL 100-S or Hitachi 7500 trans-
ed electron microscope at initial magnifications of \( \times 1000 \) to
Measurement of Acetaldehyde. Acetaldehyde was measured according
to a modification of the procedure of Eriksson et al. (1984). In brief,
were removed from anesthetized mice, blotted to
from methanol and ethanol with baseline resolution with a
detector was used. Under these conditions, acetaldehyde was sepa-
ated from methanol and ethanol with baseline resolution with a
total separation time of less than 1 min.
Measurement of Cardiac NAD\(^{+}\) and NADH. These cofactors
were measured according to spectrophotometric procedures (Klin-
genberg, 1985). Briefly, the heart was excised from anesthetized
and pulverized in liquid nitrogen. NADH was extracted with
alcoholic KOH and NAD\(^{+}\) extracted in 0.6 N perchloric acid. Extracts
were neutralized and centrifuged. NAD\(^{+}\) was measured spectropho-
tometrically by the production of NADH during the oxidation of
by yeast ADH. NADH was measured by the drop in absor-
bance at 340 nm during the NADH-dependent reduction of pyruvate
by lactate dehydrogenase.
Statistical Analysis. The difference between the mean values of
two groups was determined using two-tailed Student’s \( t \) test. Com-
parisons of multiple mean values were made with one-way ANOVA
and Scheffe’s post hoc test. Statistical analysis was performed with
the program SPSS 8.

Results

Three lines of mice constructed with the MyADH transgene
were assayed for enzyme activity. As shown in Fig. 1, ADH
activity in the hearts of transgenic mice was increased by up
to 40-fold over levels measured in nontransgenic, FVB mice. MyADH
line 239, which had both high enzyme activity and a
coat color marker for convenient identification of transgenic
was chosen for further studies. All subsequent data
were obtained from this line. We noted no differences in
fertility or body weight between MyADH 239 mice and FVB
mice. Tissue specificity of the transgene expression is specific to the heart in line 239. RNA was isolated from the indicated tissues and probed with a fragment
specific to the transgene product. As explained in the text, multiple bands
in the heart may be due to multiple polyadenylation sites in the trans-
gene. The positions of ribosomal RNAs are indicated at the left. Hrt,
heart; Liv, liver; Brn, brain; Lng, lung; and Msl, muscle.

The MyADH transgene expression is specific to the heart in line 239.
transgene one in the ADH cDNA and another derived from the
rat insulin II gene. Most important to our original aim, we found that
the elevation in cardiac ADH activity significantly increased car-
diac acetaldehyde levels after the administration of an i.p.
injection of 3 g/kg alcohol. Thirty minutes after injection,
mice were sacrificed for rapid removal of the heart and quan-
tification of acetaldehyde through gas chromatography and flame ionization detection. As shown in Fig. 3, acetaldehyde
levels measured in heart tissue of transgenic mice were four times higher than those in control mice. We also measured systemic acetaldehyde levels with the use of a new subcutaneous sampling procedure (manuscript in preparation) designed to avoid artifactual production of acetaldehyde that plagues plasma and blood sample analysis (Eriksson et al., 1984). Our preliminary assays indicate that systemic acetaldehyde levels were significantly higher in transgenic mice (p < .02 by Student’s t test, n = four per group).

Because the transgene satisfied our primary aim of increasing cardiac exposure to acetaldehyde, we started these mice on a chronic alcohol diet. Animals were initiated on a 1% alcohol (by volume) liquid diet using the Lieber DeCarli formulation (Dyets Inc.); the diet was gradually increased to 4% alcohol. The 4% maximum was chosen because control mice fed higher-percentage alcohol diets demonstrated very high mortality rates in preliminary studies. After 10 weeks on the alcohol diet, mice of each group were sacrificed to assess the onset of cardiomyopathy by measuring changes in gene expression. Increased expressions of SkActin and ANF have been reported in several cardiomyopathies (Jones et al., 1996; Colbert et al., 1997), and this provides a sensitive indicator of cardiac damage. As shown in Fig. 4, levels of these two mRNAs were significantly increased in alcohol-treated MyADH mice relative to alcohol-treated control mice. Without alcohol treatment, MyADH mice did not have increased expression of these genes. Control mice on alcohol also did not show a statistically significant increase in expression of either mRNA. However, a possible trend toward increased ANF expression was observed in alcohol-treated control mice (Fig. 4B). These results, obtained after only 10 weeks of alcohol treatment, indicated that the MyADH transgene sensitized the heart to the deleterious effects of alcohol.

