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Identification of the class-3 aldehyde dehydrogenases present in human MCF-7/0 breast adenocarcinoma cells and normal human breast tissue

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Abstract—Affinity column chromatography was used to semipurify the very small amounts of class-3 aldehyde dehydrogenase (ALDH-3) present in human MCF-7/0 breast adenocarcinoma cells and human normal breast tissue. Characterization of the semipurified enzymes revealed that each was a type-1 ALDH-3 rather than a type-2 ALDH-3 as previously reported. Although clearly a type-1 ALDH-3, the MCF-7/0 enzyme, as well as the type-1 ALDH-3 constitutively present in cultured colon C cells and induced in cultured MCF-7/0 cells by methylcholanthrene, does, however, differ from the prototypical human stomach mucosa type-1 ALDH-3 in one, perhaps pharmacologically important, way, viz. when the ability to catalyse the oxidation of aldophosphamide is normalized by the ability to catalyse the oxidation of benzaldehyde, each of these enzymes, as well as the type-2 ALDH-3 found in MCF-7/OAP cells, exhibits greater ability to catalyse the oxidation of aldophosphamide than does stomach mucosa type-1 ALDH-3; hence, although not type-2 ALDH-3s, they may be slight variants of the prototypical type-1 ALDH-3.

Key words: aldehyde dehydrogenase; breast adenocarcinoma; normal human breast; cyclophosphamide; aldophosphamide; oxazaphosphorines; drug resistance

Relatively high levels of a cytosolic ALDH-3* are found in a number of human tissues including stomach mucosa [1, 2]. The stomach mucosa enzyme has been studied extensively [1 and publications cited therein] and can be viewed as the prototype of this class of aldehyde dehydrogenases. High levels of ALDH-3 are also found in cultured human colon C carcinoma cells [3].

Very low levels of ALDH-3 are found in human normal breast tissue and in cultured MCF-7/0 breast adenocarcinoma cells but markedly elevated levels of this enzyme were shown to be present in MCF-7/0 cells made stably resistant to oxazaphosphorines such as mafosfamide and 4-hydroperoxycyclophosphamide by growing them in the presence of 4-hydroperoxycyclophosphamide for several months (MCF-7/OAP). The ALDH-3 present in the latter differed somewhat, both physically and catalytically, from the prototypical enzyme and that found in colon C cells (Table 1). Hence, it was christened type-2 ALDH-3 to distinguish it from the type-1 ALDH-3 found in stomach mucosa and in colon C cells [1–3].

As judged by relevant enzyme behavior when soluble (105,000 *g* supernatant) fractions prepared from either 1×10^7 untreated MCF-7/0 cells or 1 g of human normal breast tissue obtained from pre- and post-menopausal women were subjected to isoelectric focusing, we preliminarily concluded that the enzyme in these cells/tissues was also of the type-2 variety [1, 2]. This conclusion seemed to be consistent with all of the then existing knowledge, viz. that the MCF-7/0-derived MCF-7/OAP cells expressed a type-2 ALDH-3 [1]. Subsequently, however, doubt arose as to the correctness of this conclusion when we observed that exposure of MCF-7/0 cells to methylcholanthrene for a few days induces ALDH-3 expression (as well as transient resistance to the oxazaphosphorines), and established that this ALDH-3 was of the type-1 variety [4]. Very large amounts of total protein had to be loaded onto the gels in the isoelectric focusing experiments referred to above because the enzyme was present in such small quantities in MCF-7/0 cells and human normal breast tissue. Our concern was that the large amounts of nonspecific protein present in the 105,000 *g* supernatant fractions placed onto the gels may have affected the electrophoretic behavior of the specific protein of interest, i.e. ALDH-3, thus misleading us with regard to the identity of the enzyme expressed in MCF-7/0 cells and human normal breast tissue. In an attempt to allay our concern, we then conducted an experiment in which a mixture of a very small, but detectable, amount of purified stomach mucosa type-1 ALDH-3 and a soluble (105,000 *g* supernatant) fraction obtained from 1×10^7 MCF-7/0 cells was submitted to isoelectric focusing. The results of this experiment, however, only increased our concern since, under the conditions of this experiment, the stomach mucosa enzyme behaved as does type-2 ALDH-3. Hence, we decided to minimize/eliminate the problem of nonspecific protein interference by semipurifying the very small amounts of enzyme present in MCF-7/0 cells and human normal breast tissue and re-evaluating the issue. The results of these experiments are presented herein.

