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Expression and Localization of Human Alcohol and Aldehyde Dehydrogenase Enzymes in Skin

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Alcohol dehydrogenase (ADH; EC 1.1.1.1) and aldehyde dehydrogenase (ALDH; EC 1.2.1.3.) are important enzymes involved in the biotransformation of both alcohols and aldehydes. Today, six classes of ADH and twelve classes of ALDH have been defined in mammals. Here we report the detection and localisation of three classes of ADH and two classes of ALDH in human skin, using Western blot analysis and immunohistochemistry with class-specific antisera. Western blot analysis of human skin cytosol revealed that class I-III ADH and class 1 and class 3 ALDH enzymes are expressed, constitutively, in three different anatomical regions of human skin (foreskin, breast, abdomen). Densitometric analysis of the immunoreactive bands revealed differential constitutive expression of these enzymes in foreskin, breast, and abdomen skin. Immunohistochemistry showed the presence of class I ADH and class III ADH enzymes, predominantly in the epidermis with some localised expression in the dermal appendages of human skin. In comparison, staining for class II ADH was more faint in the epidermis with very little dermal expression. Class 1 ALDH and class 3 ALDH were predominantly localised to the epidermis with minimal, highly localised dermal appendageal expression. These cutaneous ADH and ALDH enzymes may play significant roles in the metabolism of endogenous or xenobiotic alcohols and aldehydes.

Alcohol dehydrogenase (ADH; EC 1.1.1.1) and aldehyde dehydrogenase (ALDH; EC 1.2.1.3) are key polymorphic enzymes involved in the biotransformation of a wide variety of endogenous and exogenous alcohols and aldehydes in the body. Endogenous substrates for ADH enzymes include retinol, 3β-hydroxysteroids, ω-hydroxy fatty acids and the lipid peroxidation product 4-hydroxynonenal. ALDH enzymes have been implicated in the metabolism of endogenous lipid peroxidation products such as malondialdehyde and hydroxyalkenals [1–5]. Skin contact with xenobiotic alcohols (e.g. ethanol, cinnamyl alcohol) and aldehydes (e.g. formaldehyde, acetaldehyde, furfural, cinnamaldehyde), as a result of domestic, environmental and occupational exposure, may cause local toxicity (i.e. skin sensitization or irritancy) in susceptible humans [6–11]. These xenobiotics may also be absorbed across the skin resulting in systemic exposure [12, 13]. In particular, aldehydes such as acrolein, 2,3-epoxypropanal and formaldehyde, are highly reactive chemicals that can cause cytotoxicity, mutagenicity and carcinogenicity, by binding to host macromolecules [5, 14, 15].

Six classes of mammalian ADHs, have been defined by sequence homology and substrate specificity, all of which are either homo- or hetero-dimers comprised of two 40 kDa subunits [16]. Class I ADH is abundant in the liver and primarily metabolises primary alcohols and is the main ethanol-metabolising isoenzyme [17]. Class II ADH metabolises primary aliphatic alcohols and aromatic aldehydes [18, 19] and has been identified mainly in the liver, with low mRNA levels in stomach, pancreas and small intestine [20]. Class III ADH is a formaldehyde specific isoenzyme identical to glutathione-dependent formaldehyde dehydrogenase and performs poor ethanol-metabolism [21]. Estonius *et al.* (1996) observed differential expression of mRNA for class I–IV ADH in a wide variety of human tissues, including liver, kidney and stomach, by Northern-blot analysis, but skin was not evaluated [20]. Class I, II and III ADH have been identified in human whole skin homogenates at the mRNA level by Engeland & Maret (1993) [22] but no previous studies have identified specific classes of ADH at the protein level in human skin.