Human hearts are enlarged due to AHMD. Normal mouse hearts were also enlarged after 5 months of an alcohol diet (Fig. 5). However, the MyADH transgene dramatically increased this effect. Five months of the alcohol diet produced an increase in heart-to-body weight ratio of 18% in control mice and 80% in transgenic mice. All alcohol-treated transgenic hearts were severely dilated, as is seen in human AHMD, and most alcohol-treated transgenic hearts had a large thrombus in the left atrium. This is also a common finding in human AHMD (Ferrans, 1989).

Electron microscopy revealed morphological effects of alcohol treatment that were exacerbated by the transgene. Myocardium in non-alcohol-treated control (Fig. 6A) and non-alcohol-treated transgenic (Fig. 6C) animals showed that

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**Fig. 4.** Expression of SkActin and ANF mRNAs are increased in MyADH mice treated with alcohol for 10 weeks. Levels of RNA expression are normalized to levels of GAPDH expression in each sample. Separate samples were obtained from three to six animals for each point. *p < .05 by Student’s t test, SkActin or ANF expression levels are higher in alcohol-treated ADH mice than in alcohol-treated FVB mice.

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**Fig. 5.** The ADH transgene exacerbates cardiac enlargement due to chronic alcohol. Hearts from age-matched transgenic or control FVB mice were examined after 5 months of alcohol diet or standard laboratory chow diet. *, ADH alcohol hearts are larger than FVB alcohol hearts and **, FVB alcohol hearts are larger than hearts without alcohol (p < .05 by ANOVA, n = ≥7/group).
both exhibit fine structural characteristics considered typical for heart muscle (McNutt and Fawcett, 1974). In contrast, alcohol-treated control (Fig. 6B) and alcohol-treated transgenic (Fig. 6D) animals demonstrated fine structural abnormalities that were exacerbated in the latter. Alcohol-treated control animals showed focal lesions similar to those seen in early stages of human AHMD (Ferrans, 1989); these included intracellular edema, fragmentation and loss of myofibrils, and glycogen accumulation. Focal lesions were common, and in some areas, ultrastructural changes characteristic of chronic AHMD were noted (Urbano-Marquez et al., 1989), including interstitial fibrosis, loss of myofibrils, and mitochondria with disorganized cristae.

Electron microscopic observations of the myocardium in alcohol-treated transgenic mice (Fig. 6D) showed they were the most severely affected. This degree of damage was typical of most of the myocardium in all alcohol-treated transgenic hearts. Significantly, the alterations in these animals closely mirrored those seen in chronic AHMD (Bulloch et al., 1972; Urbano-Marquez et al., 1989). In these animals, the most striking change was a loss of myofibrils and a concomitant increase in edematous sarcoplasm. Mitochondria were reduced in number and smaller and less dense than in nonalcoholic transgenic animals. In addition, loss and disorganization of contractile elements, loss of sarcoplasmic reticulum, and mitochondria swelling were evident. Interestingly, our study showed no significant change in lipid inclusions in alcohol-treated animals, which is consistent with studies of
myocardium in human chronic alcoholics (Bulloch et al., 1972).

Decreased contractility is a characteristic of human AHMD. Eighteen weeks of the alcohol diet markedly reduced contractility in both transgenic and control animals (Fig. 7). However, the residual contractility of control hearts was three times greater than the contractility of MyADH hearts. Without alcohol treatment, there was no detectable difference between control and transgenic contractility.

