Human aldehyde dehydrogenase 3A1 (ALDH3A1): biochemical characterization and immunohistochemical localization in the cornea

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ALDH3A1 (aldehyde dehydrogenase 3A1) is expressed at high concentrations in the mammalian cornea and it is believed that it protects this vital tissue and the rest of the eye against UV-light-induced damage. The precise biological function(s) and cellular distribution of ALDH3A1 in the corneal tissue remain to be elucidated. Among the hypotheses proposed for ALDH3A1 function in cornea is detoxification of aldehydes formed during UV-induced lipid peroxidation. To investigate in detail the biochemical properties and distribution of this protein in the human cornea, we expressed human ALDH3A1 in Sf9 insect cells using a baculovirus vector and raised monoclonal antibodies against ALDH3A1. Recombinant ALDH3A1 protein was purified to homogeneity with a single-step affinity chromatography method using 5'-AMP-Sepharose 4B. Human ALDH3A1 demonstrated high substrate specificity for medium-chain (6 carbons and more) saturated and unsaturated aldehydes, including 4-hydroxy-2-nonenal, which are generated by the peroxidation of cellular lipids. Short-chain aliphatic aldehydes, such as acetaldehyde, propionaldehyde and malondialdehyde, were found to be very

poor substrates for human ALDH3A1. In addition, ALDH3A1 metabolized glyceraldehyde poorly and did not metabolize glucose 6-phosphate, 6-phosphoglucono- δ -lactone and 6-phosphogluconate at all, suggesting that this enzyme is not involved in either glycolysis or the pentose phosphate pathway. Immunohistochemistry in human corneas, using the monoclonal antibodies described herein, revealed ALDH3A1 expression in epithelial cells and stromal keratocytes, but not in endothelial cells. Overall, these cumulative findings support the metabolic function of ALDH3A1 as a part of a corneal cellular defence mechanism against oxidative damage caused by aldehydic products of lipid peroxidation. Both recombinant human ALDH3A1 and the highly specific monoclonal antibodies described in the present paper may prove to be useful in probing biological functions of this protein in ocular tissue.

Key words: aldehyde dehydrogenase 3A1 (ALDH3A1), cornea, epithelium, 4-hydroxy-2-nonenal, lipid peroxidation, stromal keratocyte.

INTRODUCTION

ALDH3A1 (aldehyde dehydrogenase 3A1) is a member of the ALDH superfamily, which consists of NAD(P)⁺-dependent enzymes that catalyse the oxidation of a wide variety of endogenously produced and exogenous aldehydes to their corresponding acids [1]. ALDH3A1 is expressed at high concentrations in the cornea of several mammalian species comprising up to 40% of the total water-soluble fraction [2]. Although several hypotheses exist regarding the role of corneal ALDH3A1, the prevailing hypothesis suggests a metabolic role towards detoxification of aldehydes formed during UV-induced lipid peroxidation, including α,β -hydroxyalkenals ([3] and references therein). α,β -Hydroxyalkenals, such as 4-HNE (4-hydroxy-2-nonenal) and MDA (malondialdehyde), are metastable toxic aldehydes formed endogenously as breakdown products of lipid peroxidation of $\omega-6$ polyunsaturated fatty acids [4]. Increased levels of 4-HNE and/or MDA in ocular tissue are associated with cataracts [5], pathologic corneas [6], fibrotic proliferative retinopathies [7] and retinal detachment [8]. The cornea is constantly exposed to UV radiation, which may lead to the production of high intracellular concentrations of toxic aldehydic products. It is becoming increasingly clear that the cornea expresses several proteins as a part of a cellular defence

mechanism that protects this vital tissue from the toxic effects of UV radiation. Albumin, which is the most prevalent corneal protein, may protect the cornea against oxidative damage by serving as an antioxidant scavenger of hydrogen peroxide [9]. NADP⁺-dependent isocitrate dehydrogenase, which is expressed at high concentrations in bovine cornea, protects against UV-induced oxidative injury by preventing lipid peroxidation and oxidative DNA damage [10]. Nuclear ferritin, detected in chicken corneal epithelium, prevents UV-induced DNA oxidative damage [11]. We have recently shown that ALDH3A1 prevents UV- and 4-HNE-induced oxidative damage in HCE (human corneal epithelial) cells [3]. A possible explanation for the protective effect of ALDH3A1 is the detoxification of toxic aldehydes generated by lipid peroxidation. However, ALDH3A1 has been reported to be catalytically inactive towards MDA [12], and a controversy exists regarding the ability of this enzyme to metabolize 4-HNE. Studies with rat ALDH3A1 and an *Arabidopsis thaliana* ALDH3 protein have indicated that 4-HNE is not a substrate for this enzyme [13,14]. In contrast, ALDH3A1 purified from human cornea exhibits a K_m of 110 μ M for 4-HNE [15], whereas a K_m of ≈ 54 μ M for 4-HNE was recently reported for cell extracts from ALDH3A1-expressing HCE cell lines [3]. In addition to the substrate controversy, there are conflicting reports regarding the cellular distribution of corneal ALDH3A1. Early histochemical

Abbreviations used: ALDH, aldehyde dehydrogenase; 4-HNE, 4-hydroxy-2-nonenal; MDA, malondialdehyde; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, Nitro Blue Tetrazolium; NDRI, National Disease Research Interchange; HCE cell line, human corneal epithelial cell line; TBS, Tris-buffered saline.