Mammalian ALDH enzymes were originally categorised into three classes: class 1 includes the cytosolic forms; class 2 includes the mitochondrial forms and

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class 3 comprises the cytosolic dioxin-inducible ALDH 3 enzyme and microsomal ALDHs [23]. Today, human ALDHs are categorized into twelve different classes based primarily on gene expression and substrate specificity [24]. Class 1 ALDH is known to be present constitutively in human liver and in many other human tissues including brain, hair roots and red blood cells [25–28]. Retinal is a good substrate for class 1 ALDH [29] and retinal metabolism has previously been observed in human skin *in vitro* [30]. Class 1 ALDH is also a major soluble constituent of eye lens and has been implicated in the detoxication of peroxidic aldehydes produced by ultraviolet light absorption [31]. Class 3 ALDH preferentially metabolises aromatic aldehydes [24]. Four arbitrary bands relating to ALDH activity were observed in human fibroblasts and hair roots using isoelectric focusing gel techniques with acetaldehyde-activity staining [32–33]. However, none of the class 1-3 ALDHs have conclusively been identified to date at either the mRNA or protein level in human skin.

Since ADH and ALDH enzymes play a role in the metabolism of endogenous and xenobiotic alcohols and aldehydes, to which the skin is exposed, it is of importance to determine the presence of these enzymes in this tissue. Here, we report the presence of class I, II and III ADH and class 1 and 3 ALDH in human skin cytosol by Western blot analysis and their localisation in intact human skin by immunohistochemistry, using class-specific antisera.

MATERIALS AND METHODS

Human tissue samples. Human foreskin (11 samples), breast (10 samples) and abdomen (7 samples) skin samples were obtained from a total of 27 individuals (aged between 1 and 78 years, Caucasian or Afro-Caribbean) who underwent surgery at St. Mary's Hospital, London. All of the abdomen skin samples came from female donors and, in one case, both breast and abdomen skin was obtained from the same female 43 year old donor. Directly following excision, the tissue was either placed in ice cold 0.9% saline solution then used immediately or snap frozen in liquid nitrogen and stored at −70°C for later use. A human control liver sample was obtained from a 12 year old male traffic accident victim admitted at Addenbrookes Hospital, Cambridge. All human skin and liver samples were obtained with ethical approval from the Local Research Ethics Committees of St. Mary's Hospital and Addenbrookes Hospital, respectively.

Preparation of skin and liver cytosol fractions. All excised skin was cleaned of fat and connective tissue and weighed. The skin samples were then minced with scissors and homogenised in ice-cold sucrose buffer (~3-4 g of skin per ml of 0.25 M sucrose buffer, pH 6.8 containing 15 mM Tris and 0.1 mM EDTA) using an Ultra Turrax homogeniser (Merck, UK). All homogenates were filtered through 140 μm nylon gauze (Lockertex, UK) to remove large debris, and differential centrifugation was performed to prepare cytosol fractions as follows. The filtered crude homogenate was spun initially at 600g for 10 minutes using a Sorvall RC-5B refrigerated superspeed centrifuge (Dupont Instruments) and the resulting supernatant spun at 10,000g for 20 minutes. The supernatant from this second spin was further spun at 100,000g for 1 hour using a Beckman Combi Ultracentrifuge (Dupont Instruments) and the resulting supernatant was designated as the cytosol fraction. The total protein content of all cytosol samples was determined using the standard method by Lowry *et al.* [34].

Western blot analysis. Western blot analysis was performed using 20 μg of total skin cytosolic protein and either 0.2 μg or 1 μg of total liver cytosolic protein. Samples were prepared for electrophoresis in Laemmli's sample buffer containing 6 mg/ml dithiothreitol. SDS-PAGE was performed using the BIO-RAD Mini-Protean II gel system, with 4% acrylamide stacking and 10% acrylamide resolving minigels (run until the dye front reached the bottom of the gel) using standard techniques [35]. It was possible to run all 28 skin samples on two minigels. Human liver cytosol was run on each gel as a control. To reduce the variations resulting from electrotransfer and subsequent development with antisera, in particular for semiquantitative analysis, the central sections of the two resolving gels containing the regions of interest (between ~20 kDa and ~90 kDa) were excised. The two gels were then placed adjacent to one another on a single nitrocellulose membrane (Schleicher & Schuell, Germany), which allowed simultaneous electrotransfer and development with antisera. Transfer of the proteins from gel to nitrocellulose membrane was performed at 150 V for 1 hour at 4°C. Membranes were blocked overnight at 4°C in 10 mM Tris-HCl buffer, pH 7.6, containing 0.25% (w/v) casein, 0.15 M NaCl and 0.5 mM thimerosal. The membranes were then developed for immunoreactivity using a selection of class-specific rabbit anti-human ADH antisera [36, 37], or rabbit anti-rat ALDH antisera [38, 39]. ALDH antisera were generously provided by Prof. Ronald Lindahl, University of South Dakota. All antibodies were diluted in 10 mM Tris-HCl buffer, pH 7.6, containing 0.05% casein, 0.15 M NaCl, 0.5 mM thimerosal. Antisera were used as follows: anti-class I ADH (diluted 1:22,000), anti-class II ADH (diluted 1:5000); anti-class III ADH (1:15,000); anti-class 1 ALDH (diluted 1:3500) or anti-class 3 ALDH (diluted 1:5000). Antibody-binding was detected using a goat anti-rabbit IgG conjugated to horseradish peroxidase (Appligene-Oncor-Lifescreen, UK)) (diluted 1:10,000), visualised using enhanced chemiluminescence (ECL reagent, Pierce, Rockford, USA) and recorded on hyperfilm (Amersham, UK). The relative intensity of immunoreactive bands was determined by measuring optical density using the Quantiscan software package (Biosoft, UK). Local background subtraction was achieved for each band, separately, by using a near-by reference area.