We considered the possibility that the cardiac damage in transgenic mice was due to depletion of NAD$^+$, an essential cosubstrate in the enzymatic conversion of ethanol to acetaldehyde. To examine this, cardiac NAD$^+$ and NADH were measured 30 min after the i.p. administration of 0.4 g of ethanol/kg b.w.t. in seven MyADH and seven control mice. This dose of alcohol was chosen because it produced levels of blood alcohol that were considerably higher than that of the chronically alcohol-treated transgenic mice. The NAD$^+$/NADH ratios were similar in transgenic and control hearts (3.89 ± 0.38 versus 3.31 ± 0.79 for transgenic and control mice, respectively; \( p > .7 \) by Student’s t test). These results do not indicate that depletion of NAD$^+$ was an adequate factor to explain the cardiac damage observed in transgenic mice.

**Discussion**

To determine the role of acetaldehyde in AHMD, we developed a new transgenic model designed to increase cardiac exposure to acetaldehyde. Cardiac ADH activity was elevated 40-fold by the MyADH transgene in line 239. The resultant increase in cardiac alcohol metabolism produced a 4-fold increase in acetaldehyde exposure. Transgene expression was confined to the heart; therefore, cardiac effects are not secondary to transgene actions in other organs. The innate stability and specificity of the transgene provide a more stable and specific method than do pharmacological approaches (Preedy and Richardson, 1994) for increasing acetaldehyde exposure.

By several criteria, it was clear that the MyADH transgene produces a very rapid, alcohol-dependent cardiomyopathy. After only 10 weeks of the alcohol diet, transgenic hearts demonstrated changes in gene expression for ANF and SkActin that are characteristic of cardiomyopathy (Colbert et al., 1997). After 18 weeks on the alcohol diet, hearts were grossly enlarged, the cardiac ultrastructure was disrupted, and contractility was reduced. The very rapid onset of cardiac damage emphasizes the potency of acetaldehyde and provides alcohol researchers with a savings of many months for the induction of full-blown AHMD.

The damage in transgenic hearts appeared to be an exacerbation of typical alcohol damage seen in control mice. In normal mice, alcohol enlarged the heart and produced focal ultrastructural damage. In transgenic animals, hearts were even larger and ultrastructural damage was more global. Also, the morphological changes seen in the alcohol-treated transgenic mice are similar to many of those seen in human AHMD (Bulloch et al., 1972; Klein and Harmjanz, 1975; Ferrans, 1989; Urbano-Marquez et al., 1989). At the gross level, these changes include enlarged hearts, dilated ventricles, and intra-atrial thrombi. At the level of ultrastructure, they include loss of contractile element-sarcoplasmic reticulum, disorganization of myofilaments/sarcromeres, sarcoplasmic edema, and swollen disrupted mitochondria. Functional damage to the heart was also more severe in transgenic mice. Consistent with previous studies, chronic alcohol significantly reduced cardiac contractility in control animals (Thomas et al., 1994; Brown et al., 1998), and this decrement was more severe in the transgenic mice. More specific contractile deficits that have been characterized in chronic alcohol studies in several species (Thomas et al., 1994) were not addressed in the present study.

These results support the hypothesized role of acetaldehyde in alcohol-induced cardiac damage. In our transgenic mice, cardiac damage was secondary to acetaldehyde exposure. Alcohol was required for the effect of the transgene. Changes in NAD$^+$/NADH ratios were not significant, and organs outside of the heart were unaffected by the transgene. Therefore, the most parsimonious explanation for cardiac damage was direct exposure to acetaldehyde. Encouraged by these data, we are producing additional transgenic mice that will reduce cardiac exposure to acetaldehyde by overexpressing acetaldehyde dehydrogenase. We predict that this new transgene will protect mouse hearts from the damaging effects of alcohol and establish the hypothesis that acetaldehyde plays a crucial role in the development of AHMD.

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**References**


