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ALDEHYDE DEHYDROGENASES AND CELL PROLIFERATION

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ABSTRACT

Aldehyde dehydrogenases (ALDHs) oxidize aldehydes to the corresponding carboxylic acids using either NAD, or NADP as a coenzyme. Aldehydes are highly reactive aliphatic, or aromatic molecules which play an important role in numerous physiological, pathological and pharmacological processes. ALDHs have been discovered in practically all organisms and there are multiple isoforms, with multiple subcellular localizations. More than 160 *ALDH* cDNAs, or genes have been isolated and sequenced to date from various sources, including bacteria, yeast, fungi, plants and animals.

The eukaryote *ALDH* genes can be subdivided into several families; the human genome contains 19 known *ALDH* genes, as well as many pseudogenes. Noteworthy is the fact that that elevated activity of various ALDH families, namely ALDH1A2, ALDH1A3, ALDH1A7, ALDH2*2, ALDH3A1, ALDH4A1, ALDH5A1, ALDH6, and ALDH9A1, has been observed in normal and cancer stem cells. Consequently, ALDH may be not only be considered a marker of these cells, but also may well play a functional role in terms of self-protection, differentiation and/or expansion of stem cell populations.

The *ALDH3* family includes enzymes able to oxidize medium-chain aliphatic and aromatic aldehydes, such as peroxidic and fatty aldehydes. Moreover, these enzymes also have non-catalytic functions, including antioxidant functions and some structural roles. The gene of the cytosolic form, *ALDH3A1*, is localized on chromosome 17 in human beings and on the 11th and 10th chromosome, in the mouse and rat, respectively. ALDH3A1 belongs to the phase II group of drug-metabolizing enzymes and is highly expressed in the stomach, lung, keratinocytes and cornea, but poorly, if at all, in normal liver. Cytosolic ALDH3 is induced by polycyclic aromatic hydrocarbons, or chlorinated compounds, such as 2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin in rat liver cells and increases during carcinogenesis. It has been observed that this increased activity is directly correlated to the degree of deviation in hepatoma and lung cancer cell lines, as is the case in chemically-induced hepatoma in rats. High ALDH3A1 expression and activity have been correlated with cell proliferation, resistance against aldehydes derived from lipid peroxidation and resistance against drug toxicity, such as oxazaphosphorines. Indeed, cells with a high ALDH3A1 content are more resistant to the cytostatic and cytotoxic effect of lipidic aldehydes than are those with a low content. A reduction in cell proliferation can be observed when the enzyme is directly inhibited by the administration of synthetic specific inhibitors, antisense oligonucleotides or siRNA, or indirectly inhibited by the induction of PPAR γ (Peroxisome Proliferator-Activated Receptor) with polyunsaturated fatty acids, or **PPAR γ transfection**. Conversely, cell proliferation is stimulated by the activation of ALDH3A1,

whether by inhibiting PPAR γ with a specific antagonist, antisense oligonucleotides, siRNA, or a **medical device (i.e. composite polypropylene prosthesis for hernia repair)** used to induce cell proliferation. To date, the mechanisms underlying the effects ALDH has on cell proliferation are not yet fully clear. A likely hypothesis is that the regulatory effect is mediated by the catabolism of some endogenous substrates deriving from normal cell metabolism, such as 4-hydroxynonenal, which have the capacity to either stimulate, or inhibit the expression of genes involved in regulating proliferation.

Keywords: ALDH families; ALDH3A1; stem cells; normal and cancer cells; breast; liver; lung; skin; Ah gene battery; drug resistance; cell proliferation; PPAR; polyunsaturated fatty acids; lipid peroxidation; 4-hydroxynonenal.