Preparation of skin for immunohistochemistry. Full thickness skin samples were placed in 4% paraformaldehyde (buffered with sodium phosphate to pH 7.4) and fixed overnight at 4°C before processing into paraffin-wax embedded blocks. Sections (5 μm) were cut and placed onto poly-L-lysine coated slides. Processing and sectioning was performed by the Department of Histopathology at St. Mary's Hospital, London.

Immunohistochemistry. All immunohistochemistry procedures were performed at room temperature. Skin sections were deparaffinised in xylene and rehydrated in six ethanol washes of decreasing percentage (100-70%). Endogenous peroxidase was quenched by incubation in 3% hydrogen peroxide:TBS (Tris buffered saline) and non-specific sites blocked by 25% normal goat serum:TBS. Sections were then probed with the respective antisera (diluted with TBS): anti-class I ADH (1:400); anti-class II ADH (1:250); anti-class III ADH (1:400); anti-class 1 ALDH (1:250); anti-class 3 ALDH (1:250) or non-immune sera (1:250). Antibody-binding was detected using a goat anti-rabbit IgG conjugated to biotin (DAKO, UK) (diluted in TBS to 1:500) with subsequent bound biotin detected using a horseradish peroxidase streptavidin conjugate (Vector Laboratories, Peterborough, UK) (diluted in TBS to 1:200). Bound peroxidase activity was observed by developing with diaminobenzidine (DAB) (Vector Laboratories, Peterborough, UK). Sections were counterstained with Gill's haematoxylin and visualised with light microscopy.

Caucasian skin sections were used for immunohistochemistry, as the signal resulting from DAB development, may be confused with

