

# Class I Alcohol Dehydrogenase Is Highly Expressed in Normal Human Mammary Epithelium but not in Invasive Breast Cancer: Implications for Breast Carcinogenesis<sup>1</sup>

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## ABSTRACT

Detoxification of ethanol can contribute to oxidative cellular and DNA damage and, thereby, to carcinogenesis. The potential relevance of this to breast carcinogenesis is suggested by evidence that alcohol consumption is a risk factor for breast cancer. It is, however, not known whether ethanol can be metabolized in breast parenchyma. The goal of this study was to determine whether class I and/or IV alcohol dehydrogenase (ADH), medium chain ADHs that can catalyze oxidation of ethanol, are expressed in human breast parenchyma. Normal and neoplastic human breast tissue specimens were examined for class I and IV ADH mRNA by reverse transcription-PCR, for protein by immunocytochemistry and Western analysis, and for their potential to catalyze NAD<sup>+</sup>-dependent oxidation of ethanol. Together, the findings provide evidence that: (a) class I ADH is the medium-chain ADH that is expressed in human breast parenchyma, specifically in the mammary epithelium; (b) human breast parenchyma can support ADH-mediated oxidation of ethanol; and (c) the expression of class I ADH is dramatically reduced or abrogated in invasive breast cancers. Expression of class I ADH in normal human breast parenchyma was confirmed by probing a multiple human tissue polyA<sup>+</sup>RNA. The unexpected finding of virtual abrogation of expression of class I ADH in invasive breast cancer suggests that the enzyme has some "tumor suppressor" function in the mammary epithelium. The one property of class I ADH fitting this designation is its potential to catalyze the oxidation of the micronutrient/prohormone retinol to retinal, the first step in the biosynthesis of retinoic acid, the principal known mediator of the actions of retinoids important for maintaining epithelia in a differentiated state.

## INTRODUCTION

Consumption of alcohol has been identified as a risk factor for breast cancer in epidemiological studies (1). Analysis of pooled data from six prospective large cohort studies suggests a linear relationship between breast cancer risk and consumption of EtOH<sup>3</sup> in alcoholic beverages over the range reported by most women (<60 g of EtOH/day) with increments of 10 g of EtOH/day (0.75 l g/drink) being associated with a 9% increase in breast cancer risk (2). A link between EtOH and breast carcinogenesis has been identified also in animal studies (1). The majority of studies of mechanisms underlying this phenomenon have focused on systemic effects of EtOH, such as effects on hormone levels and nutrition (1). The question whether and how *in situ* metabolism of EtOH could contribute to breast carcinogenesis has been addressed in a single publication and only at a

theoretical level (3). Yet oxidation of EtOH, the first step in its detoxification, is a known potential contributor to a pro-oxidant state and, thereby, to carcinogenesis (4). Specifically, the product of the reaction, acetaldehyde, is a reactive electrophile implicated as a co-carcinogen, and its detoxification can result in the generation of potentially mutagenic reactive oxygen species and drain cellular antioxidant defenses (4, 5). There is also growing evidence implicating a state of chronic oxidative stress in breast parenchyma in the high incidence of breast cancer in countries of the industrialized West, a state to which EtOH metabolism could contribute (6-9).

We reasoned that because of the potential harmful effects of EtOH on the offspring, the mammary epithelium would be equipped with enzymes needed to detoxify EtOH. Therefore, this study was initiated to determine whether class I and/or class IV ADHs, two members of the medium chain ADH/reductase superfamily that can metabolize EtOH, are expressed in normal and neoplastic human breast parenchyma.

Class I and IV ADH have been studied extensively because of their importance in the detoxification of exogenous EtOH in the gastrointestinal tract and liver, and their role in the cellular oxidative stress and oxidative DNA damage associated with alcoholism. In humans, class I ADH is composed of three genes (*ADH1A*, *ADH1B*, and *ADH1C*), whereas class IV ADH is represented by a single gene (*ADH4*; Refs. 10-12). The three class I ADH proteins are 96% homologous. They function as homo- or heterodimers of the three polypeptide gene products (designated  $\alpha$ ,  $\beta$ , and  $\gamma$ , or ADH1A, ADH1B, and ADH1C). Although differences in the amino acid composition of the isoenzymes are small, they are associated with marked differences in their affinity for their substrates and in the efficiency of the reactions they catalyze, including oxidation of EtOH (13, 14). Class IV ADH is 69% homologous with class I ADH isoforms and functions as a homodimer of the single gene product ( $\sigma$  or ADH4A). Here, we present molecular biological, immunochemical, and biochemical evidence that class I ADH is the ADH with a potential to metabolize EtOH that is expressed in the normal human mammary epithelium, and that its expression is suppressed in invasive breast cancers. The latter unexpected finding suggests some additional function(s) for class I ADH in the mammary epithelium, one that is linked to "tumor suppression." Of the many reactions that class I ADH has the potential to catalyze, the one that fits this designation is the oxidation of retinol to retinal, the first step in the biosynthesis of RA, the retinoid that plays an essential role in the maintenance of epithelia in a differentiated state.

## MATERIALS AND METHODS

### Tissue

Normal and neoplastic breast parenchyma were obtained from a tissue bank maintained in the laboratory of one of the investigators (J. W.; Ref. 15). Tissues, obtained from fresh surgical specimens from patients with simple macromastia (normal) and breast cancer, were processed by, or under the

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<sup>3</sup>The abbreviations used are: ADH, alcohol dehydrogenase; EtOH, ethanol; RA, retinoic acid; 4-MP, 4-methylpyrazole; RT-PCR, reverse transcription-PCR.

supervision of one of the investigators (J. W.). The specimens were freed from excess fat cells by blunt dissection and by trimming with surgical scissors. Some portions were snap-frozen at  $-80^{\circ}\text{C}$  and stored at  $-80^{\circ}\text{C}$ , whereas others were fixed in 10% neutral buffered formalin for 12–24 h and then embedded in paraffin. Particular care was taken to minimize contamination with fat the specimens that were snap-frozen for future use in molecular biological or biochemical studies. The age of the patients with macromastia ranged from 17 to 51 years, and of those with breast cancer, from 35 to 59 years. Four of the patients with breast cancer were postmenopausal and one was at 33 weeks of gestation. All of the cancers used in this study were at least 1.5 cm in size and were diagnosed as invasive. Authorization for the use of these tissues for research was obtained from the Institutional Review Board.

#### Analysis of Normal and Neoplastic Breast Tissue for Class I and IV ADH mRNA

**Preparation of RNA.** Total RNA was obtained from snap-frozen breast tissue using Trizol Reagent (Life Technologies, Inc., Grand Island, NY) according to manufacturer's instructions. Briefly, breast tissue was kept on dry-ice until the addition of Trizol reagent (1 ml/100 mg tissue) and then was immediately homogenized (Polytron, Brinkman). The tissue homogenate was centrifuged at  $12,000 \times g$  for 10 min at  $5^{\circ}\text{C}$  and any fat collected at the surface was removed. The Trizol layer was transferred to a new tube, chloroform was added, and the phases were separated by centrifugation. The RNA in the aqueous phase was precipitated using isopropyl alcohol and resuspended in diethylpyrocarbonate-treated water.

