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THE NOVEL ALDEHYDE DEHYDROGENASE GENE, *ALDH5*, ENCODES AN ACTIVE ALDEHYDE DEHYDROGENASE ENZYME

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The mRNA for the novel aldehyde dehydrogenase 5 (*ALDH5*) gene was detected in HuH7 hepatoma cells. The cells also expressed cytosolic aldehyde dehydrogenase (ALDH1) mRNA, but no mitochondrial aldehyde dehydrogenase (ALDH2) mRNA. Extracts of the hepatoma cells contained an enzymatic activity with an isoelectric point similar to that of ALDH1. This enzyme activity was insensitive to inhibition by disulfiram, a potent inhibitor of ALDH1. The enzyme was active with short chain aldehydes (acetaldehyde and propionaldehyde) and NAD^+, but not with $NADP^+$, and the activity was higher in the mitochondrial pellet than other cell fractions. These studies demonstrate the expression of *ALDH5* mRNA in a human hepatoma and suggest that the gene product is enzymatically active and probably resides in the mitochondria.

A large number of aldehydes are encountered in normal metabolic pathways and from exposure to the environment. Some of these aldehydes are metabolized by fairly specific enzymes; others are handled by a family of broad specificity aldehyde dehydrogenases (ALDH , E.C.1.2.3.1) (1). These latter enzymes share common sequence motifs and catalytic mechanisms. All are multimeric enzymes that utilize NAD^+ (or in some cases $NADP^+$) (2,3). These enzymes are of biomedical interest because of their participation in several metabolic pathways, including metabolism of certain aldehyde intermediates of drug metabolism (e.g., cyclophosphamide) as well as acetaldehyde generated from ethanol. The concentration of acetaldehyde in the bloodstream is near the limit of detection during ethanol oxidation, and the concentration in the liver cell itself is no more than 100 μM (4,5). The maintenance of this

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Abbreviations used: ALDH1, cytosolic aldehyde dehydrogenase; ALDH2, mitochondrial aldehyde dehydrogenase; ALDHE3, E3 isozyme of aldehyde dehydrogenase; ALDH5, aldehyde dehydrogenase 5; mRNA, messenger RNA; cDNA, complementary DNA; bp, base pairs; kb, kilobase pairs.

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low level appears to be necessary to reduce the chances of formation of acetaldehyde adducts with other cellular components, and argues for the need for highly efficient enzyme systems for the removal of this compound.

It has been suggested that three different enzymes could participate in the oxidation of acetaldehyde generated from ethanol: cytosolic ALDH1 (6), cytosolic ALDHE3 (7), and mitochondrial ALDH2 (6). These enzymes are tetramers with 54 kD subunits, and have quite low K_ms for acetaldehyde (about 30 μM for ALDH1 and ALDHE3, and < 1 μM for ALDH2). cDNAs and genes for these enzymes have been cloned (8-10). The enzymes show an amino acid identity of about 65% between ALDH1 and ALDH2, and about 40% between ALDHE3 and ALDH1 or ALDH2 (10). Additional ALDHs have been cloned by screening libraries at reduced stringency. One interesting example is *ALDH$_x$*, now designated *ALDH5*. It has 72% identity to ALDH2 and its deduced N-terminal sequence resembles the amphipathic helix of a mitochondrial leader peptide. It differs from all known ALDHs in that it is encoded by an intron-less gene. This suggested the possibility that it was in fact a processed retro-pseudogene (11); however, mRNA corresponding to this gene was detected in the poly-A^+ mRNA of liver, testis, and brain (11). To date, no evidence has been presented to show that this mRNA encodes an enzymatically active protein, and if so, what the characteristics of the enzyme are. We report the preliminary characterization of ALDH5 enzyme activity in a human hepatoma cell line.