Materials and Methods and Results

4-Hydroperoxycyclophosphamide was provided by Dr. J. Pöhl, Asta-Werke AG, Bielefeld, Germany. Aldophosphamide was generated in aqueous solution by chemical reduction of 4-hydroperoxycyclophosphamide using methyl sulfide as the reducing agent [1, 5]. Human MCF-7/0 breast adenocarcinoma cells were originally obtained from Dr. B. Teicher, Dana-Farber Cancer Institute, Boston, MA, and were cultured as described previously [1, 4]. Human stomach mucosa type-1 ALDH-3, human colon C carcinoma type-1 ALDH-3, human breast adenocarcinoma MCF-7/MC type-1 ALDH-3, and human breast adenocarcinoma MCF-7/OAP type-2 ALDH-3 were purified to homogeneity, and anti-stomach mucosa type-1 ALDH-3 IgY was prepared, as described previously [1, 3, 4]. Preparation of subcellular fractions, the aldehyde dehydrogenase assays, kinetic constant determinations, protein determinations, isoelectric focusing, SDS–PAGE, immunoblot analysis and data analysis were as described previously [1, 4].

Semipurification of class-3 aldehyde dehydrogenases present in MCF-7/0 cells (1.5×10^{10}) and in human normal

* Abbreviation: ALDH-3, class-3 aldehyde dehydrogenase.

Table 1. Physical and catalytic properties of type-1 and type-2 ALDH-3: Differences*

Parameter	ALDH-3 Type-1	ALDH-3 Type-2
Source	MCF-7/MC Colon C Stomach mucosa	MCF-7/OAP
Specific activity† (mIU/mg)	~32,900	16,667
NAD K_m‡ (μM)	~50	550
Esterolytic activity (mIU/mg)	~9,000	3,350
pI Range (No. of bands)	5.75–6.35 (4–5)	6.0–6.45 (4)
Subunit M_r (kDa)	54.5	40
Recognition of subunit by anti-stomach mucosa ALDH-3 IgY	Yes	No
NADP-dependent catalysis of acetaldehyde oxidation	++	–

* From Refs. 1–4.

† Substrate and cofactor were 4 mM benzaldehyde and 1 mM NAD, respectively.

‡ The NADP K_m value is 940 μM for type-2 ALDH-3 and about the same for type-1 ALDH-3 [1, 3, 4].

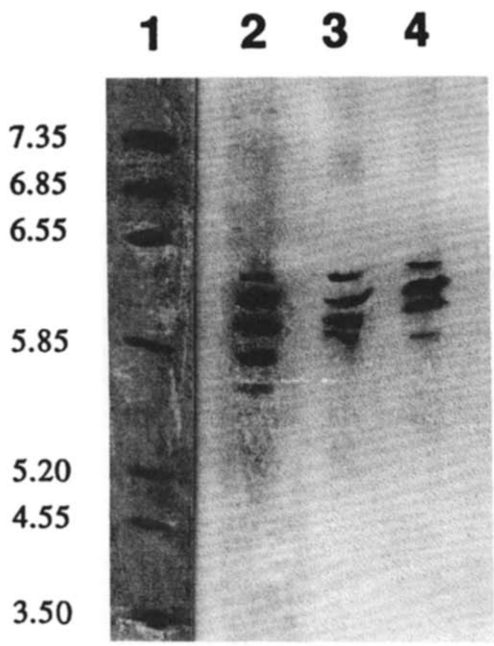

Fig. 1. Isoelectric focusing of the class-3 aldehyde dehydrogenases present in MCF-7/0 cells and human normal breast tissue. Semipurification of class-3 aldehyde dehydrogenases present in MCF-7/0 cells and human normal breast tissue was as described in Materials and Methods and Results. Electrofocusing of pI standards (lane 1) and aliquots of purified stomach mucosa type-1 ALDH-3 (lane 2), semipurified human normal breast ALDH-3 (lane 3) and semipurified MCF-7/0 ALDH-3 (lane 4) sufficient to generate 10–15 nmol NADH/min when the substrate and cofactor are benzaldehyde (4 mM) and NAD (1 mM), respectively, was as described in Materials and Methods and Results. Lane 1 was stained with Coomassie Brilliant Blue R-250 for the presence of proteins. Lanes 2–4 were stained for aldehyde dehydrogenase activity as described in Materials and Methods and Results; benzaldehyde (4 mM) and NAD (4 mM) were used as the substrate and cofactor, respectively.