**RT-PCR.** One  $\mu\text{g}$  of total RNA from normal and neoplastic breast tissue was subjected to reverse transcription at  $42^{\circ}\text{C}$  for 1.5 h using M-MLV reverse transcriptase (Promega, Madison, WI) and oligo(dT) primer. PCR was performed using one-tenth of the reverse transcription product, 1.25 units of Gold Taq, and primers specific for either ADH class I or ADH class IV and the following cycling conditions:  $94^{\circ}\text{C}$  for 10 min, then 35 cycles of 30 s at  $94^{\circ}\text{C}$  denaturing, 30 s at  $60^{\circ}\text{C}$  annealing, and 1 min at  $72^{\circ}\text{C}$  elongation. The primer sequences used were ATCCACACCTCCATCAGTCATTTCC and AGAATTGTAAAAACCCGGAGAGCAACTAC for class I ADH and ATGCTTGTCGCAACCCAGATGGCA and ATCATGGTTTCAAGATGCCCAATA for class IV ADH. One fifth of the PCR product was electrophoresed on a 2% agarose gel. RNA from human liver was used as a positive control for class I ADH and from human stomach and skin as positive controls for class IV ADH. Samples that had not been subjected to reverse transcription were used as negative control.

**Multiple Tissue Expression Array.** Northern analysis of total RNA from normal and neoplastic human breast parenchyma, using standard methodology and the probes specific for class I and class IV ADH, yielded only a faint band in preparations from normal tissue and only for class I ADH (data not shown). Because a high proportion of human breast parenchyma is composed of extracellular matrix (generally  $>90\%$ ; see Ref. 15), it is a tissue from which it is difficult to obtain polyA<sup>+</sup> RNA in the quantities required for Northern analysis. Therefore, we used a commercially available Multiple Tissue Expression Array (Clontech, Palo Alto, CA) to analyze the relative expression of class I and IV ADH mRNA in normal human breast tissue. The array contained polyA<sup>+</sup> RNA from 68 normal adult and fetal human tissues, each of which was normalized to RNA expression levels of eight housekeeping genes (16). The membranes were probed, according to the manufacturer's instructions, with two probes specific for either class I ADHs (5'-ATGCATTCAGTGGCACCCAACTCTT-3' and 5'-ATTGATTCTGTCTTCTCTGCAGACC-3') or class IV ADH (5'-ACTCCACGACCAGTAATATCGCTCC-3' and 5'-ATCATGTTTCAAGATGCCCAATA-3'), labeled at the 5' end with  $^{32}\text{P}$  according to Woods (17). Expression array data were analyzed using Intelligent Quotient software (Genomic Solutions, Ann Arbor, MI). Briefly, the volume of expression for a given tissue was determined by multiplying the measured area of the tissue dot on the array by the pixel density within the digitized dot and then was expressed as a percentage of the total array volume.

#### Immunocytochemical Analysis of Normal and Neoplastic Breast Tissue for Class I and IV ADH Protein

**Antibodies.** The antibody used for immunocytochemistry was raised in rabbits against ADH purified from adult human liver tissue by 4-(3-aminopropyl) pyrazole-affinity chromatography according to Miller *et al.* (18). Analysis

of crude human liver extracts by immunoblot demonstrated that the polyclonal antibody recognized a single  $M_r$  40,000 band corresponding in size to purified class I ADH and class IV ADH (data not shown). Because this antibody was available only in a limited amount, the confirmation of immunocytochemical findings by immunoblot analysis was carried out using a second polyclonal antibody. This second antibody, was obtained from W. F. Bosron (Indiana University School of Medicine, Indianapolis, IN). It was developed in rabbits against  $\beta_2\beta_3$  expressed protein (ADH1B3, according to current nomenclature; Ref. 12) and was found to be suitable for immunoblot analysis but not for immunocytochemistry. This antibody has been shown to cross-react with both class I and class IV ADH.<sup>4</sup> Therefore, findings obtained using either antibody will be referred to as class I/IV ADH.

**Immunocytochemistry.** Immunocytochemistry was carried out on tissue from 12 patients with macromastia and 9 patients with breast cancer. Sections ( $5 \mu\text{m}$ ), cut from tissue that had been fixed in neutral buffered formalin and embedded in paraffin, were placed on ProbeOn<sup>1</sup> slides (Fisher Biotech, Atlanta, GA), dewaxed, and rehydrated in decreasing concentrations of EtOH. Endogenous alkaline phosphatase was inhibited by incubating the sections in 0.2 N HCl for 5 min. Nonspecific binding was blocked by incubating sections for 20 min at room temperature in 5% normal goat serum and 10% fetal bovine serum in PBS containing 0.3% Triton X-100. This was followed by incubating the section overnight with the primary antibody in a humid chamber at  $4^{\circ}\text{C}$  and then with biotin-conjugated goat-antirabbit secondary antibody (Promega) at 1:1000 dilution in PBS for 1 h at room temperature. The signal was amplified using Vectastain ABC reagent with alkaline phosphatase serving as the reporter and Vector Red as the chromogenic substrate (Vector, Burlingame, CA). The reaction was arrested by immersing the slides in distilled water. The sections were then dehydrated in EtOH, cleared in xylene, and mounted with Permount (Fisher Biotech). Sections were examined and recorded using an Olympus BX60 microscope (Lake Success, NY) with a SPOT, Jr. digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) and Image Pro Plus version 3.0 software, or a Nikon Digital Camera DXM 1200 and Nikon ACT1 software (version 2). All of the sections were reviewed by two of the investigators (E. A. T. and J. W.) independently and together. Tissue sections from breast cancer cases were reviewed with a consulting pathologist (E. F.).

**Immunoblot Analysis.** Frozen tissue from eight macromastia patients and nine cancer patients was pulverized or minced and then homogenized in 25 mM HEPES (pH 7.4), 0.5 mM EDTA, 5 mM  $\text{MgCl}_2$ , and 10% glycerol containing 0.6  $\mu\text{M}$  leupeptin, 2.0  $\mu\text{M}$  pepstatin, 2 mM 1,10 *O*-phenanthroline, and 1 mM phenylmethylsulfonyl fluoride, 1 mM *N*- $\alpha$ -*p*-tosyl-L-lysine chloromethyl ketone (Sigma Chemical Co., St. Louis, MO) at  $5^{\circ}\text{C}$ . Homogenates were centrifuged at  $12,000 \times g$  for 30 min at  $5^{\circ}\text{C}$ . The concentration of protein in the supernatants was determined according to Bradford, using BSA as standard (Bio-Rad Laboratories, Inc., Hercules, CA; Ref. 19). Proteins (3  $\mu\text{g}$ ) were resolved using 8.0% SDS-PAGE and then were transferred to Hybond-P membrane (Amersham Pharmacia Biotech, Arlington Heights, IL). Antibody was used at a 1:1000 dilution in a standard immunoblot procedure (20) and was detected using enhanced chemiluminescence (Amersham Pharmacia Biotech, Arlington Heights, IL).