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studies demonstrated expression of ALDH3A1 in the epithelium, stroma and endothelium of bovine, baboon, porcine and ovine corneas [16,17]. More recently, Kays and Piatigorsky [18] reported that expression of ALDH3A1 was restricted to the epithelium of mouse, monkey and human cornea. Further, King et al. [15] reported expression of ALDH3A1 in the human corneal and lens epithelium.

As conflicting evidence exists in the literature regarding ALDH3A1 substrate specificities and the localization of the enzyme in the corneal tissue, we sought to characterize the biochemical properties of human ALDH3A1 and to develop a monoclonal antibody to determine the cellular distribution in the human cornea. In the present study, we describe a baculovirus-mediated expression system for human ALDH3A1 and the purification of the recombinant protein by affinity chromatography. We report catalytic properties of the recombinant protein, the development of a highly-specific monoclonal antibody and the cellular distribution of ALDH3A1 in the human cornea determined by immunohistochemistry.

MATERIALS AND METHODS

Materials

The baculovirus expression vector, pBlueBac4.5, was purchased from Invitrogen Life Technologies (Carlsbad, CA, U.S.A.). Restriction endonucleases were obtained from either Invitrogen or New England Biolabs (Beverly, MA, U.S.A.). T4 DNA ligase was purchased from Stratagene (La Jolla, CA, U.S.A.). 5'-AMP-Sepharose 4B was obtained from Amersham Biosciences (Piscataway, NJ, U.S.A.). PVDF membranes were purchased from Millipore (Bedford, MA, U.S.A.). Chicken polyclonal antibody against human ALDH3A1 was kindly provided by Dr Norman Sladek (University of Minneapolis, Minneapolis, MN, U.S.A.). Horseradish peroxidase-conjugated chicken anti-human IgG secondary antibody was obtained from Jackson Immuno Research (West Grove, PA, U.S.A.). BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (Nitro Blue Tetrazolium) were purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). SyproRuby Dye reagent was obtained from Molecular Probes (Eugene, OR, U.S.A.). Alkaline phosphatase-conjugated donkey anti-mouse IgG and rhodamine-conjugated donkey anti-mouse IgG were purchased from Chemicon International (Temecula, CA, U.S.A.). Reagents for chemiluminescence were purchased from NEN Life Science Products (Boston, MA, U.S.A.). Aldehyde substrates (except where noted), NADP⁺, sodium pyrophosphate, sodium phosphate, potassium phosphate, EDTA and dithiothreitol were purchased from Sigma (St. Louis, MO, U.S.A.). 4-HNE was purchased from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). MDA was synthesized according to a method described previously [4]. The BCA (bicinchoninic acid) protein assay reagent was obtained from Pierce Chemical Company (Rockford, IL, U.S.A.). All chemicals were of analytical grade. Unless otherwise specified, all tissue-culture media, supplements, growth factors, assay reagents and buffers were from Gibco-BRL (Grand Island, NY, U.S.A.).

Cell culture

The human lung adenocarcinoma cell line (A549) was grown in minimal essential medium supplemented with 10 % fetal bovine serum, 2.2 g/l NaHCO₃ and 100 units/ml penicillin/100 µg/ml streptomycin solution at 37 °C in 5 % CO₂ in air. The generation of an HCE cell line stably transfected with ΔpCEP4Δ/hALDH3A1 (ALDH3A1-1/D) is reported elsewhere [3]. This cell line was

grown at 37 °C in Dulbecco's modified Eagle's medium/Ham's F12 supplemented with 15 % fetal bovine serum, 0.5 % (v/v) DMSO, 0.1 µg/ml cholera toxin, 10 ng/ml epidermal growth factor, 5 µg/ml insulin, 40 µg/ml Gentamycin, 20 mM glutamine and 0.1 mg/ml hygromycin under 5 % CO₂ in air. Cells in exponential growth (about 70 % confluent) were used for all experiments.

Construction and expression of recombinant baculovirus

The previously cloned human ALDH3A1 cDNA [19] was used to obtain the coding region of ALDH3A1. This was excised as an *Xho*I fragment from the ΔpCEP4Δ/hALDH3A1 mammalian expression vector and subcloned into baculovirus expression vector pBlueBac4.5. Restriction and sequence analyses were performed to verify the correct insertion of the coding region of ALDH3A1 into pBlueBac4.5 vector. Recombinant viruses were plaque-purified and amplified in Sf9 insect cells (from *Spodoptera frugiperda*) as described previously [20]. Ten independently isolated plaques were amplified in Sf9 cells and tested for expression of the recombinant protein by Western blot analysis. More than 50 % of the isolated plaques produced ALDH3A1 protein. ALDH3A1 antigen was not detected in uninfected cells. Plaques with the highest ALDH3A1 expression were used for subsequent infection of insect Sf9 cells. Sf9 cells (at a density of 1 × 10⁶) were infected with human ALDH3A1 baculovirus at a multiplicity of 1 for 48 h. Infected cells were harvested by centrifugation at 1000 g for 5 min and washed with PBS; cell pellets were lysed for subsequent processing.