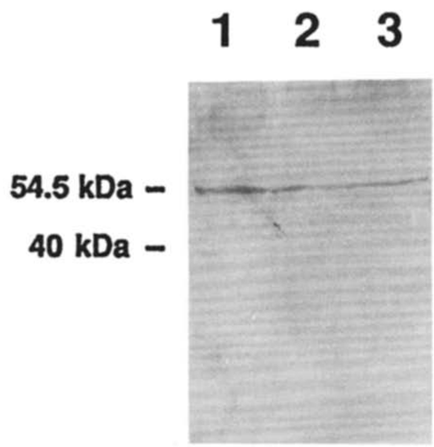

Fig. 2. Immunoblot visualization of denatured semipurified class-3 aldehyde dehydrogenases present in MCF-7/0 cells and human normal breast tissue. Semipurification of class-3 aldehyde dehydrogenases present in MCF-7/0 cells and human normal breast tissue was as described in Materials and Methods and Results. Anti-stomach mucosa type-1 ALDH-3 IgY was generated and used as described in Materials and Methods and Results to visualize purified stomach mucosa type-1 ALDH-3 (lane 1), semipurified MCF-7/0 ALDH-3 (lane 2) and semipurified human normal breast ALDH-3 (lane 3) after these enzymes had been submitted to SDS–PAGE and electrotransferred onto Immobilon-PVDF transfer membranes. Placed on gels were 5 μg of purified stomach mucosa type-1 ALDH-3 and semipurified MCF-7/0 and human normal breast ALDH-3 sufficient to generate 98 and 85 nmol NADH/min, respectively, when the substrate and cofactor are benzaldehyde (4 mM) and NAD (1 mM), respectively.

Table 2. Kinetic properties of class-3 aldehyde dehydrogenases semipurified from MCF-7/0 cells and human normal breast tissue*

Source	K_m (μM)	
	NAD	NADP
MCF-7/0	40	952
Normal breast	49	Not determined

* Kinetic constants were determined as described in Materials and Methods and Results. Benzaldehyde (4 mM) was the substrate. Each value is the mean of 4 (MCF-7/0) or 2 (normal breast) determinations.

breast tissue (14.5 g; pool of samples obtained from six women ranging in age from 25 to 79) was by Reactive Blue 2-Sepharose CL 6B affinity column chromatography as described previously [1, 4]. Specific activities of the semipurified enzymes were 2667 (MCF-7/0) and 3058 (normal breast) mIU/mg protein when benzaldehyde (4 mM) and NAD (1 mM) were used as substrate and cofactor, respectively, to quantify catalytic activity. These values were 6668- and 4531-fold greater than those obtained with the respective starting preparations, viz. 105,000 *g* supernatant fractions. Yields were 89 and 95%, respectively. As judged by isoelectric focusing followed by staining for NAD-linked aldehyde dehydrogenase-catalysed oxidation of acetaldehyde and benzaldehyde, each of these enzyme preparations was completely free of ALDH-1, ALDH-2 and all other aldehyde dehydrogenases; however, protein staining after both isoelectric focusing and SDS–PAGE revealed the presence of several nonspecific proteins (data not shown).

As judged by pI values and isoelectric focusing patterns (Fig. 1) and subunit molecular weight as well as recognition by anti-stomach mucosa ALDH-3 IgY (Fig. 2), the cytosolic class-3 aldehyde dehydrogenase present in MCF-7/0 cells and human normal breast tissue was of the type-1 variety. Further supporting this notion were the observations that (1) the K_m values for NAD were about 50 μM (Table 2), (2) NAD was the much preferred cofactor (Table 2), and (3) each enzyme catalysed the NADP-linked oxidation of acetaldehyde (data not shown).

The MCF-7/0 enzyme, like the ALDH-3 constitutively present in cultured colon C cells and induced in cultured MCF-7/0 cells by methylcholanthrene, differed from the prototypical human stomach mucosa type-1 ALDH-3 in that it and the other two enzymes, as well as the type-2 ALDH-3 found in cultured MCF-7/OAP cells, exhibited greater ability to catalyse the oxidation of aldophosphamide than did stomach mucosa type-1 ALDH-3 when the ability to catalyse the oxidation of aldophosphamide was normalized by the ability to catalyse the oxidation of benzaldehyde (Table 3). Human normal breast tissue type-1 ALDH-3 was not evaluated in this regard because (1) normal breast tissue expresses very small amounts of this enzyme, and (2) none of the ALDH-3s catalyse the oxidation of aldophosphamide at very great rates; thus, very large amounts of normal breast tissue (*ca.* 100 g) would have been needed to yield sufficient semipurified enzyme to do this experiment. This amount of human normal breast tissue was not available to us.