#### Analysis of Normal and Neoplastic Human Breast Tissue for NAD-dependent EtOH Dehydrogenase Activity: Enzyme Assays

Homogenates from normal breast parenchyma ( $n = 23$ ) and breast cancer tissue ( $n = 10$ ) were prepared as for immunoblot analyses (see above). Enzyme activity was measured by monitoring the rate of generation of NADH in the course of oxidation of EtOH. Initial comparisons of enzyme activity of normal and neoplastic breast tissue were carried out in a reaction mixture containing 2.4 mM NAD<sup>+</sup>, 33 mM EtOH, 1 mM  $\text{ZnCl}_2$ , and 100  $\mu\text{l}$  of tissue homogenate in HEPES buffer (pH 7.4) in a total volume of 1 ml in the absence or presence of 4-MP, an inhibitor of oxidation of EtOH by class I and IV ADH (21, 22). The reaction was initiated by adding NAD to the samples. The generation of NADH was monitored at 340 nm in a Perkin-Elmer Lambda 4B dual beam spectrophotometer (Norwalk, CN) for 10 min at  $25^{\circ}\text{C}$  against a reaction mixture containing no substrate, using a molar absorptivity of  $6220 \text{ cm}^{-1}$ . Except for the inclusion of  $\text{ZnCl}_2$ , these incubation conditions are ones in routine use for measuring ADH-mediated EtOH-dehydrogenase activity in

<sup>1</sup> W. F. Bosron, personal communication.

liver, purified or expressed enzyme. The rationale for including  $ZnCl_2$  in the incubation medium and in experiments carried out to confirm its role as a source of halide ion needed to raise the level of enzyme activity to detectable levels (23), are described under "Results."

### Statistical Analysis

Statistical significance of differences in NAD-dependent oxidation of EtOH by homogenates of normal breast parenchyma incubated in medium containing 1 mM  $ZnCl_2$  and a different concentration of NaCl was tested for by one-way ANOVA. Statistical significance of differences between EtOH dehydrogenase activity of normal human breast parenchyma incubated in the presence of 1 and 2 mM NaCl was tested by the Student *t* test.

## RESULTS

**RT-PCR.** By RT-PCR, transcripts of class I ADH were identified in 9 of 10 specimens of total RNA prepared from histologically normal tissue from mammoplasty (normal) and mastectomy (normal from cancer; Fig. 1). Under the same conditions, class I ADH transcripts were identified in two of five specimens of RNA prepared from breast cancers. The sequence of the amplicon was shown to correspond to the expected sequence for class I ADH. In contrast, no transcripts of class IV ADH were identified in preparations of either normal or neoplastic breast tissue. Under the same conditions, a strong band was identified for class IV ADH in RNA from human stomach, a tissue with a known high level of expression of class IV ADH (24), and a weak band in RNA from skin, a tissue reported to express class IV ADH (25).

**Multiple Tissue Expression Array.** Class I ADH was readily detected in polyA<sup>+</sup>RNA from normal human mammary tissue in the multiple tissue expression array (Table 1). After 2 h, the average volume of expression of class I ADH in mammary tissue was 1.65% of total array expression as compared with 13.75% for liver, a tissue known to express class I ADH at a very high level, and similar to that from descending colon, pancreas, and lung, three extrahepatic tissues known to express class I ADH (26, 27). In contrast, class IV ADH expression was undetectable after 2 h exposure of the membrane to the probes, an exposure time that was sufficient to detect class IV ADH in stomach and esophagus. With exposure times increased to 6 h, class IV ADH became detectable and represented 0.81% of total array expression as compared with 4.45% for stomach and 3.85% for esophagus, two tissues with known high levels of class IV ADH expression (24). The significance of these low levels of expression of

Table 1. Relative expression of class I ADH and class IV ADH on a multiple-tissue expression array

A commercial array of polyA<sup>+</sup>RNA from 68 normal adult and fetal human tissues (Clontech), was probed with <sup>32</sup>P-labeled oligonucleotides specific for either class I or class IV ADH, according to the manufacturer's directions. No hybridization of either probe was detected with negative controls for nonspecific binding or binding to repetitive or oligo(dT) sequences. Expression array data were analyzed using Intelligent Quotient software (Genomic Solutions) as follows. The relative expression level of class I ADH or IV ADH was determined by multiplying the measured area of the tissue dots generated by the array by pixel density to determine the volume of each tissue dot. Individual tissue volumes were then divided by the total volume for all tissues and were expressed as percentages of the total volume. Thus, the results from class I ADH and IV ADH are relative to the other values for that probe, and the values for the two probes cannot be directly compared. The blots were exposed for varying lengths of time to determine the linear range of detection for each probe. The values presented are at 1 and 2 h of exposure for class I ADH and at 2 h and 6 h of exposure for class IV ADH. The 1- and 2-h exposures of class I ADH are in the linear range. The 2-h exposure of class IV ADH is below the linear exposure time for this probe and cannot be directly compared with the 6-h time exposure, which was in the linear exposure range based on values obtained after 12- and 24-h exposure.

Tissue	Class I ADH		Class IV ADH	
	% relative expression		% relative expression	
	1 h	2 h	2 h	6 h
Mammary	1.62	1.65	ND*	0.81
Liver	13.83	13.75	ND	1.75
Descending colon	1.49	1.54	ND	1.07
Pancreas	1.99	1.94	ND	1.87
Lung	1.38	1.38	ND	0.98
Stomach	0.42	0.47	37.60	4.45
Esophagus	0.38	0.37	31.83	3.85

\*ND, not detectable.

class IV ADH in breast tissue could have functional significance if they reflect expression of class IV ADH in some specific cell type(s), such as in blood vessels. Both class I and class IV have been identified by others in human blood vessels (28, 29). However, the findings clearly indicate the predominance of class I ADH in breast tissue.

**Immunocytochemistry.** Fibrocystic changes were commonly seen in many tissue sections, whether obtained from patients undergoing mammoplasty or from sites distant to the cancers in mastectomy specimens. Such fibrocystic changes are considered benign, and their presence in a high percentage of specimens is the norm, at least in tissue obtained from women in the industrialized West (see Ref. 30).

In normal tissue, luminal and basal epithelial cells lining the mammary ductal system were immunopositive for ADH, including cells that lined dilated cysts and ducts (Fig. 2). There were no systematic differences in the intensity of immunostaining as a function of the type of duct or lobule (type I, type II, and type III lobule), nor of the age or parity of the patient. Immunostaining of the distinct myoepithelium of larger interlobular ducts and of intra- and interlobular stromal cells was minimal (Fig. 2). In blood vessels, the second immunoreactive tissue compartment, immunostaining was especially prominent in larger blood vessels. This finding confirms reports by others of expression of class I and class IV ADH in blood vessels (28, 29).