Purification of ALDH3A1

ALDH3A1 was purified using affinity chromatography, similar to that described previously for the isolation of tumour-associated ALDH3A1 from rat hepatoma HTC cells [21] and the baculovirus-expressed rabbit and human ALDH1A1 [20] with minor modifications. Briefly, the Sf9 cell pellets were suspended in 100 mM potassium phosphate, pH 7.4, containing 1 mM EDTA, 1 mM 2-mercaptoethanol and 0.02 % Triton X-100. Cell suspensions were sonicated on ice for 30 s followed by a 30 s interval of rest to prevent over-heating for a total of six sonication cycles. The resulting homogenate was centrifuged at 100 000 g for 1 h and the supernatant was applied to a 5'-AMP-Sepharose 4B affinity column equilibrated with binding buffer (100 mM potassium phosphate, pH 7.4, containing 1 mM EDTA, 1 mM 2-mercaptoethanol and 0.01 % Triton X-100). The bound ALDH3A1 was eluted with binding buffer containing 0.25 mM NAD⁺. Active fractions were pooled and concentrated with an Amicon® concentrator (30 kDa cutoff; Millipore) under nitrogen. To test the homogeneity of the affinity-purified ALDH3A1, the pooled fractions were subjected to electrophoresis on SDS/PAGE (12.5 % gels) and processed for either silver staining or Western blot analysis. For Western blot analysis, the protein samples were transferred to a PVDF membrane, which was subsequently incubated in blocking buffer (50 mM Tris/150 NaCl, pH 7.5, containing 0.1 % Tween-20 and 5 % non-fat milk) for 2 h at room temperature. Chicken anti-human ALDH3A1 was then added at a dilution of 1:2000 in blocking buffer. Labelled proteins were visualized using horseradish peroxidase-conjugated rabbit anti-chicken secondary antibody (1:5000) and detected by chemiluminescence.

ALDH3A1 enzyme activity and kinetic studies

The enzymic activity of recombinant ALDH3A1 was measured spectrophotometrically (using an Beckman DU-640 instrument)

by monitoring the production of NADPH (340 nm) during the enzymic oxidation of aldehyde substrates. The specific activity was determined at 25 °C in a 1 ml reaction mixture composed of 100 mM sodium pyrophosphate buffer (pH 8.0), 2.5 mM NADP⁺, 1 mM pyrazole and 5–40 µg of ALDH3A1 recombinant protein. The enzymic reaction was initiated by the addition of various concentrations of aldehyde substrates. Aldehydes were prepared in 20 % methanol and the final concentration of methanol in the reaction mixture was less than 1 %. To determine the apparent K_m and V_{max} values, ALDH3A1 enzymic activities were measured with various concentrations of substrate ranging from one-tenth of the apparent K_m to 10 times the K_m for each substrate. A total of 9–12 concentrations were assayed for each substrate and included at least three concentrations below the apparent K_m , three above the K_m , and three within the saturation portion of the kinetics curve. Substrates that did not show activity with ALDH3A1 were tested in the following ranges: 25 mM–25 µM for glucose 6-phosphate, 100 mM–10 µM for 6-phosphoglucono-δ-lactone and 10 mM–1 µM for 6-phosphogluconic acid. All enzyme assays were performed in triplicate in three separate experiments. Rates were fitted to the Michaelis–Menten equation using SigmaPlot® software for enzyme kinetics (version 7.0, 2001). Enzyme specific activities are expressed as either nmol of NADPH/min per mg of protein or µmol of NADPH/min per mg of protein.

Production of monoclonal antibody against human ALDH3A1

Male BALB/c mice were immunized three times with 100 µg of recombinant human ALDH3A1. Spleen cells from the immunized mice were fused with P3/U1 murine myeloma cells and cultured in HAT (hypoxanthine/amethopterin/thymidine) selection medium [22]. Culture supernatants of the hybridoma were screened using ELISA, employing pairs of wells of microtitre plates on which cells were adsorbed with recombinant human ALDH3A1 as the antigen (1 µg of protein/well). After incubation with 100 µl of hybridoma supernatants, and with intervening washes with TBS (Tris-buffered saline), pH 7.8, containing 0.05 % Tween 20 (TBS-Tween), the wells were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG, followed by a substrate solution containing 1 mg/ml *p*-nitrophenyl phosphate. Hybridoma cells, corresponding to the supernatants that were positive for recombinant human ALDH3A1, were then cloned by limiting dilution. After repeated screening, five clones were obtained. Among them, one clone (247 H9D4F5) yielded IgG1 antibodies that recognized the recombinant antigen either by Western blotting or immunohistochemistry.