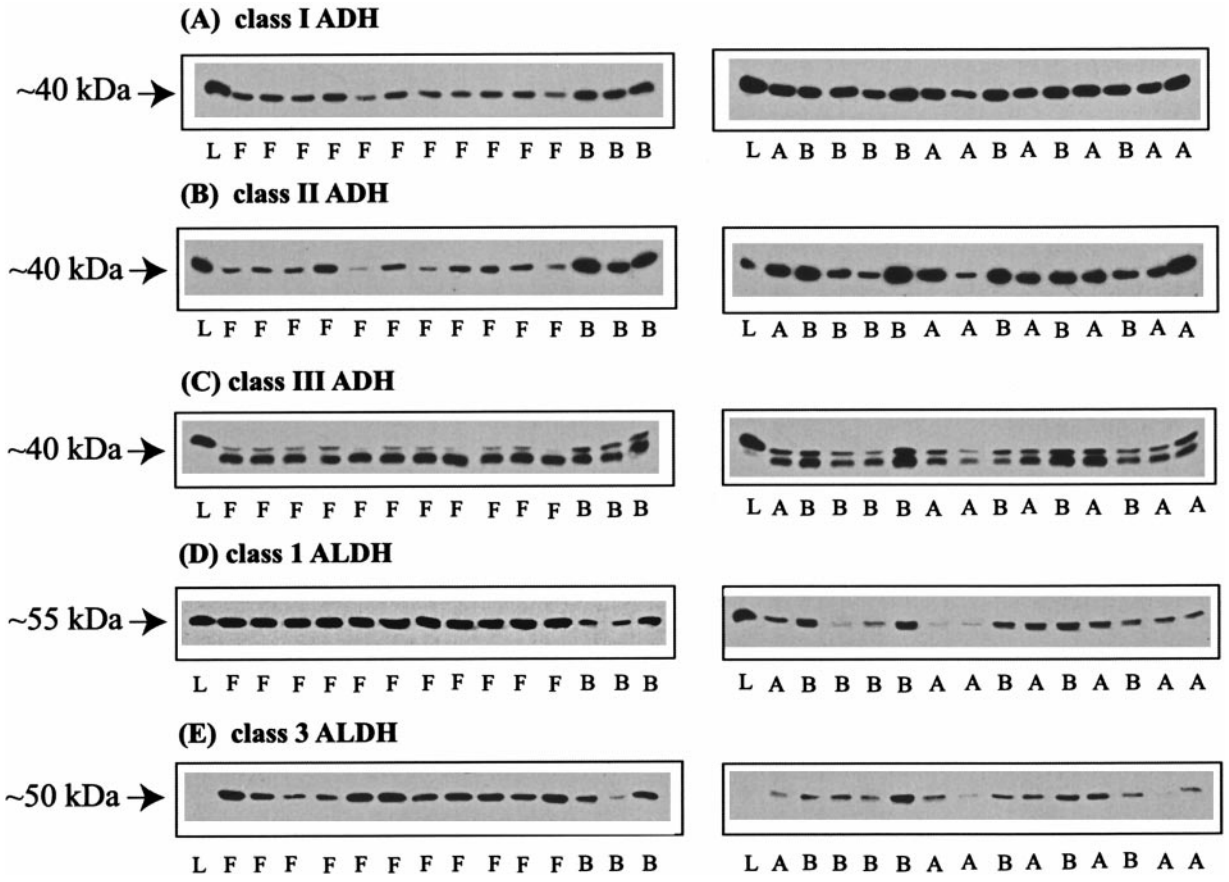

FIG. 1. Western blot analysis of ADH and ALDH enzymes in human skin cytosol fractions. Nitrocellulose membranes were probed with antisera containing antibodies to (A) class I ADH (diluted 1:22 000), (B) class II ADH (diluted 1:5000), (C) class III ADH (diluted 1:12 000), (D) class 1 ALDH (diluted 1: 3500) and (E) class 3 ALDH (diluted 1:5000). L = human liver cytosol fraction (all 1 μg total protein loaded except for (B) 0.2 μg loaded). Lanes 1 to 28 represent different human skin cytosol fractions (all 20 μg total protein loaded), loaded with increasing age, from 1 to 78 years of age. Lanes labelled with F represent foreskin samples, age range 1 to 11 years with median age 5, (n = 11); lanes labelled with B represent breast samples, age range 23 to 59 years with median age 35.5, (n = 10); lanes labelled with A represent abdomen samples, age range 30 to 78 years with median age 43, (n = 7).

the layer of brown melanin at the epidermal-dermal junction, characteristic of Afro-Caribbean skin. Serial sections of skin were investigated to allow ease of comparison between non-immune and immune sera.

RESULTS

Western Blot Analysis

Western blot analysis, using the class-specific anti-human ADH and anti-rat ALDH antisera, revealed immunoreactive bands corresponding to the subunit molecular masses of ADH (~40 kDa) and ALDH (~50–55 kDa) monomers, respectively (Fig. 1). In all cases, there was no indication of cross-reactivity with other proteins in any of the human samples, under the conditions used.

The class I and II ADH antisera revealed single immunoreactive bands at ~40 kDa, in all 28 human skin samples (Figs. 1A and 1B) and the human liver sample. For both of these proteins, densitometric analysis revealed the level of expression was similar in breast and abdominal skin, but was significantly ($P < 0.001$) lower in foreskin compared to that in breast and abdominal skin (Figs. 2A and 2B). The class III ADH antisera revealed a doublet immunoreactive band at ~40 kDa, in all 28 human skin samples, but a single band in the human liver sample (Fig. 1C). The upper immunoreactive band of the doublet in the skin sample comigrates with the single band in the human liver sample. Densitometric analysis of both bands of the doublet revealed a comparable level of class III ADH protein expression in foreskin, breast and abdomen skin (Fig. 2C). Accounting for differences in total protein loaded, the levels of class I ADH, class II ADH and class III ADH protein in the liver sample were shown to be approximately 30-fold, 80-fold and 22-fold higher than the mean level from all 28 skin samples, respectively.