In all of the tissue sections from cancers, immunoreactivity overall was markedly reduced as compared with normal mammary epithelium (Fig. 3). The reduction of immunostaining was especially striking in invasive components of the cancers (Fig. 3, D, E, F, and H). The heterogeneity in histology characteristic of breast cancers was paralleled by heterogeneity of immunostaining: an occasional immunopositive histologically normal ductal element was seen in the midst of immunonegative cancer cells and some clusters of immunonegative cancer cells were seen the midst of more immunopositive ones (Fig. 3, A-C'). In sections from one of the nine patients, there were larger foci of cancer cells with features suggesting *in situ* cancers. In these lesions, immunoreactivity approached that of normal mammary epithelium (Fig. 3C'). Immunoreactivity associated with blood vessels

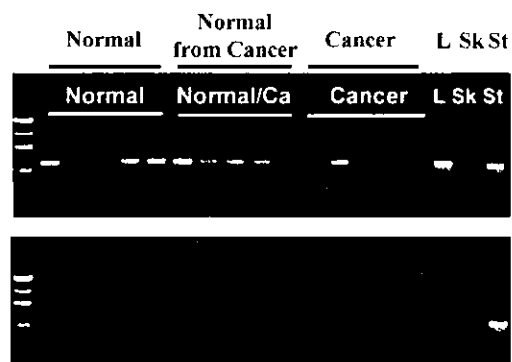
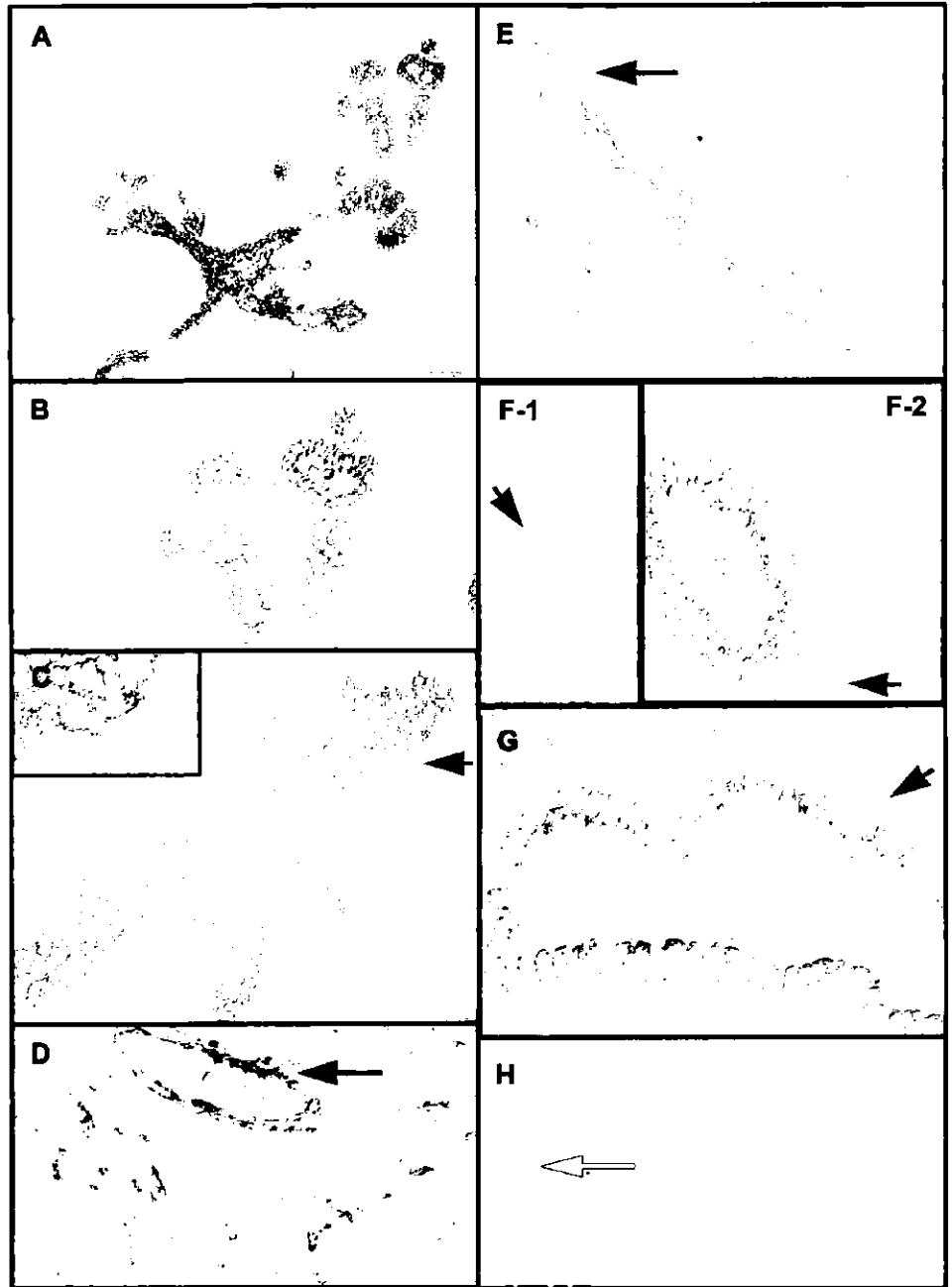


Fig. 1. Nonquantitative RT-PCR for total RNA from normal and neoplastic breast tissue for transcripts of class I and class IV ADH. RNA was prepared from tissues obtained at reduction mammoplasty (Normal; *n* = 5), from mastectomy specimens from patients with breast cancer from histologically normal tissue from sites distant from the cancer (Normal Ca; *n* = 5), and from the primary cancers (Cancer; *n* = 5). Total RNA from liver (L) and from stomach (St) were used as positive controls for class I ADH; RNA from stomach (St) was also used as a positive control for class IV ADH. RNA from skin (Sk), a tissue reported to express class IV ADH, was included as a second positive control.

Fig. 2. Immunocytochemistry of nonneoplastic human mammary parenchyma using an antibody cross-reactive with class I and class IV ADH. Immunocytochemistry was carried out as described in "Materials and Methods," using alkaline phosphatase as the reporter and Vector Red Alkaline Phosphatase Substrate as the substrate and chromogen. *A*, an interlobular duct and associated terminal ductules showing uniform strong immunostaining of mammary epithelial cells. *B*, higher magnification of terminal ductal unit from *A*, showing immunostaining of both luminal and basal mammary epithelial cells. *C*, interlobular duct with a distinct myoepithelial layer showing minimal immunostaining surrounding strongly immunopositive epithelial cells. *Insert*, region indicated by *arrow*, at higher magnification. *D*, blood vessel showing immunostaining of what appears to be the muscular layer. *E*, a mammary duct with luminal epithelial cells that are strongly immunopositive, and myoepithelial cells (*arrow*) that are only minimally immunopositive. *F-1* and *F-2*, weakly immunopositive stromal cells (*arrows*) next to strongly immunopositive ducts. *G*, a dilated duct lined with immunopositive luminal epithelial surrounded by weakly immunopositive myoepithelial cells, similar to that seen in a normal duct shown in *C* and *E*. *H*, control section incubated with PBS in place of the primary antibody. Immunoreactivity is limited to noncellular deposit similar to that within the lumen of the duct in *F*. Sections were not counterstained and images were captured using Nomarski optics, as described in "Materials and Methods."



surrounding some neoplastic foci was intense but was minimal in others (Fig. 3. *E* and *H*). Because of the heterogeneity in the level of immunostaining in the cancers, the scoring of intensity of was not attempted. Controls were uniformly negative (Fig. 2*H*). Immunopositive deposits seen within the lumen of some ducts and cysts are attributable to proteinaceous, acellular material that could not be blocked by agents such as goat or calf serum, albumin, or nonfat dried milk (30).