Western blot analysis of human corneas using the monoclonal antibody

Normal human corneas ($n=2$) were obtained via the NDRI (National Disease Research Interchange) within 36 h of enucleation. Neither individual was noted to have abnormal ocular histories. Upon arrival in our laboratory, tissues were rinsed thoroughly in cold PBS, pH 7.2, frozen under liquid nitrogen and ground into a fine powder using a mortar and pestle. The frozen powder was extracted at 4 °C with a buffer containing 50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 1.0 % Nonidet P40, 0.5 % sodium deoxycholate, 0.1 % SDS, 100 µg/ml aprotinin, 2 µg/ml leupeptin and 100 µM sodium vanadate. Equal amounts of total protein from each sample were subjected to SDS/PAGE. Immediately after electrophoresis, the gel was stained using the SyproRuby Dye reagent and the fluorescence (excitation at 450 nm and emission at 610 nm) was visualized

with the aid of an FMBIO III imaging device (MiraiBio, Alameda, CA, U.S.A.). Following image capture, the proteins were transferred to PVDF membranes. The membranes were blocked in TBS supplemented with 5 % non-fat dried milk. The membranes were then incubated in primary antibody, diluted 1:1 with antibody buffer (TBS containing 5 % BSA and 0.1 % Tween 20), overnight at 4 °C. After extensive washing, the membranes were incubated with alkaline phosphatase-conjugated donkey anti-mouse IgG at a 1:5000 dilution in antibody buffer for 1 h. The sections were then extensively washed with TBS, and the antibody visualized by incubation with BCIP/NBT as described previously [23]. The membrane was directly scanned and digital images captured.

Immunohistochemistry of human corneas using the monoclonal antibody

Normal corneas ($n=10$) were obtained via NDRI within 24–48 h of enucleation. None of these individuals were noted to have abnormal ocular histories. Upon arrival in our laboratory, tissues were rinsed thoroughly in cold PBS, pH 7.2, and embedded in Tissue Tek OCT compound (Sakura Finetek USA, Torrance, CA, U.S.A.). The blocks were frozen under nitrogen vapour and 5 µm sections were cut on a cryostat (Leica, model CM 1850). Tissue sections were incubated in antibody buffer (PBS, pH 7.2, containing 5 % BSA and 0.01 % Triton X-100) for 30 min. Primary antibody, diluted 1:1 in antibody buffer, was applied to the tissue and then incubated in a moist chamber for 1 h. The tissue sections were washed with PBS and incubated with rhodamine-conjugated donkey anti-mouse IgG at a 1:40 dilution in antibody buffer for 1 h. The sections were then extensively washed with PBS, examined using a Nikon epifluorescence microscope and digital images captured using a CoolSnap CCD (Photometrics).

Other methods

Cell lysates were prepared as followed. Cells were washed twice with ice-cold PBS and sonicated on ice for 30 s, followed by a 30 s interval of rest to prevent over-heating, for a total of six sonication cycles in lysis buffer (25 mM Tris, pH 7.4, 0.25 M sucrose, 0.5 µg/ml leupeptin, 0.5 µg/ml aprotinin, 1.0 µg/ml pepstatin and 100 µg/ml PMSF). The suspension was centrifuged at 100 000 *g* for 1 h at 4 °C. Protein concentrations in the cell lysates were measured using a BCA kit according to the manufacturer's instructions.

RESULTS

Baculovirus-mediated expression and purification of human ALDH3A1

The ALDH3A1 cDNA was inserted into a baculovirus, and Sf9 cells were infected with the recombinant viruses. Ten independently isolated plaques were amplified and tested for expression of the recombinant protein using Western blot analysis. More than 50 % of the viral plaques produced a 54 kDa protein that immunoreacted with ALDH3A1 antibody, which is consistent with the predicted size of ALDH3A1 (Figure 1). Two recombinant viruses with the highest ALDH3A1 expression were used for subsequent infection of insect Sf9 cells at a multiplicity of 1. Cells were harvested 48 h post-infection and the cytosolic fraction was used for the purification of the human ALDH3A1 protein. Expression of ALDH3A1 in infected Sf9 cells was compared with that of a human lung adenocarcinoma cell line (A549) and a HCE cell line stably transfected with the human ALDH3A1 cDNA

Figure 1 Expression of ALDH3A1 antigen by Sf9 cells infected with baculovirus recombinants

Sf9 cells (5×10^5) infected with recombinant baculoviruses (lanes 2–11) were assayed for ALDH3A1 antigen by Western blotting following SDS/PAGE. Sf9 cells in lane 1 are the uninfected control.

Figure 2 Levels of human ALDH3A1 expression in baculovirus-infected Sf9 cells

ALDH3A1 expression in human lung adenocarcinoma cells (A549), HCE cells stably transfected with human ALDH3A1 (ALDH3A1-1/D) and Sf9 insect cells infected with ALDH3A1/baculovirus (ALDH3A1-Sf9). Different amounts of proteins from total cellular extracts were subjected to SDS/PAGE and analysed by Western blotting as described in the Materials and methods section.

Table 1 ALDH3A1 enzymic activities in cell lines

Specific activities are expressed as nmol of NADPH/min per mg (means $\pm$ S.D., $n = 4$) and were assayed as described in the Materials and methods section. A549, human lung adenocarcinoma cells; ALDH3A1-1/D, HCE cells stably transfected with human ALDH3A1 cDNA; ALDH3A1-Sf9, insect Sf9 cells transfected with human ALDH3A1 cDNA.