MATERIALS AND METHODS

Cell lines and culture conditions. HuH7 cells (12) were a gift from Dr. Chao-Hung Lee, Department of Pathology, Indiana University School of Medicine. The cells were grown in Dulbecco's minimal essential medium supplemented with 5% fetal bovine serum. For all activity assays, the cells were split 1:4 and grown for 2 days before harvest when approximately confluent. The cells were harvested for enzyme analyses by washing the monolayer with phosphate buffered saline, then scraping the cells and centrifuging them in a bench top centrifuge. The cell pellet was made 10 mM in dithiothreitol (except for the studies on disulfiram sensitivity) and the cells were disrupted by sonication at 0° C. A supernatant was prepared by centrifuging the sonicated cell extract at 14,000 x g for 10 minutes. Cell fractionation studies were carried out using the method of Koivusola (13). The cells were scraped in 0.25 M sucrose buffered with 10 mM sodium phosphate, pH 7.4, containing 2 mM dithiothreitol. The cells were then disrupted with a Dounce homogenizer and centrifuged at 700 x g for 5 min. This pellet contained undisrupted cells and nuclei. The supernatant was then centrifuged for 10 min at 4500 x g and the mitochondrial pellet recovered. The supernatant was centrifuged for 1 h at 100,000 x g and the microsomal pellet and cytosolic supernatant were recovered. The particulate fractions were disrupted by sonication before enzyme assay.

RNA preparation and analysis. Cellular total RNA was isolated by dissolving the cells in guanidinium isothiocyanate following the acid-phenol extraction protocol (14). Twenty μg of

total RNA was loaded on a 1% agarose gel containing 1.8 M formaldehyde and electrophoresed as described (15). The RNA was transferred to nitrocellulose by capillary blotting. The filters were probed with radiolabelled probes corresponding to the human liver ALDH2 cDNA (which we cloned using the rat cDNA as the probe (16)), the 3' untranslated region of human ALDH1 (9), and the cloned human ALDH5 gene (11). The latter two probes were generated by polymerase chain reaction of human genomic DNA using primers based upon published sequences, and their identity was confirmed by restriction mapping and partial sequencing. Hybridization was carried out in 50% formamide-containing buffers at 37° or 42° and the final wash of the blot was with 0.1 x SSC (1 x SSC = sodium chloride 150 mM, sodium citrate 15 mM, pH 7.4), 0.1% SDS at 55° for 20-30 min.

Protein analyses. ALDH activity was determined in buffer containing 50 mM sodium pyrophosphate, pH 8.8, 0.1 mM 4-methylpyrazole, 1 mM EDTA, and 2.5 mM NAD^+. The rate of formation of NADH was followed by measuring absorbance at 340 nm. After determining a blank rate, the reaction was initiated by adding propionaldehyde at 3 mM. Proteins fractionated in SDS-PAGE gels or by isoelectric focusing were transferred to nitrocellulose filters by electroelution or capillary blotting, respectively. The filters were blocked with PBS containing 0.1% Triton X-100 and 5% BSA, then incubated with polyclonal antiserum raised against purified human liver ALDH2 (17). After washing, the filters were developed by incubating them with [^{125}-I]-labelled protein A. Isoelectric focusing was performed using 1% agarose gels containing 0.5 ml of pH 3-10 Ampholines, 1.5 ml of pH 4-6 Ampholines, and 20% glycerol in a total volume of 34 ml. The anode and cathode buffers were 0.5 M acetic acid and 0.5 M NaOH, respectively. The gel was prefocused at 5 W for 1 h, then the protein samples were applied and the focusing was continued at 10 W for 2 h. The gel was then stained for enzyme activity in a solution of 10 mM propionaldehyde, 1 mM NAD^+, 0.1 mM 4-methylpyrazole, 0.3 mM 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, and 0.14 mM phenazine methosulfate in 50 mM pyrophosphate, pH 8.8.

RESULTS AND DISCUSSION

The aldehyde dehydrogenase gene family is characterized at the sequence level by certain conserved motifs, especially that surrounding the active site cysteine (18). This has permitted cloning of new members of the family, such as *ALDH5,* based upon screening libraries with oligonucleotides corresponding to these motifs (11). This interesting gene has high sequence homology with ALDH1 and ALDH2, suggesting that the gene product would be a broad specificity, NAD^+-dependent aldehyde dehydrogenase. The gene is actively transcribed in liver and testis (11), as well as kidney, skeletal muscle, and fetal tissues (MJ Stewart, unpublished results).