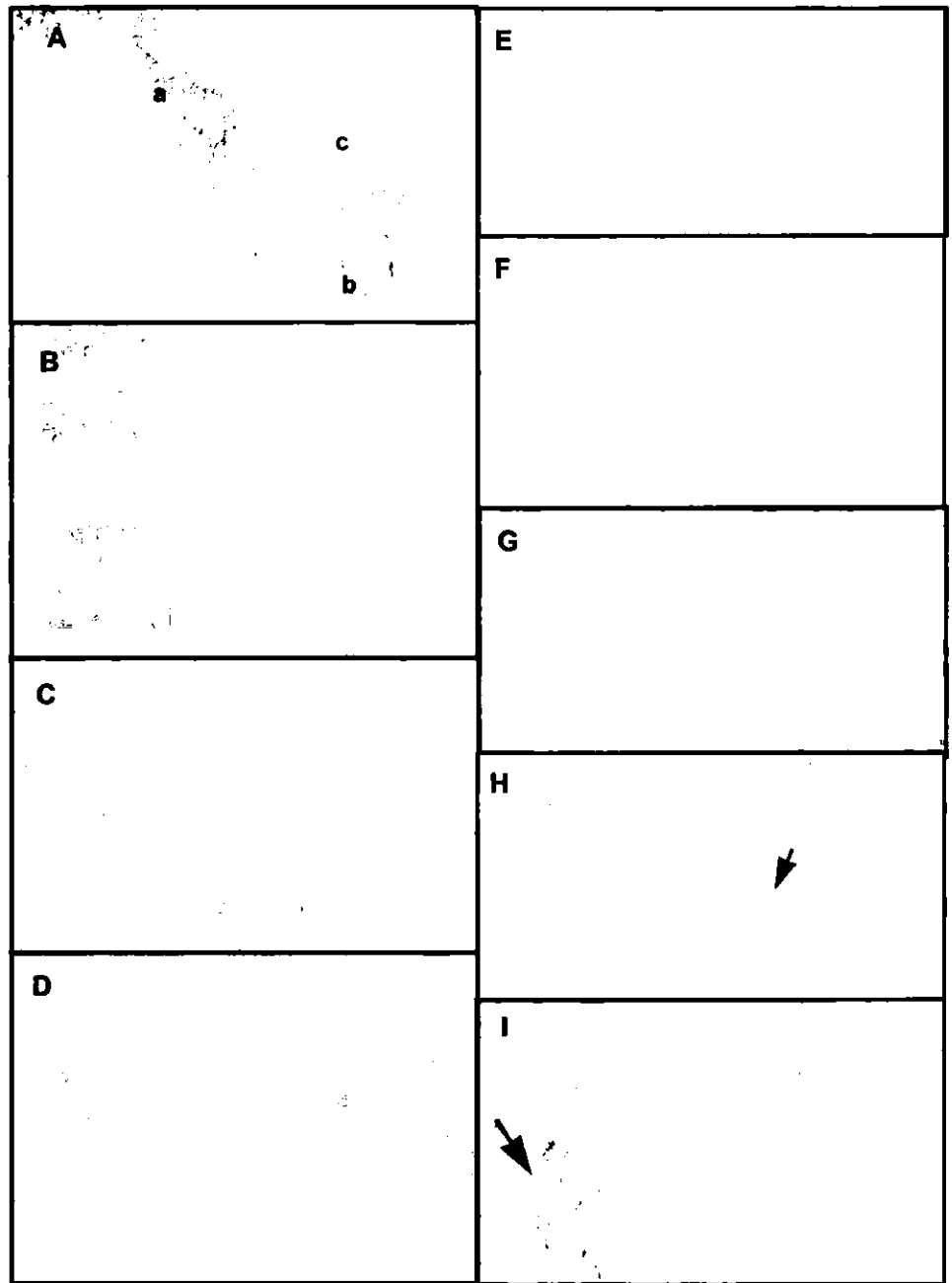
**Immunoblot Analysis.** In immunoblots from all of the normal tissue samples, the class I/IV ADH antibody identified a single immunoreactive band that comigrated with a  $M_r$  40,000 standard (Fig. 4). This size corresponds to that of class I and class IV ADH, two proteins that differ from each other in length by a single amino acid. Data from analysis of the tissues at the level of transcripts, however, warrant the conclusion that the immunoreactive protein identified in

normal breast parenchyma by immunoblot analysis is principally class I ADH.

The intensity of the immunoreactive band varied among the samples. This is attributable, at least in part, to differences in the percentage of tissue occupied by mammary ductal elements in the different specimens. In normal breast parenchyma, the percentage of tissue occupied by mammary epithelium is small and variable, with most of the tissue being occupied by collagenous extracellular matrix (15).

In summary, immunoreactive protein was identified consistently in normal mammary epithelium but not in invasive breast cancers. Although the antibody used did not differentiate between class I and IV ADH, data obtained by RT-PCR and multiple tissue array indicate that class I ADH is the protein identified by immunocytochemistry and immunoblot analysis in the mammary epithelium.

Fig. 3. Immunocytochemistry of neoplastic human breast tissue using an antibody cross-reactive with class I and class IV ADH. Immunocytochemistry was carried out as described in "Materials and Methods," using alkaline phosphatase as the reporter and the Vector Red Alkaline Phosphatase Substrate as the substrate and chromogen. *A*, a strongly immunoreactive, histologically normal interlobular duct (*a*) and a cluster of cancer cells with reduced immunostaining (*b*) are seen separated from each other by essentially immunonegative cancer cells (*c*). *B*, the three clusters of neoplastic cells in a ductal configuration (*on the right*) are only minimally immunoreactive, whereas in the larger cluster of neoplastic cells (*on the left*) there are a few strongly immunopositive cells embedded in minimally immunopositive cancer (*insert*, at higher magnification). *C*, three clusters of cancer cells ranging in immunoreactivity from weakly positive to essentially immunonegative. *D*, a cluster of cancer cells with reduced immunoreactivity *on the left* is seen close to a strongly immunopositive one *on the right* that has features of an *in situ* lesion. *E* and *F*, examples of invasive cancers showing minimal immunostaining. *G*, a lobular cancer with reduced immunostaining. *H* and *I*, examples of variability in immunostaining of blood vessels (*arrows*) associated with neoplastic foci. The blood vessels in *H* are only weakly immunopositive, as are the surrounding cancer cells. In contrast, blood vessels surrounding minimally immunoreactive cancer cells in *I* show strong immunostaining. Sections were not counterstained and images were captured using Nomarski optics, as described in "Materials and Methods."