Cell line	Specific activity
A549	162 ± 5.3
ALDH3A1-1/D	1655 ± 95.3
ALDH3A1-Sf9	2711 ± 83.4

(ALDH3A1-1/D) by Western blot analysis. Different amounts of cytosolic proteins from A549 (10, 30 and 50 μ g), ALDH3A1-1/D (10, 30 and 50 μ g) and infected Sf9 cells (1, 2.5 and 5 μ g) were run on SDS/PAGE and transferred to PVDF membrane. Immunodetection of ALDH3A1 revealed a 54 kDa protein band with the intensity corresponding to the amount of the loaded protein (Figure 2). Comparison of the ALDH3A1 levels in all three cell extracts showed that Sf9 cells express ALDH3A1 approx. 5 times and 20 times more than the HCE-ALDH3A1-1/D and A549 cells, respectively. ALDH3A1 enzymic activity in these cell lines was proportional to the amount of ALDH3A1 protein expressed in each cell line (Table 1).

Purification of ALDH3A1 was achieved by 5'-AMP-Sepharose 4B affinity chromatography. With this single-step procedure, 18–20 mg of ALDH3A1 can be purified from 500 ml of Sf9 cell culture. The purification of the ALDH3A1 expressed in Sf9 cells is summarized in Table 2. ALDH3A1 activity was monitored spectrophotometrically, using benzaldehyde as the substrate. Purification of ALDH3A1 produced an 8.6-fold increase in ALDH3A1 specific activity and an approx. 6.3% yield. The purification resulted in a single protein band at 54 kDa (Figure 3, lane 2), which was immunostained as an ALDH3A1 protein (Figure 3, lane 3).

Table 2 Purification of human ALDH3A1 expressed in Sf9 cells

The specific activity of human ALDH3A1 was determined using benzaldehyde as the substrate and NADP⁺ as the cofactor at 25 °C as described in the Materials and methods section.

Step	Total protein (mg)	Specific activity (μ mol of NADPH/min per mg)	Yield (%)	Purification (-fold)
Cell lysate	304	3.2	100	—
100 000 g soluble fraction	53.3	16.4	17.5	5.2
5'-AMP-Sepharose 4B eluate	19.2	27.4	6.3	8.6

Figure 3 Purification of human ALDH3A1 expressed in baculovirus-infected Sf9 cells

Lane 1, protein standards on SDS/PAGE (12.5% gel); lane 2, SDS/PAGE (12.5% gel) of 1 μ g of recombinant purified human ALDH3A1 (silver staining); lane 3, immunoblot analysis of 1 μ g of recombinant purified human ALDH3A1.

Kinetic properties of human ALDH3A1

The purified ALDH3A1 protein was used for kinetic studies. Table 3 shows the apparent K_m (μ M) and V_{max} (nmol of NADPH/min per mg of protein) values of homogeneous recombinant ALDH3A1 for several conventional substrates of this enzyme as well as for lipid-derived aldehydes and other substrates. The recombinant enzyme was capable of oxidizing medium-chain (six carbons and higher) saturated and unsaturated aldehydes, such as hexanal, octanal, nonanal, dodecanal, *trans*-2-hexenal, *trans*-2-octenal, *trans*-2-nonenal and 4-HNE, as well as the aromatic aldehyde, benzaldehyde. Short-chain aldehydes (acetaldehyde and propionaldehyde) were found to be poor substrates for ALDH3A1, which showed a greater specificity towards the medium-chain aldehydes than the short-chain aldehydes (two or three carbons), as determined by the catalytic efficiency constant V_{max}/K_m . It appears that ALDH3A1 specificity increases with the chain length (C_6 – C_{12}) of aliphatic aldehydes. Interestingly, the saturated aldehydes used in this study (hexanal, octanal and nonanal) had lower apparent K_m values than the unsaturated aldehydes of the same carbon length (*trans*-2-hexenal, *trans*-2-octenal and *trans*-2-nonenal). Although 4-HNE appeared to be a very good substrate for ALDH3A1 (K_m approx. 45 μ M), MDA proved to be a poor one (K_m approx. 6550 μ M).

Because the cornea exhibits high activity of the pentose phosphate pathway [24], we wanted to determine whether aldehyde intermediates of this pathway were metabolized by ALDH3A1.

Table 3 Kinetic properties of recombinant human ALDH3A1

Apparent K_m and V_{max} values were determined by fitting the data to the Michaelis–Menten equation using SigmaPlot®. V_{max}/K_m represents aldehyde-oxidizing capacity (expressed as nmol of NADPH produced/min per mg of protein per nmol of aldehyde per ml). Data represent means $\pm$ S.E.M. from three separate experiments.

Substrate	V_{max} (nmol of NADPH/ min per mg)	K_m (μ M)	V_{max}/K_m
Acetaldehyde	5445 $\pm$ 133	7034 $\pm$ 635	0.8
Propionaldehyde	426 $\pm$ 24.3	4736 $\pm$ 400	0.1
Hexanal	2570 $\pm$ 59	18 $\pm$ 1.6	142.7
Octanal	251 $\pm$ 11	1 $\pm$ 0.2	251.0
Nonanal	734 $\pm$ 44	0.5 $\pm$ 0.2	1468.0
Decanal	535 $\pm$ 26	0.8 $\pm$ 0.2	669.0
Dodecanal	500 $\pm$ 28	0.5 $\pm$ 0.1	1000.0
<i>trans</i> -2-Hexenal	1676 $\pm$ 67	155 $\pm$ 26	10.8
<i>trans</i> -2-Octenal	7303 $\pm$ 482	35 $\pm$ 7.0	208.7
<i>trans</i> -2-Nonenal	5150 $\pm$ 261	12 $\pm$ 3.0	429.2
4-HNE	963 $\pm$ 140	45 $\pm$ 18	21.4
MDA	685 $\pm$ 31	6550 $\pm$ 501	0.1
Benzaldehyde	19574 $\pm$ 724	200 $\pm$ 30	97.9
Glyceraldehyde	87 $\pm$ 17.4	10500 $\pm$ 3769	0.008
Glucose 6-phosphate	0	0	0
6-Phosphoglucono- δ -lactone	0	0	0
6-Phosphogluconate	0	0	0