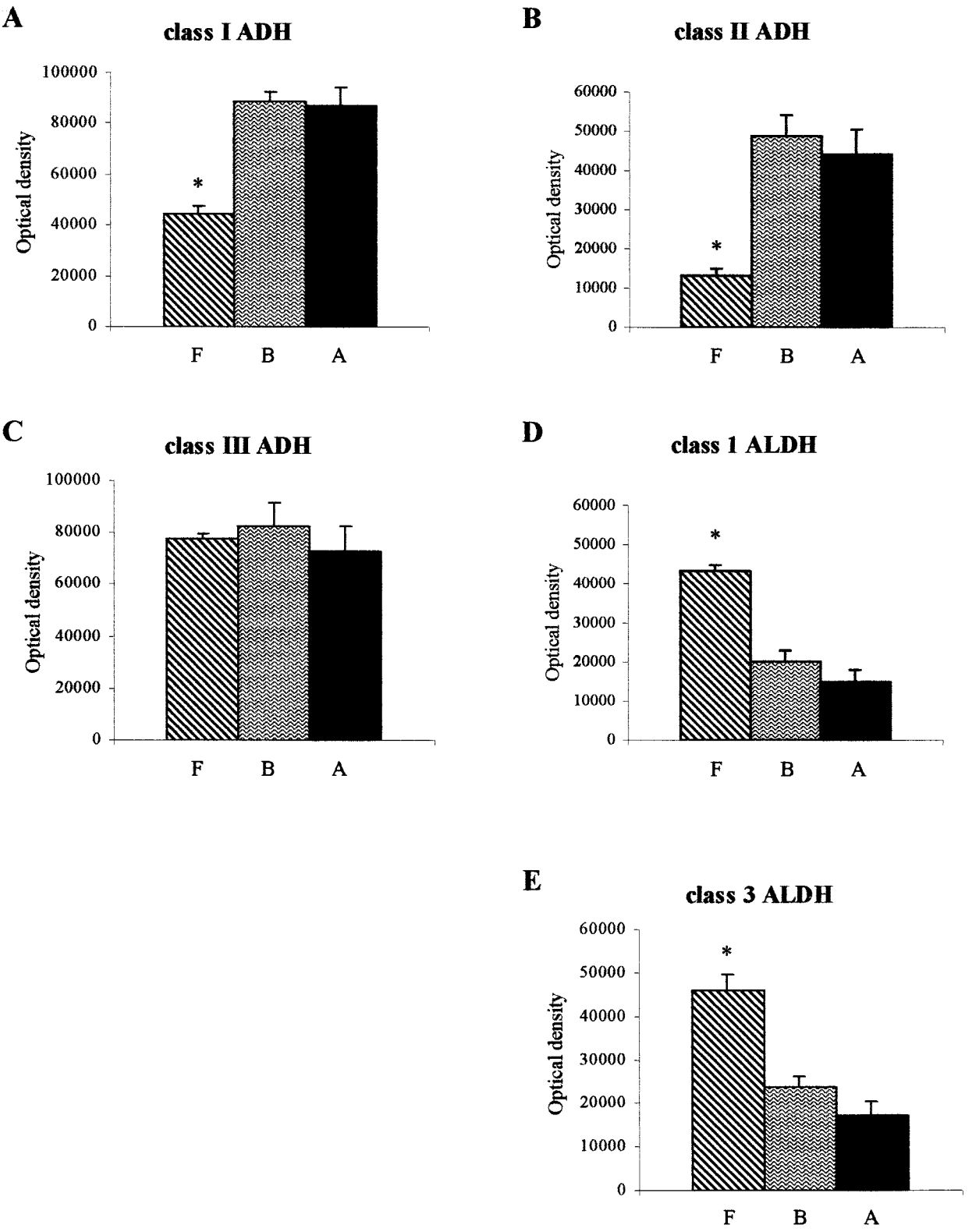

FIG. 2. Semiquantitative densitometric analysis of immunoreactive bands resulting from Western blot analysis of human skin cytosol with class specific ADH and ALDH antisera. Densitometric analysis of immunoreactive bands resulting from detection with antisera to (A) class I ADH, (B) class II ADH, (C) class III ADH, (D) class 1 ALDH and (E) class 3 ALDH, was performed using Quantiscan (Biosoft). Values are means (± SEM) of the optical density (net volume of pixels per specified area), in arbitrary units. Diagonal lined bars represent male foreskin (n = 11), wavy lined bars represent female breast skin (n = 10) and solid bars represent abdominal skin (n = 7). * $P < 0.001$, highly significant difference in optical density readings from foreskin compared to breast skin or abdomen skin, using ANOVA parametric analysis (Instat 2.03).

The anti-class 1 ALDH and anti-class 3 ALDH antisera revealed single immunoreactive bands at ~55 kDa and ~50 kDa respectively in all 28 human skin samples (Figs. 1D and 1E). The class 1 ALDH antisera detected a single 55 kDa immunoreactive band in the human liver sample, but the class 3 ALDH antisera did not detect any immunoreactivity in this sample. Densitometric analysis showed the levels of class 1 ALDH and class 3 ALDH expression were comparable in breast and abdomen skin, whereas the levels were significantly higher ($P < 0.001$) in foreskin compared to those in breast and abdominal skin (Figs. 2D and 2E). Accounting for differences in total protein loaded, the level of class 1 ALDH protein in the liver sample was calculated to be approximately 22-fold higher than the mean density for all 28 skin samples.

Immunohistochemistry

Classes I–III ADH and classes 1 and 3 ALDH were detected in all human skin sections (Fig. 3). Skin sections incubated with non-immune sera showed no immunoreactive staining (Fig. 3A).