**Enzyme Assays.** The protein identified by immunocytochemistry and immunoblot analysis was characterized functionally by measuring the rate of NAD-dependent oxidation of EtOH in the absence and presence of 4-MP, an inhibitor of oxidation of EtOH by class I and IV ADH (22). In the absence of  $ZnCl_2$ , there was either no oxidation or only minimal oxidation of EtOH by homogenates of either normal or neoplastic breast tissue ( $<0.1$  mIU/mg protein/min). This was the

40 kDa — — — — —  
 N N C C N C C C C N N

Fig. 4. Immunoblot of  $12,000 \times g$  supernatant from tissue homogenates of nonneoplastic (N) and neoplastic (C) human breast parenchyma, using an antibody cross-reactive with class I and IV ADH. Three  $\mu g$  total protein was loaded into each lane and resolved as described in "Materials and Methods." A single immunoreactive band that comigrated with a  $M_r$  40,000 standard was detected in all of the samples of normal tissue. This band was reduced or absent in samples from breast cancers.

case whether the incubations were carried out in phosphate buffer at either pH 7.5 or 10.4, or at either 25°C or 30°C. Including 1 mM  $ZnCl_2$  in the medium resulted in consistent and reproducible oxidation of EtOH in homogenates of normal breast parenchyma (Table 2A). We were prompted to use  $ZnCl_2$  by a suggestion in the literature that  $Zn^{2+}$  may function not only as an integral part of the enzyme but as a cofactor in the reaction. Only subsequently did we become aware of evidence of the role of certain halide ions, including  $Cl^-$ , for optimal ADH enzyme activity (23).

Total NAD-dependent metabolism of the EtOH by homogenates of normal breast parenchyma, using 33 mM substrate in the presence of 1 mM  $ZnCl_2$ , ranged from 3.89 to 9.17 mIU/mg protein/min (mean  $\pm$  SE =  $5.49 \pm 1.25$ ; Table 2A). From 75 to 99% of this activity was inhibited by 33  $\mu M$  4-MP. Because mammary ductal elements occupy only a small proportion of the breast parenchyma

Tables 2 A and B. *NAD*-dependent ethanol-dehydrogenase activity of normal and neoplastic human breast tissue in the presence or absence of 4-methylpyrazole (4-MP), an inhibitor of oxidation of ethanol (EtOH) by class I and IV ADH

Supernatants of homogenates of normal and neoplastic breast tissue, obtained from reduction mammoplasty and mastectomy specimens, respectively, and L:OH-dehydrogenase activity of these supernatants was measured by monitoring the rate of generation of NADH in the course of oxidation of L:OH as described in "Materials and Methods." Incubations were carried out in the absence and presence of 33  $\mu$ M 4-MP. All samples were assayed in duplicate. Controls contained no L:OH. **In A**, the incubation mixture contained 2.4 mM NAD<sup>+</sup>, 33 mM L:OH, 1 mM ZnCl<sub>2</sub> and 100  $\mu$ l of tissue homogenate, in a total volume of 1 ml of 10 mM HEPES buffer, pH 7.4. **In B**, 2.0 mM NaCl was substituted for the 1 mM ZnCl<sub>2</sub> in the incubation mixture and the incubation was carried out in 200 mM sodium phosphate buffer, pH 10.5. Difference between L:OH-dehydrogenase activity in the absence and presence of 4MP ( $\Delta$ ) can be attributed to class I ADH since 4-MP at the low concentration used (33  $\mu$ M) can be assumed to be a specific inhibitor of class I ADH.

A: 1 mM ZnCl <sub>2</sub> , pH 7.4					Breast Cancer				
#	4-MP		$\Delta$	% Inhibition	#	4-MP		$\Delta$	% Inhibition
	Minus	Plus				Minus	Plus		
N1	3.89 <sup>a</sup>	0.56	3.33	86	C1	0.76	0.08	0.68	89
N2	4.02	1.02	3.00	75	C2	ND <sup>b</sup>	ND		
N3	4.89	ND	4.80	98	C3	ND	ND		
N4	9.17	0.08	8.92	99	C4	ND	ND		
Mean $\pm$ SE	5.49 $\pm$ 1.25	0.42 $\pm$ .24	5.01 $\pm$ 1.36	90 $\pm$ 5.66					

B: 2 mM NaCl, pH 10.5					Breast Cancer				
#	4-MP		$\Delta$	% Inhibition	#	4-MP		$\Delta$	% Inhibition
	Minus	Plus				Minus	Plus		
N5	1.92 <sup>a</sup>	ND <sup>b</sup>	1.92	100	C5	ND <sup>b</sup>	ND		
N6	3.38	ND	3.38	100	C6	ND	ND		
N7	3.47	ND	3.47	100	C7	ND	ND		
N8	9.31	0.08	9.23	99	C8				
Mean $\pm$ SE	4.52 $\pm$ 1.64		4.5 $\pm$ 1.61	99.75 $\pm$ 0.25					

<sup>a</sup> Enzyme activity is expressed as mIU/mg protein/min.

$\Delta$  = difference between L:OH-dehydrogenase activity in the absence and presence of 4-MP.

<sup>b</sup> ND, not detectable.

(generally <10%; Ref. 15), using tissue protein for normalizing the data, as in this study, underestimates the enzyme activity of mammary epithelial cells, the principal cell type expressing class I ADH.

Under the same assay conditions, only one of the four homogenates of breast cancer samples had detectable, albeit a low level, of NAD-dependent EtOH metabolism. The total enzyme activity of this sample was only 0.76 mIU/mg protein/min, whereas that inhibited by 33  $\mu$ M 4-MP was 0.68 mIU/min/mg protein (Table 2A). The potential of normal breast parenchyma to catalyze NAD-dependent oxidation of EtOH, and a loss of this potential in breast cancers, was confirmed in incubations carried out at pH 10.5, the presumed optimum for ADH-catalyzed reaction (Table 2B). The reduction of enzyme activity in breast cancers close to or below the limit of sensitivity of the assay, despite their much higher cellularity than that of normal breast parenchyma, paralleled the reduction in immunoreactive protein identified by immunocytochemistry and immunoblot analysis.

Isoforms of class I and class IV ADH differ greatly in their affinity for EtOH, their EtOH saturation profiles as well as in their sensitivity to inhibition ( $K_i$ ) by 4-MP (14, 22, 31, 32): the concentration of 33 mM EtOH used in these assays is one that would detect activity by isoenzymes of both class I and class IV ADH, whereas the finding that most or all of the enzyme activity could be inhibited by 33  $\mu$ M concentration of 4-MP is consistent with class I ADH expression in normal mammary epithelium. This conclusion is supported also by the saturation profile of EtOH-dehydrogenase activity (Fig. 5). Enzyme activity increased sharply as EtOH concentration was increased from 1 mM to 10 mM, and then decreased as substrate concentration was increased further (Fig. 5). This pattern of substrate dependence, indicating a very high affinity for EtOH and saturation at  $\sim$ 10 mM substrate, is similar to that reported for human class I ADH  $\beta\beta$  isoenzymes, specifically, the  $\beta_1\beta_1$  homodimer ( $V_{max}$ , 9.2 mM; Refs. 31, 33).