In these experiments, ALDH3A1 failed to demonstrate any oxidizing activity with glucose 6-phosphate, 6-phosphoglucono- δ -lactone or 6-phosphogluconate in a wide range of concentrations (Table 3).

Production of monoclonal antibodies against ALDH3A1

Recombinant human ALDH3A1 was used as an immunoantigen to generate monoclonal antibodies in mice. Mouse immunization yielded monoclonal antibody-secreting hybridoma clones, from which three reacted strongly against the homologous immunogen (results not shown). One of these monoclonal antibodies was subsequently used to detect the ALDH3A1 protein in human corneal extracts. The proteins in the corneal extracts from two individuals were subjected to Western blot analysis. Detection using the monoclonal antibody that we developed for ALDH3A1 revealed one strong protein band at the expected molecular mass (54 kDa; Figure 4B, lanes 1 and 2).

Immunohistochemistry on human corneas

Using the monoclonal antibody, we localized the ALDH3A1 immunohistochemically in human corneal tissues (Figure 5). Figure 5(A) shows the intense fluorescence associated with keratocytes throughout the stroma as well as epithelial cells. Staining did not appear to be associated with the endothelium (Figure 5B, arrows). Figure 5(C) shows a higher magnification of the epithelium and the enhanced intensity in the suprabasal cells. No staining was observed after staining with non-specific mouse IgG (Figure 5D, negative control).

DISCUSSION

ALDH3A1 is one of the most abundant proteins expressed in cornea of most mammalian species studied to date and it accounts for 5–40% of the total water-soluble proteins [2]. This

Figure 4 Western blotting of human ALDH3A1 in human corneal extracts using the monoclonal antibody

(A) Proteins in extracts from two different human corneas (lanes 1 and 2) were separated by SDS/PAGE and stained with SyproRuby. (B) The separated proteins in the extracts from two different human corneas after SDS/PAGE (lanes 1 and 2) were transferred on to a PVDF membrane and probed using our monoclonal ALDH3A1 antibody as described in the Materials and methods section. Lane M, molecular-mass markers with sizes indicated in kDa. Arrow on the left indicates the ALDH3A1 protein.

enzyme is believed to play a protective role in the cornea and multiple hypotheses have been postulated to address the nature of such a protective mechanism. These include the metabolism of toxic aldehydes produced during lipid peroxidation under oxidative conditions, direct absorption of UV light, scavenging of free radicals, chaperone-like activity and maintaining corneal transparency [2,25]. Although markedly reduced levels of ALDH3A1 have been detected in mouse opaque cornea [26,27], the direct involvement of ALDH3A1 as a structural element in corneal transparency is uncertain because both SWR/J mice (ALDH3A1-deficient [28,29]) and transgenic *Aldehyde 3A1*-knockout mice [30] have structurally normal corneas with no apparent abnormalities. However, SWR/J mice are more prone to ocular hazing after exposure to UVB than are other inbred mouse strains with wild-type ALDH3A1 expression [31]. The latter observation supports the notion that corneal ALDH3A1 protects this vital tissue from the deleterious effects of UV radiation. We have recently shown that ALDH3A1 protects corneal epithelial cells against UV- and 4-HNE-induced apoptosis [3]. We have also shown UV radiation induces lipid peroxidation in HCE cells with the order of effectiveness being UVA < UVB < UVC [32]. The cornea is constantly exposed to environmental stressors, which may induce oxidative stress and subsequent aldehydic toxicity due to lipid peroxidation. For example, accumulation of α,β -unsaturated aldehydes has been observed in certain eye disorders [5–8]. 4-HNE is the most toxic and the most abundant aldehyde produced by lipid peroxidation and displays a variety of cytotoxic and genotoxic consequences [4]. It is produced during β -scission of alkoxy radicals derived from $\omega - 6$ polyunsaturated fatty acids, which is the most significant event in radical reactions [4]. 4-HNE is highly reactive and it has been suggested to mediate and amplify the cellular effects of its radical precursors [4]. We and others have reported that ALDH3A1 protects against 4-HNE-induced protein adduction, possibly due to metabolism of 4-HNE by this enzyme [3,33]. The major biochemical pathways of 4-HNE metabolism are outlined in Scheme 1 and include: (i) aldehyde

Figure 5 Immunofluorescence of ALDH3A1 in normal human corneas

Normal human corneas ($n = 11$) were obtained from NDRI, embedded in Tissue Tek OCT compound and frozen under liquid nitrogen. Sections of 5 μm were obtained and processed as described in the Materials and methods section using the monoclonal ALDH3A1 antibody (**A–C**) or mouse IgG (**D**), followed by rhodamine-conjugated goat anti-mouse IgG to visualize the primary antibodies. Shown are the representative staining patterns obtained. Scale bars, 50 μm . s, stroma; e, epithelial cells. Small arrows indicate the endothelium area.