Expression of ALDH mRNA and protein in hepatoma cells

In a survey of hepatoma cell lines, we found that HuH7 and PLC/PRF/5 cells expressed a 3.1 kb mRNA that hybridized with a rat ALDH2 cDNA probe, but was larger than that expected for ALDH2 (2.1 kb, (9)). The rat hepatoma cell lines H4IIE and H4IIE-C3 cells expressed RNA of the expected size for ALDH2 mRNA (Figure 1). The cell extracts were then Western blotted with antiserum raised against purified human liver ALDH2 (17) to

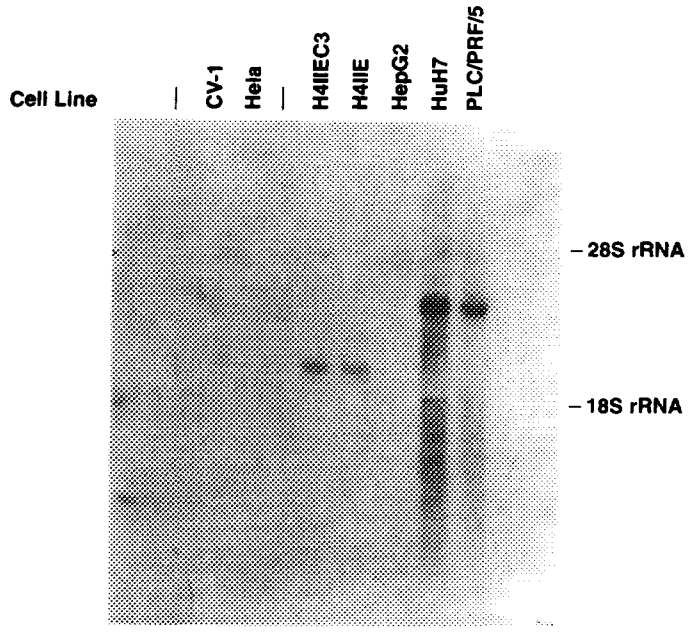

Figure 1. Northern blots of RNA isolated from various cell lines.
20 μg of total RNA was electrophoresed and Northern blotted. The filter was hybridized in 50% formamide buffer with a radiolabelled rat *ALDH2* cDNA at 37°. The location of the 28S and 18S rRNA bands are shown. HeLa and CV-1 cells had no ALDH2 mRNA, while H4IIEC3 and H4IIE cells contain ALDH2 mRNA of the expected size. HuH7 and PLC/PRF/5 cells contained an approximately 3.1 kb RNA that cross-hybridized with this probe.

determine the levels of ALDH2 protein (Figure 2). The rat hepatoma cells expressed high levels of immunoreactive protein; however, the human hepatomas expressing the 3.1 kb mRNA expressed low levels of immunoreactive protein that appeared to be a doublet of two similar sized proteins. The disparity between the abundance of the HuH7 ALDH mRNA and the protein level would be consistent with the protein being related but not identical to ALDH2. The identity of the smaller immunoreactive protein in HeLa cells is not known. We then probed HuH7 RNA for ALDH1 and ALDH5 mRNAs. A probe for ALDH1 was generated by polymerase chain reaction to correspond to the 3' untranslated region, which does not share sequence homology with ALDH2 or ALDH5. Both the 2.1 kb *ALDH1* mRNA (9) and 3.1 kb *ALDH5* mRNA (11) were detected in the HuH7 cells (Figure 3). Thus the two proteins detected on the Western blot are likely to represent ALDH1 and ALDH5 proteins cross-reacting with the antiserum.

Properties of the ALDH expressed in HuH7 cells

The ALDH activity in HuH7 cells was 3.9 ± 0.2 nmol/min/mg protein (n=6 plates of cells) using 3 mM propionaldehyde and NAD^+ as substrates. The ALDH activity was

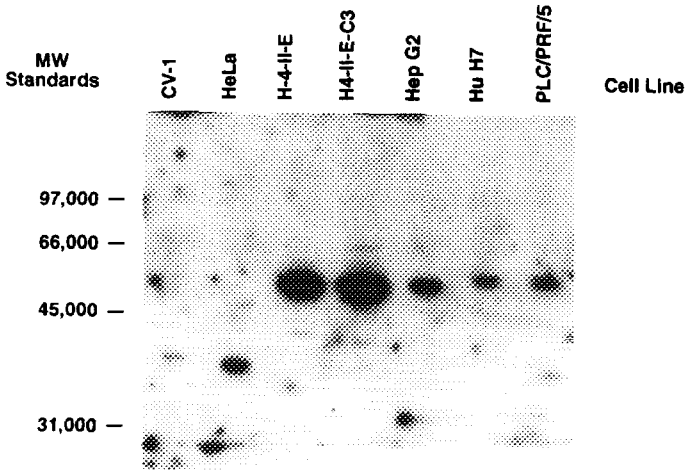

Figure 2. Western blot analysis of extracts from various cell lines for aldehyde dehydrogenase protein.