Using class 1 ADH antisera, immunostaining of class I ADH protein was observed throughout the epidermis with equivalent expression in all keratinocyte layers; dermal expression was highly localised, particularly in the microvasculature and sweat ducts (Fig. 3B). Class II ADH antisera revealed immunostaining throughout the epidermis, with slightly higher staining at the keratinocyte layer directly beneath the stratum corneum, and very little in the dermis (Fig. 3C). Class III ADH antisera immunostaining was observed at the basal epidermis and in the dermis, associated with the dermal appendages/microvasculature (Fig. 3D). Similarly, class 1 ALDH and class 3 ALDH antisera immunostaining was evenly distributed throughout the epidermis, with low level staining in selected dermal cells (Figs. 3E and 3F).

DISCUSSION

In this study, the expression and localisation of ADH and ALDH enzymes in human skin has been observed by Western blot analysis and immunohistochemistry. Using specific antisera, we have shown the constitutive presence of classes I-III ADH and classes 1 and 3 ALDH in all 28 human skin samples analysed.

The detection of ADH and ALDH enzymes in tissues has, until recently, been hampered by the availability of methodologies to discriminate between the various classes. Previously, different ADH and ALDH enzymes were identified in human cultured fibroblasts and hair roots using isoelectric focusing (IEF) techniques with ethanol- and acetaldehyde-activity staining [32]. Spectrophotometric activity assays had also been used to analyse ADH activity in human whole skin homogenates [40–42]. However, overlapping substrate specificity and a lack of specific inhibitors provided limitations in identifying specific classes with these methods. The advent of class-specific antibodies has now enabled individual classes to be conclusively identified in a variety of tissue samples [36–39]. Western blot analysis for ADH, as reported in this study, extends the Northern blotting studies performed by Engeland and Maret (1993), who identified classes I, II and III ADH mRNA in human whole skin homogenates [22]. However, no previous studies have been performed to show the presence of specific ALDH classes in human skin at either the mRNA or protein level.