**Role of Halide Ion on EtOH-dehydrogenase Activity.** Additional experiments were performed when we became aware of an

older, largely forgotten, report of the importance of certain halide ions in ADH-catalyzed oxidation of EtOH. Specifically, certain halide ions have been shown to facilitate the dissociation of NADH from the binary complex, a rate-limiting step in the enzyme reaction (23). A comparison of EtOH-dehydrogenase activity in medium supplemented with either ZnCl<sub>2</sub> or NaCl confirmed that the stimulation of enzyme activity that we observed was attributable to the Cl<sup>-</sup> ion and not to the Zn<sup>+</sup> ion. As shown in Table 3A, enzyme activity of normal breast tissue in medium supplemented with 1, 2, or 10 mM NaCl was comparable with that obtained in medium containing 1 mM ZnCl<sub>2</sub>. Mean enzyme activity measured in the presence of 2 mM NaCl was somewhat higher and the variance somewhat lower than that obtained with 1 or 10 mM NaCl. However, by ANOVA, there was no statistical difference in the values for enzyme activity obtained using the three

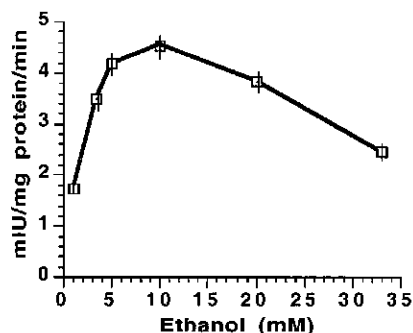


Fig. 5. Effect of concentration of L:OH (*Ethanol*) on NAD-dependent L:OH-dehydrogenase activity by normal human breast parenchyma. Oxidation of L:OH was measured in supernatants of homogenates of nonneoplastic human breast parenchyma obtained from reduction mammoplasty specimens as described under "Materials and Methods." The assays were carried out on duplicate samples prepared from tissue from four donors ( $n = 4$ ). The incubation mixture contained 2.4 mM NAD<sup>+</sup>, 1 mM ZnCl<sub>2</sub>, L:OH at the concentrations indicated on the horizontal axis and 100  $\mu$ l of tissue homogenate, in a total volume of 1 ml of 10 mM HEPES buffer (pH 7.4).

Table 3 A and B. Effect of ion species and buffer (A) and pH (B) on NAD-dependent ethanol-dehydrogenase activity by normal breast parenchyma

**A:** Effect of substituting NaCl for ZnCl<sub>2</sub> and of buffer (10 mM HEPES vs. 200 mM NaPO<sub>4</sub>). The assay was carried out on duplicate samples, (*n* = 5) as described in "Materials and Methods." Incubation mixture contained 100 μl supernatants of homogenate of normal breast parenchyma, 2.5 mM NAD<sup>+</sup>, 33 mM ethanol (EtOH) in either HEPES or phosphate (NaPO<sub>4</sub>) buffer, both at pH 7.4, in a total volume of 1 ml containing either 1, 2, or 3 mM NaCl or 1 mM ZnCl<sub>2</sub>, the highest concentration at which this salt could be used without it precipitating. By one-way ANOVA, there were no significant differences in values obtained for EtOH oxidation in incubations containing either ZnCl<sub>2</sub> or NaCl or in incubations carried out in the two buffers in the presence of either ion. Values obtained in incubations carried out in medium not supplemented with Cl<sup>-</sup> were significantly different from those obtained in medium supplemented with Cl<sup>-</sup> ion (*P* = < 0.01), irrespective of the buffer used. **B:** The effect of pH. The assays were carried out on duplicate samples (*n* = 7), incubation mixture contained 100 μl supernatants of homogenate of normal breast parenchyma, 2.5 mM NAD, 33 mM EtOH, 200 mM NaPO<sub>4</sub> buffer pH 7.4 or pH 10.5, in a total volume of 1 ml, in the absence or presence of 33 mM 4-methylpyrazole (4-MP), an inhibitor of class I and IV ADH. Under the same assay conditions there was no detectable EtOH dehydrogenase activity in incubations containing homogenates of breast cancer tissue (*n* = 3). Tissue homogenates were prepared and enzyme was measured as described in "Materials and Methods."

A: Effect of ion species and buffer								
Buffer	10 mM HEPES				200 mM NaPO <sub>4</sub>			
	Ion	None	ZnCl <sub>2</sub>		NaCl		None	NaCl
1 mM			1 mM	2 mM	10 mM	1 mM		2 mM
Mean ± SE	0.96 ± 0.22	3.68 ± 0.15	2.74 ± 0.46	3.83 ± 0.19	3.45 ± 0.035	0.79 ± 0.14	3.53 ± 0.19	3.22 ± 0.25
B: Effect of pH								
pH	7.4			10.5				
	Inhibitor	4MP	4MP	Δ (%)	4MP	4MP	Δ (%)	
Mean ± SE	4.11 ± 0.44*	0.57 ± 0.12	3.64 ± 0.53 (89)	5.70 ± 0.63	0.34 ± 0.10	5.36 ± 0.61 (97)		
Range	2.4-5.58	0-0.12	1.61-5.58	3.84-7.63	0-0.69	3.49-7.63		

Enzyme activity is expressed as mIU/mg protein/min.

Δ = difference between EtOH-dehydrogenase activity in the absence and presence of 4-MP.

concentrations of NaCl or between NaCl- and ZnCl<sub>2</sub>-supplemented samples. In medium not supplemented with Cl<sup>-</sup>, oxidation of EtOH by supernatants of normal breast tissue homogenates was undetectable. The need for the halide ion for optimal enzyme activity could become critical when measuring EtOH metabolism by extrahepatic tissues, such as normal breast parenchyma, in which cell populations expressing the enzyme represent only a small percentage of the total tissue (see Ref. 15). In such cases, not supplementing the incubation medium with a halide ion may lead to a failure to recognize the potential of the tissue to metabolize EtOH.

Substituting NaCl for ZnCl<sub>2</sub> allowed us to determine the effect of raising the pH of the incubation medium from 7.4 to 10.5 on enzyme activity; although pH 7.5 is frequently used in assays of NAD-dependent metabolism of EtOH, the pH optimum for this activity is reported to be 10.5, a pH at which ZnCl<sub>2</sub> precipitates. Substituting 200 mM NaPO<sub>4</sub> buffer for 10 mM HEPES buffer, required to assay enzyme activity at the higher pH, had no significant effect on enzyme activity (Table 3A). Enzyme activity of homogenates of normal breast parenchyma measured in 200 mM NaPO<sub>4</sub> buffer at pH 10.5 was ~1.5 times higher than that obtained at pH 7.4 (Table 3B). We compared NAD-dependent oxidation of EtOH by normal and neoplastic breast tissue in incubation medium containing 2 mM NaCl<sub>2</sub>, 10 mM EtOH, and at pH 10.5. Under these conditions, enzyme activity of the four additional normal breast tissue samples ranged from 1.92 to 9.31 mIU/mg protein/min, and 99-100% of this activity was inhibited by 33 μM 4-MP, whereas EtOH metabolism by homogenates from the four additional breast cancer samples remained undetectable (Table 2B).