Extracts from the cell lines analyzed in Figure 1 were subjected to SDS-PAGE and Western blotted to nitrocellulose filters. The filters were then probed with polyclonal antiserum raised against human ALDH2 (17), followed by washing and incubation with ^{125}I - labelled protein A and autoradiography.

further characterized by isoelectric focusing on wide pH range gels (pH 3-10). A band of activity was seen in the mid-range of this gradient (not shown). The activity was exhibited with propionaldehyde or acetaldehyde and NAD^+ as substrates. When parallel gels were stained with $NADP^+$ in place of NAD^+, no activity was observed. This nucleotide cofactor preference of the HuH7 ALDH is similar to that for the class 1 and 2 ALDHs, although ALDH1 has been reported to be active with $NADP^+$ with a K_m of 1.5 mM (19). Narrower range gradients were then used to determine the relationship between the HuH7 ALDH and liver ALDHs (Figure 4). HuH7 ALDH migrated cathodal to ALDH2 at virtually the same pI as ALDH1. Since the electrophoretic behavior of the enzyme did not permit differentiation between ALDH1 and the putative ALDH5 enzymes, the sensitivity of the enzyme to disulfiram was also examined. ALDH1 is inhibited over 90% by exposure to 10 μM disufiram *in vitro* (20), with a reported K_i of 0.2 μM (6), whereas other ALDHs are less sensitive (20). The extracts of HuH7 cells or autopsy human liver samples were incubated at room temperature for 10 minutes with vehicle alone (dimethylsulfoxide), 10 or 50 μM disulfiram, then loaded on the isoelectric focusing gel (Figure 4). The intensity of the ALDH1 enzyme activity band in the liver samples was progressively reduced by increasing concentrations of disulfiram, while the activity at the same pI in HuH7 cells was insensitive to inhibition by these concentrations. The intensity of staining of the more anodal ALDH2 or the cathodal ALDH3 bands were similar in each lane containing human liver extract, indicating equal

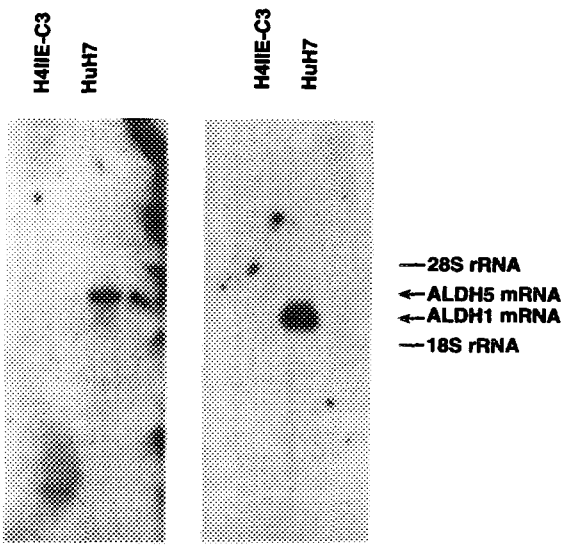

Figure 3. Northern blot analysis of RNA from H4IIEC3 and HuH7 cells.
20 μg of total RNA from the noted cell lines was electrophoresed and blotted to nitrocellulose filters. The filter on the left was probed with radiolabelled *ALDH5* gene, and the filter on the right was probed with the radiolabelled 3' untranslated region of the human *ALDH1* cDNA in 50% formamide buffers at 42°. The locations of the 28S and 18S rRNAs are shown in the margin.

loading of the extracts. Although the Western and Northern blots indicated that ALDH1 was likely to be expressed in the cells, the isoelectric focusing gel suggested that the majority of the enzyme activity in HuH7 cells corresponded to ALDH5. This could result from ALDH5 having a high specific activity relative to ALDH1. The residual enzyme activity at the pI of ALDH1 in the human sample may represent uninhibited ALDH1 or, alternatively, ALDH5.