Densitometric analyses of the Western blots revealed lower levels of class I and class II ADH, and higher levels of class 1 and class 3 ALDH in foreskin, compared to the respective levels in breast or abdomen skin. These results may represent anatomical site variations in cutaneous ADH and ALDH expression, an observation previously not reported for these enzymes, although documented for others [43–45]. Alternatively, they may be due to gender differences: all breast and abdomen skin samples were from females and, naturally, all foreskin samples were from males, although no evidence of sex-dependent variation in human hepatic ADH activity has been documented [46]. A further potential variable in this study was age: all foreskin samples were from children (1–11 yrs) whilst breast and abdomen samples were from adults (23–59 and 30–78 yrs, respectively). However, Wynne *et al.* (1992), concluded that age (45–88 yrs) had no effect on ADH activity in human liver [46], although age-related variations in human hepatic activity of other enzymes, such as cytochrome P450, have been documented [47].

Interestingly, classes I, II and III ADH were present at higher levels (ranging from 22 to 80-fold) in the control liver sample compared to the mean level in skin. Also, the level of class 1 ALDH was shown to be approximately 22-fold higher in liver than in skin. This is in accordance with comparative expression levels between other skin and liver enzymes in rodents, including cytochrome P450s [48], esterases [49] and glutathione S-transferase [48].

The absence of constitutive expression of class 3 ALDH, the aromatic aldehyde metabolising enzyme, in human liver cytosol is in agreement with a number of authors who have shown a low or undetectable expression of this enzyme in normal liver [5, 24]. However, class 3 ALDH enzyme expression has been observed to be inducible in rodent liver, following exposure to tumourigenic chemicals, such as dioxin, or during neoplastic transformation [39, 50]. Also, class 3 ALDH is strongly expressed in about 70% of poorly differentiated and 30% of well differentiated human hepatocellular carcinomas [51]. Such studies may suggest that class III ALDH induction provides the means to increase the detoxication of tumour-causing xenobiotics.