Scheme 1 Routes of 4-HNE metabolism in ocular tissues

Abbreviations: ALDH1A1, aldehyde dehydrogenase 1A1; GST, glutathione S-transferase; AR, aldose reductase; ADH, alcohol dehydrogenases; GS-HNE, glutathione conjugate of HNE.

Table 4 Kinetic properties for ocular enzymes towards 4-HNE

Aldose reductase catalyses both the reduction of unconjugated 4-HNE to dihydroxynonene (K_m value is shown) as well as the reduction of conjugated GST (glutathione S-transferase)-4-HNE to glutathione dihydroxynonene (K_m approx. 34 μM ; see Scheme 1).

Enzyme	K_m (μM)	Reference
Human corneal ALDH3A1	110	[12]
Baboon corneal ALDH3A1	220	[43]
Bovine corneal ALDH3A1	400	[44]
Human ALDH3A1 (stable transfected cells)	54	[3]
Human ALDH3A1 (recombinant)	45	This study
Human corneal ALDH1A1	2	[12]
Mouse corneal alcohol dehydrogenase	300	[45]
Human ALDH1A1 (recombinant)	18	[20]
Rabbit ALDH1A1 (recombinant)	2	[20]
Human corneal GST 5.8	80	[46]
Bovine lens GST 5.8	57	[47]
Bovine lens aldose reductase	9	[48]

dehydrogenase-catalysed oxidation to 4-hydroxy-*trans*-2-nonenoic acid, (ii) conjugation with GSH (GS-HNE), (iii) the alcohol dehydrogenase-catalysed reduction to 1,4-dihydroxy-2-nonene (DHN) and (iv) aldose reductase-catalysed reduction of GS-HNE to GS-DHN. The relative contribution of each pathway remains an area of active research. The published kinetic properties of ocular enzymes involved in 4-HNE metabolism are summarized in Table 4. Because controversy surrounds the 4-HNE substrate specificity of ALDH3A1, full characterization of the protein is important, particularly if valid conclusions are to be made regarding the physiological role of ALDH3A1 in ocular tissues. Therefore, a main objective of the present study was to express sufficiently high levels of recombinant human ALDH3A1 using the baculovirus system. The recombinant protein was then subsequently used for biochemical characterization and generation of monoclonal antibodies.

Using the baculovirus system, we obtained Sf9 cells that expressed substantial amounts of a catalytically active human ALDH3A1. We have recently used the baculovirus system for high-level expression of rabbit and human ALDH1A1 proteins [20]. As was the case for ALDH1A1 proteins, ALDH3A1 was readily purified by a single affinity-chromatography step and we obtained 18–20 mg per 500 ml of Sf9 cell culture. Development of such an efficient expression and purification system is an important tool to facilitate the study of catalytic properties of ALDHs, particularly given that the catalytic activities of several human ALDHs are unknown (e.g. ALDH3B1, ALDH3B2 and ALDH7A1) [1]. In addition, the Human Genome Project and the Expressed Sequence Tag (EST) projects have revealed several splice variants for ALDH genes, including ALDH3A1, that eventually should be studied (A. Pappa and V. Vasiliou, unpublished work). The baculovirus system should provide an ideal model for the expression of these proteins.

The K_m of recombinant human ALDH3A1 for 4-HNE reported here is in agreement with that found in human corneal cells stably transfected with the human ALDH3A1 cDNA (K_m approx. 54 μM) [3], and for purified human corneal ALDH3A1 (K_m approx. 110 μM) [12]. Intracellular concentrations of 4-HNE are in the low micromolar range (1–10 μM) [4]. Nevertheless, they may well reach much higher levels (up to the millimolar range) in pathological states [4]. Accordingly, the apparent K_m of recombinant ALDH3A1 for 4-HNE (45 μM) bears pathophysiological significance and favours the notion that human corneal ALDH3A1 plays an essential role in the detoxification of per-

oxidic aldehydes produced during lipid peroxidation. The K_m value of the recombinant human ALDH3A1 for MDA was found to be approx. 6 mM, which is in agreement with that reported for the recombinant rat ALDH3A1 [13], suggesting that MDA is not a good substrate for ALDH3A1. MDA is metabolized by bovine, rat, human and rabbit ALDH1A1 proteins with a K_m in the low micromolar range ([20], and references therein). Ocular ALDH1A1 is predominantly expressed in the lens epithelium and, to a lesser extent, in the cornea [12]. We have recently demonstrated that human ALDH1A1 metabolizes 4-HNE with an apparent K_m of 17.9 μM [20], which is lower than that of ALDH3A1 (K_m 45 μM). However, ALDH1A1 exhibits a lower catalytic efficiency for 4-HNE ($V_{\max}/K_m$ 14.2 nmol of NADH produced/min per mg of protein per nmol of aldehyde per ml) [20] than does ALDH3A1 ($V_{\max}/K_m$ 21.4 nmol of NADPH produced/min per mg of protein per nmol of aldehyde per ml). Based on these data, it may be speculated that these two ALDHs play complementary roles in the protection of the ocular tissue from the deleterious effects of 4-HNE and MDA. Rabbit, which is the only mammalian species known to lack ALDH3A1 expression in cornea, expresses ALDH1A1 in the cornea at levels comparable with those found for ALDH3A1 in other mammalian species [26]. We have recently shown that the catalytic efficiency of rabbit ALDH1A1 for 4-HNE ($V_{\max}/K_m$) is 53.0 nmol of NADH produced/min per mg of protein per nmol of aldehyde per ml [20], which is greater than those of both human ALDH1A1 and ALDH3A1. Accordingly, this protein may compensate for the lack of ALDH3A1 expression in the rabbit, at least in the case of 4-HNE metabolism.