To further characterize the ALDH5 enzyme activity, crude extracts of the cells were used to estimate the Michaelis constant for propionaldehyde. The K_m was approximately 30 μM on several determinations using different cell extracts. This must be accepted as a very preliminary estimate. The ALDH5 sequence contains an N-terminal extension that could represent a mitochondrial leader sequence; we therefore fractionated HuH7 cells by differential centrifugation and assayed the activity of the ALDH in each fraction. Because of the effect of diluting the cells in the the fractionation buffer, we cannot comment upon activity in the cytosolic fraction. There was variability in the activity as expressed as nmol/min/mg cell protein; however, the activity was found almost exclusively in the nuclear and mitochondrial pellets. The nuclear fraction also would contain undisrupted cells, which might account for the activity in that fraction. The ratio of activity in the mitochondrial pellet to that in the nuclear pellet was 2.5 ± 0.6 (n=3), suggesting that the HuH7 ALDH was mainly contained

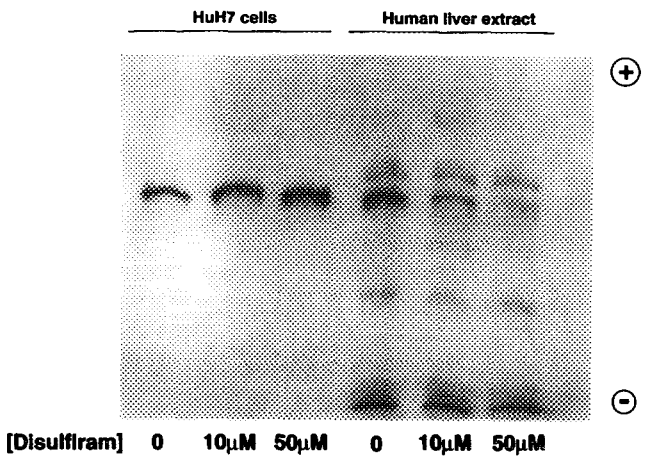

Figure 4. Electrophoretic mobility and sensitivity to disulfiram of aldehyde dehydrogenases present in extracts of HuH7 cells and human liver.

Cell extracts were incubated with the noted concentrations of disulfiram or vehicle (dimethylsulfoxide) for 10 minutes at room temperature before loading on an agarose isoelectric focusing gel. After focusing, the gel was stained for aldehyde dehydrogenase activity. The locations of the anode and cathode are marked by + and -, respectively. The bands of activity in the liver extracts correspond to ALDH2, ALDH1, and ALDH3 moving from acidic (anodal) to basic (cathodal) isoelectric points. The HuH7 aldehyde dehydrogenase migrated to the same isoelectric point as the human ALDH1 enzyme but was insensitive to disulfiram.

in mitochondria or other organelles co-sedimenting with them. Because of the small amount of starting material, we were not able to measure marker enzymes in the fractions to estimate their purity, so this conclusion is tentative. These experimental findings are in good agreement with predictions based on the amino acid sequence deduced from the gene.

It might be asked why this enzyme activity has not previously been identified in human tissues. We presume that it has been misidentified because its isoelectric point coincides with that of ALDH1. One laboratory attempting to purify the inactive ALDH2 from an ALDH deficient human liver sample purified an enzyme that bound to AMP-sepharose and had the same isoelectric point as ALDH1 (21). It is now known that the mutant ALDH2 enzyme does not bind to this chromatographic medium because of a markedly decreased affinity for NAD^+ (22), suggesting that ALDH5 may have been unintentionally purified by these investigators. A group studying ALDH in cornea found an enzyme activity at the position of ALDH1 on isoelectric focusing, and identified a partial sequence of ALDH5 by reverse transcription-polymerase chain reaction (23). Thus, several lines of evidence lead to the conclusion that ALDH5 is indeed expressed as an active enzyme. This second mitochondrial ALDH may play a role in the disposal of ethanol-generated acetaldehyde and it will be interesting to examine associations of this enzyme activity with alcoholism and its complications.

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