Discussion

Physical and kinetic characterization of the cytosolic class-3 aldehyde dehydrogenases semipurified from cultured human MCF-7/0 breast adenocarcinoma cells and normal breast tissue, as reported herein, revealed that each is a type-1 ALDH-3.

Like the colon C and MCF-7/MC type-1 ALDH-3s, however, the MCF-7/0 enzyme appears to be a slight variant of the prototypical stomach mucosa type-1 ALDH-3 since the ability of each of these enzymes to catalyse the oxidation of aldophosphamide (normalized by the ability to catalyse the oxidation of benzaldehyde) was substantially greater than was the ability of stomach mucosa enzyme to do so. Given that, thus far, the apparent type-1 variant has been found only in malignant cells, an attractive idea is that it is tumor-specific. An alternative possibility is that it may be tissue-specific. Each of these notions is being explored in our laboratory.

Ramifications of the finding that the cytosolic class-3 aldehyde dehydrogenase constitutively expressed in MCF-7/0 cells is a type-1 ALDH-3 include the following.

Constitutive expression of the type-1 ALDH-3 is very low in MCF-7/0 cells [1]. MCF-7/0 cells made stably resistant to the oxazaphosphorines by growing them in the presence of increasing concentrations of 4-hydroperoxycyclophosphamide for several months [6] constitutively express relatively large amounts of ALDH-3, but, in this case, it is of the type-2 variety [1]. One mutational event could account for constitutive expression of the atypical enzyme (in addition to the usual constitutive expression of the typical enzyme) in the latter, viz. a mutation that results in the constitutive expression of a gene, one that codes for type-2 ALDH-3, present, but not expressed, in the parent line. Alternatively, two mutations may have occurred, one in the coding region of the type-1

Table 3. Catalysis of aldophosphamide and benzaldehyde oxidation by (semi)purified NAD-dependent class-3 aldehyde dehydrogenases: Relative rates*

Source	(nmol aldophosphamide oxidized/min/mg) (1000) / (nmol benzaldehyde oxidized/min/mg)
Stomach mucosa	0.29
Colon C	2.85
MCF-7/0	2.20
MCF-7/MC	3.63
MCF-7/OAP	8.08

* Aldehyde dehydrogenase activity was determined as described in Materials and Methods and Results, using aldophosphamide (160 μM) or benzaldehyde (4 mM) as the substrate and NAD (1 mM) as the cofactor.

ALDH-3 gene so that now an atypical enzyme is produced in place of the typical enzyme, and a second allowing the constitutive, relatively elevated, expression of the atypical enzyme. 4-Hydroperoxycyclophosphamide is a demonstrated mutagen [7]. Seemingly germane, constitutive glutathione *S*-transferase and NAD(P)H:quinone oxidoreductase [NAD(P)H:menadione oxidoreductase; DT-diaphorase] activities are also elevated in MCF-7/OAP cells (Sreerama L and Sladek NE, unpublished observations).

Exposure of MCF-7/0 cells to polycyclic aromatic hydrocarbons such as methylcholanthrene, benzpyrene or 9,10-dimethyl-1,2-benzanthracene for a few days also results in a markedly increased expression of ALDH-3, but, in this case, increased expression is of the same enzyme, viz. type-1 ALDH-3, and is transient in that enzyme activity returns to basal levels when the polycyclic aromatic hydrocarbon is removed from the culture medium ([2, 4] and Sreerama L and Sladek NE, unpublished observations). The polycyclic aromatic hydrocarbons are known ligands for the so-called Ah receptor [reviewed in Ref. 8], and it is likely that, in the case of these agents, induction is via this receptor and the xenobiotic (directly) and/or antioxidant (indirectly) responsive elements (XRE and ARE, respectively) present in the 5'-flanking region of the gene coding for type-1 ALDH-3 [9]. Consistent with this notion, exposure of MCF-7/0 cells to Ah receptor ligands results in the induction of glutathione *S*-transferase, NAD(P)H:quinone oxidoreductase, cytochrome P450 IA1/2 and UDP-glucuronosyl transferase activities as well [2, 4, 10–12]; these enzymes are also believed to be induced via the Ah receptor as described above [reviewed in Ref. 13].

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