In summary, in incubations augmented with Cl<sup>-</sup> ion, NAD-dependent EtOH metabolism was demonstrable in all of the homogenates prepared from normal breast parenchyma (*n* = 23). In contrast, in only 1 of 10 breast cancer homogenates was there measurable, albeit very low, EtOH metabolism. The characteristics of enzyme activity are consistent with class I ADH-mediated metabolism of EtOH.

## DISCUSSION

This study provides evidence that class I ADH is expressed consistently throughout the normal human mammary epithelium. This finding suggests that the enzyme fulfills some "housekeeping" function(s) in this tissue compartment. One such function is likely to be the

metabolism of EtOH, a normal by-product of cellular metabolism and of fermentation activity of intestinal flora. The concentrations of EtOH in tissues from these sources are estimated to be of the order of 0.1-1 mM (see Ref. 34), values that are within the range of the affinity of isoforms of class I ADH for EtOH (0.05-4 mM; Refs. 14, 31-33).

A need to metabolize and detoxify larger amounts of EtOH can, however, contribute to pathophysiology. Acetaldehyde, the product of oxidation of EtOH, is a reactive intermediate implicated as a cocarcinogen, and increased demand for its detoxification can deplete antioxidant defenses and lead to the generation of potentially mutagenic reactive oxygen species (4, 35). Hence, increased requirement for the metabolism of EtOH, or reduced availability of enzymes or cofactors needed for its detoxification, can lead to redox changes and metabolic disorders that, in their totality, can result in a pro-oxidant state and contribute to carcinogenesis (4). An additional, less well-characterized mechanism by which EtOH may contribute to pathology and to carcinogenesis is suggested by studies that implicate EtOH in disruption of retinoid homeostasis. Specifically, EtOH has the potential to inhibit ADH-mediated oxidation of retinol (vitamin A) to retinal, the rate-limiting step in RA biosynthesis (see Ref. 10). In addition, consumption of EtOH may lead to increased metabolic inactivation of RA by inducing cytochrome P4502E1 (36).

The concentration of EtOH in breast parenchyma after ingestion of alcoholic beverages is not known. That it is likely to be similar to that in blood is suggested by reports that concentrations of EtOH in milk after administration of EtOH reach levels that approach those in blood (37-39). After social drinking, these concentrations range from 2 to 10 mM. Our finding that EtOH metabolism by breast tissue homogenates is inhibited by concentrations of EtOH above 10 mM implies that the contribution of class I ADH to the oxidation of EtOH is likely to be significant only after social drinking. After consumption of larger amounts of EtOH, other mechanisms for detoxification of EtOH would need to come into play. Of these other mechanisms, the oxidation of EtOH mediated by P4502E1 is of special interest. This member of the cytochrome P450 superfamily has been shown to be expressed in human breast tissue (40) and is inducible by EtOH, and reactions catalyzed by this enzyme are particularly prone to generate reactive oxygen species (4, 41). Hence, any dependence on P4502E1 for detoxification of EtOH in breast epithelium, because of the limited

capacity of class I ADH expressed in this tissue to oxidize EtOH, would be expected to contribute to oxidative stress and, thereby, to carcinogenesis. Together, the findings point to a need to consider, in addition to any systemic effects of EtOH, its metabolism *in situ* as one of the mechanisms by which alcohol consumption could contribute to breast cancer risk, and to characterize additional enzymatic mechanisms in the mammary epithelium available for the detoxification of EtOH.

A second property of class I ADH isoenzymes relevant to tissue redox status and carcinogenesis is their ability to reduce aldehyde breakdown products of lipid hydroperoxides, such as 4-hydroxynonenal that is generated in the course of lipid peroxidation (42, 43). This free radical-driven process is thought to occur at a low level under normal physiological conditions as a consequence of normal metabolic processes. An association between excess aldehyde products of lipid peroxidation and breast carcinogenesis is suggested by several studies (7, 8). In this capacity, ADH isoenzymes may serve a protective function by reducing the highly electrophilic aldehyde by-products of lipid peroxidation to their less toxic alcohols.

The unexpected, dramatic reduction in class I ADH expression in invasive breast cancers, however, suggests some additional function(s) for the class I ADH isoenzyme(s) in the mammary epithelium, one that is linked to tumor suppression. Class I ADH has the potential to catalyze the oxidation/reduction of a wide range of endobiotics besides EtOH and aldehyde products of lipid peroxidation (see Ref. 34). Substrates of class I ADH include products of catecholamine and serotonin metabolism, as well as the micronutrient/prohormone retinol (10, 44–49). Among these, the only one with obvious relevance to tumor suppression is the oxidation of retinol to retinal, the first and presumed rate-limiting step in the biosynthesis of RA, a principal mediator of actions of retinoids required for maintaining epithelia in a differentiated state (50).

The requirement of extrahepatic tissues for RA is thought to be met largely by RA generated *in situ* from retinol (51). The growing number of enzymes being identified that can catalyze the oxidation of retinol to retinal suggests that this essential step in RA biosynthesis is supported by a redundant system of multifunctional enzymes. It is a catalytic property that class I and IV ADH isoenzymes share with several retinol dehydrogenases (RoDHs) that are members of the short chain ADH superfamily, as well as some members of the cytochromes P450 superfamily (10, 52–56). A role for ADH isoenzymes in RA biosynthesis has been questioned (52). The notion that they can contribute significantly to RA biosynthesis is supported by embryological studies and by findings from studies of ADH-null mice (10, 57). Additional studies are needed to define the role of these diverse enzymes in RA homeostasis in the adult organism, in different cell populations and under different physiological and pathophysiological conditions.

Disruption of elements of retinoid homeostatic system has been identified in diverse cancers (15, 58–63). The majority of studies of this phenomenon have focused on receptors that mediate the actions of RA or on retinoid-binding proteins. However, findings from a few studies, including two from our laboratories, suggest that the disruption of enzymatic steps that regulate availability of RA in target cells is one of the mechanisms by which the restraining influence of retinoids on carcinogenesis is compromised (15, 64–68). That this may result in a local RA deficiency in cancers of epithelial origin is suggested by one study (69). Of direct relevance to the findings presented here is an older report of a marked reduction in ADH-mediated metabolism of EtOH in colon cancer tissue, as compared with adjacent normal tissue (65). The reduced expression of class I ADH in breast cancer identified in this study fits into the scenario suggested by these findings. Together, these observations warrant a

further, systematic examination of enzymatic mechanisms present in breast parenchyma both for the detoxification of EtOH and the biosynthesis of RA.

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Dr. Elise A. Triano succumbed to metastatic breast cancer while the manuscript was under review. The remaining authors dedicate this article to her memory.

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