The catalytic efficiency of the human recombinant ALDH3A1 for 4-HNE reported here is consistent with the involvement of this enzyme in the cellular defence against 4-HNE-induced ocular toxicity. HCE cells that lack ALDH3A1 [3] but express ALDH1A1 (A. Pappa and V. Vasiliou, unpublished work) are vulnerable to 4-HNE-induced damage [3]. Transfection of these cells with human ALDH3A1 cDNA protected them against 4-HNE-induced protein adduction and subsequent apoptosis [3]. Similar results have been reported for the mouse alveolar macrophage cells (RAW 264.7) and hamster lung fibroblast cells (V79) [33,34]. Collectively, these results lend further support to the notion that ALDH3A1 plays a significant role in detoxifying 4-HNE *in vivo*.

Kinetic constants of human recombinant ALDH3A1 obtained with other substrates used in the present study demonstrated high substrate specificities for medium-chain aliphatic aldehydes (six carbons and higher, e.g. hexanal, octanal, nonanal and dodecanal). The α,β -unsaturated aldehydes (e.g. *trans*-2-hexenal, *trans*-2-octenal and *trans*-2-nonenal) used in this study exhibited very good substrate specificities but lower affinities for the ALDH3A1 than saturated aldehydes of the same carbon length. Benzaldehyde was found to be a good substrate for ALDH3A1, whereas short-chain aldehydes (acetaldehyde and propionaldehyde) were found to be poor substrates. Similar data have been reported for rat recombinant ALDH3A1 [13] and also for the rat and human liver microsomal ALDH3A2 [35,36].

Besides ALDH3A1, mammalian cornea expresses high concentrations of transketolase, an enzyme involved in the non-oxidative branch of the pentose phosphate pathway [37]. Increased expression of transketolase may accommodate an increased need for NADPH or general metabolic needs of corneal cells. NADP⁺ may serve as a coenzyme for ALDH3A1- and also for glucose-6-phosphate dehydrogenase-mediated reactions, which generate NADPH that can be used in the synthesis of GSH from its oxidized form, GSSG. In this respect, we examined whether ALDH3A1 metabolizes any of the substrates of the oxidative branch of the

pentose phosphate pathway. Our data showed that ALDH3A1 does not metabolize glucose 6-phosphate, 6-phosphoglucono- δ -lactone or 6-phosphogluconate. Furthermore, glyceraldehyde, a glycolytic intermediate, was found to be a poor substrate (K_m approx. 10.5 mM). In the light of these data, one may conclude that human ALDH3A1 is not involved in either the pentose phosphate pathway or glycolysis.

Another important aspect of this study is the immunohistochemical localization of ALDH3A1 in human cornea. Using the monoclonal antibodies described here, we show for the first time that, in addition to epithelial cells, ALDH3A1 is also strongly expressed in stromal keratocytes of human cornea (Figure 5). ALDH3A1 was not detected in endothelial cells. We have observed a similar pattern of ALDH3A1 expression in human, pig and mouse corneas using a polyclonal antibody against this protein (A. Pappa and V. Vasilou, unpublished work). As discussed above, the cornea is constantly exposed to UV light and absorbs most wavelengths below 300 nm [38]. UV light is known to induce lipid peroxidation through generation of reactive oxygen species [39]. Our data indicate that ALDH3A1, a 4-HNE-metabolizing enzyme, is present in both epithelial cells and stromal keratocytes of human cornea, supporting the notion that this protein is a part of the cellular defence mechanism of this tissue against oxidative damage. Elimination of 4-HNE is crucial for maintaining cellular integrity, since 4-HNE-modified proteins have been found to be resistant to degradation by the proteasome and act also as potent inhibitors of the proteasome [40,41]. Inhibition of proteasomal function leads to accumulation of protein aggregates that are ultimately cytotoxic [42].

In summary, we have expressed and purified human ALDH3A1 from a baculovirus expression system and the recombinant protein was used for conducting biochemical studies and generating highly specific monoclonal antibodies against human ALDH3A1. The kinetic studies suggest that human ALDH3A1 may play an important metabolic role in detoxifying aldehydes produced from lipid peroxidation. The immunohistochemical studies revealed the regional distribution of ALDH3A1 in the human cornea. ALDH3A1, expressed at a high level employing the baculovirus system, can be purified in a single step and, together with the monoclonal antibodies described here, will be useful in providing further insight into the relationship between ALDH3A1 expression and eye pathologies.

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