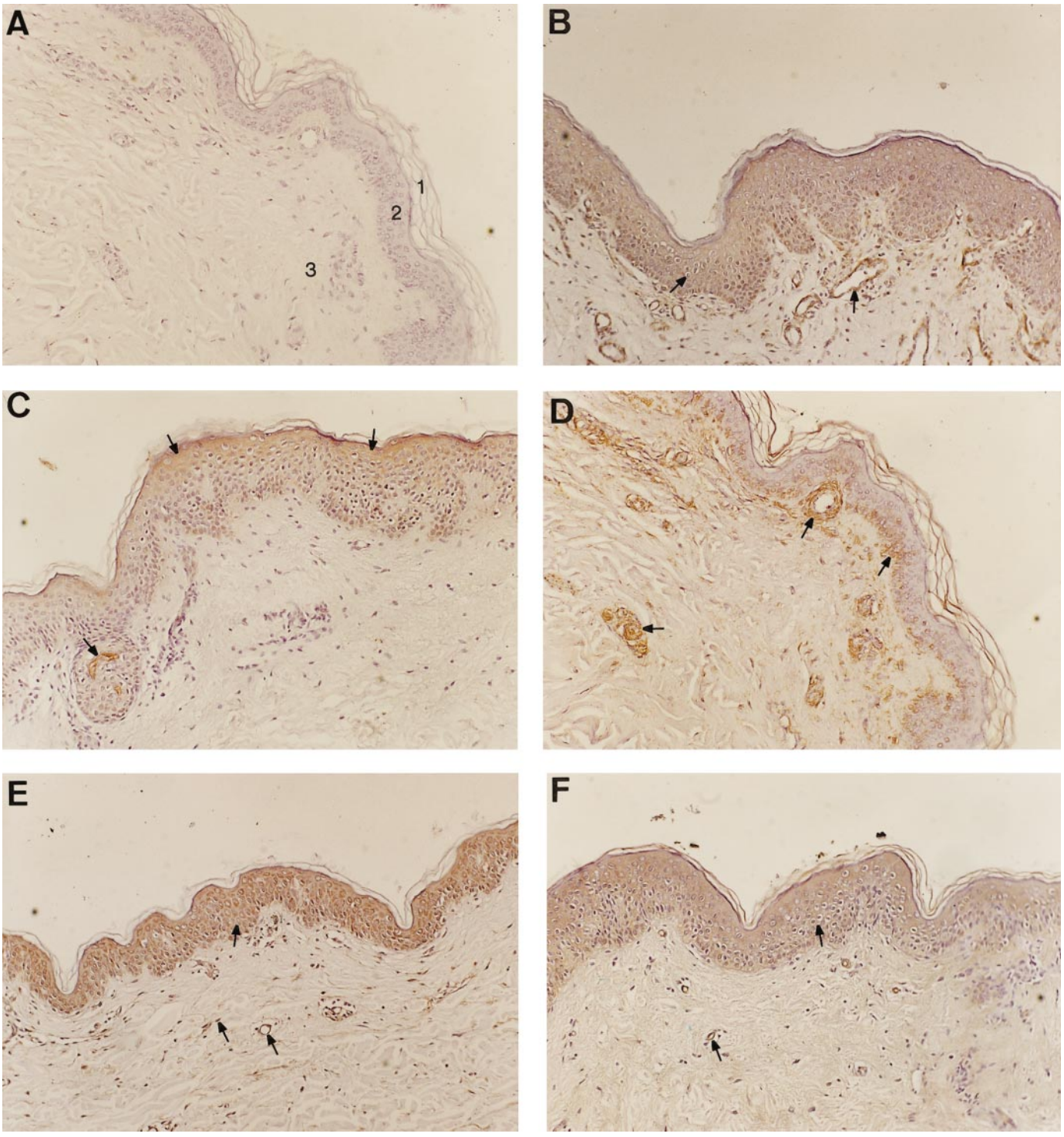

FIG. 3. Immunohistochemical analysis of ADH and ALDH proteins in human skin sections. Human skin sections (5 μm) were probed with antisera containing antibodies specific to different ADH and ALDH classes. Section (A) was incubated with non-immune sera as a control. Sections were incubated with antisera to (B) class I ADH (diluted 1:400); (C) class II ADH (diluted 1:250); (D) class III ADH (diluted 1:400); (E) class 1 ALDH (diluted 1:250) and (F) class 3 ALDH (diluted 1:250). Sections (A) and (D) were taken from a 78 year female abdomen skin sample; section (B) was taken from a 5 year male foreskin sample; sections (C) and (F) were taken from a 24 year breast skin sample and section (E) was taken from a 39 year breast skin sample. All immunostaining was from diaminobenzidine (DAB) development (brown colour) and all sections were counterstained with Gill's haemtoxylin. Magnification $\times$ 125. Labels on section (A) are as follows: (1) stratum corneum; (2) epidermis; (3) dermis. Arrows on all sections indicate specific immunostaining.

The constitutive expression of class 3 ALDH in the skin, as reported in this study may provide a role in the metabolism and toxicity of xenobiotic and endogenous chemicals. In addition, class 3 ALDH in skin may also aid detoxication of commonly encountered xenobiotic aromatic aldehydes, to which the skin is exposed in the form of industrial and environmental chemicals and consumer products.

In terms of the distribution and localisation of these ADH and ALDH enzymes, immunohistochemistry revealed classes I, II and III ADH and classes 1 and 3 ALDH proteins to be present, constitutively, predominantly within the epidermis of human skin. Class I and class III ADH were additionally localised to specific dermal cells and structures such as the microvasculature and sweat ducts. The presence of all ADH and ALDH enzymes in the epidermis is consistent with its role as a major site of xenobiotic metabolism and its position at the interface between the external environment and internal body organs [52]. The dermal cell staining with class I and class III ADH antisera may be in fibroblasts as Buehler *et al.* (1982) detected ADH in human skin fibroblast cultures, using immunohistochemical analysis with anti-ADH antiserum [53]. This distribution is in agreement with that of other enzymes; the cytochrome P450 isoforms CYP 1A1/2 and CYP3 have also been observed to be predominantly localised to the epidermis by immunohistochemical analyses with some dermal staining for CYP3A in human skin [54, 55].

In conclusion, the enzymes classes I, II and III ADH and classes 1 and 3 ALDH have been seen to be expressed, constitutively, in human skin, with localisation in specific regions predominantly within the epidermis. Presupposing that these enzymes are catalytically active, it might be hypothesised that they play important roles in the cutaneous metabolic activation or detoxication of endogenous and xenobiotic alcohols and aldehydes.

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