Introduction

Aldehyde dehydrogenase (ALDH) is a family of enzymes involved in the maintenance of cellular homeostasis by metabolizing both endogenous and exogenous reactive compounds [1,2]. Indeed, they are known to modulate several cell functions, including proliferation, differentiation and survival, as well as the cellular response to oxidative stress. ALDHs exhibit a rather broad substrate specificity and many of them are able to oxidize several highly-reactive aliphatic and aromatic aldehydes that play an important mediating role in numerous physiological, pathological and pharmacological processes. Generally speaking, aldehydes derived from either exogenous, or endogenous precursors, may be either beneficial, or harmful to human health. Exogenous aldehydes, whether intermediates, or products, may derive from the metabolism of drugs (e.g. ethanol, cyclophosphamide, ifosfamide), or substances present in the environment (i.e. smog, cigarette smoke and vehicle exhaust fumes), or may even be introduced as such [1,3-6]. Endogenous aldehydes derive from the metabolism of amino acids, biogenic amines, vitamins, steroids, or lipids. Different aldehydes perform various different functions e.g. retinal is required for vision, whereas its oxidation product from ALDH, retinoic acid, is involved in tissue differentiation. Betaine, derived from oxidation of betaine aldehyde, an intermediate product of choline metabolism, has a crucial defence role against osmotic stress. Whilst γ -aminobutyric acid, semi-aldehyde succinic dehydrogenase is involved in its catabolism [1,3,5,7]. Aldehydes are also potent electrophiles formed during oxidative stress and may interact with cellular nucleophiles, such as proteins and nucleic acids [8,9]. ALDHs are able to detoxify such cytotoxic and cytostatic aldehydes into less reactive forms [10,11].

By using NAD, or NADP as coenzymes, ALDHs oxidize aldehydes to their corresponding carboxylic acids. Although interest in the role of ALDH was initially concentrated on the metabolism of ethanol, where acetaldehyde is transformed into acetic acid, subsequently, the metabolism of other important compounds has also been examined. Since then, numerous studies have demonstrated the presence of ALDHs in practically all organisms, as well as in multiple isoforms [12-14] and their presence in the cytoplasm, nucleus, mitochondria and the endoplasmic reticulum. Depending on the enzyme family and subfamily, ALDH enzyme levels vary, as does tissue and organ distribution [3].

They also exhibit additional, non-enzymic functions, including the capacity to bind to some hormones and other small molecules, as well as that of attenuating the effect ultraviolet irradiation has on the cornea. Mutations in ALDH genes, that lead to a defective aldehyde metabolism, are the molecular basis of several diseases, including gamma-hydroxybutyric aciduria, pyridoxine-

dependent seizures, Sjögren-Larsson syndrome and type II hyperprolinaemia and may even contribute to the etiology of complex diseases, including cancer and Alzheimer's disease. Interestingly, several ALDH enzymes appear to be markers for both normal and cancer stem cells [1]. More than 160 *ALDH* cDNAs, or genes have been isolated and sequenced from various sources e.g. bacteria, yeast, fungi, plant and animals [15-17] and current knowledge reports 19 putatively functional genes and many pseudogenes in the human genome [1].

ALDH families

A total of 86 distinct cDNAs, or genes of *ALDH* have been cloned in eukaryotes and have been divided into several families, including subfamilies, showing a sequence similarity of 60 % or more (from <http://www.aldh.org/superfamily.php>) [18,19]. At present, *family 1* has 11 subfamilies, *families 3* and *5* each have 3 subfamilies, *families 4, 6, 7, 8* and *9* have two subfamilies and the other families have one subfamily each [18,19]. Every single *ALDH* gene subfamily is a genetically segregating distinct unit, or cluster of genes, located close together in the same chromosomal region. For example, mouse *Ahd3a1* and *Ahd3a2* genes are localized in the same mapping position on chromosome 11 and human *ALDH3A1* and *ALDH3A2* are localized to the same mapping position on chromosome 17. To date, the human *ALDH* superfamily is currently known to have 19 putatively-functional genes, clustered in 11 families and 4 subfamilies [19, 20] with distinct chromosomal locations. ALDH families have some specific functions such as:

- the *ALDH1* family, present especially in the cytosol of various tissues, includes enzymes able to oxidize retinal and aliphatic aldehydes. It also plays a role in the detoxification of peroxidic aldehydes produced by ultraviolet light absorption, protecting the lens of the eye [21]. Moreover, it exhibits high activity for oxidation of aldophosphamide and has a role in the detoxification of some commonly used anticancer drugs, such as oxazaphosphorines [22,23]. It has been demonstrated that cancer cell acquired drug resistance is associated to the transcriptional activation of *ALDH1* expression [24];
- the *ALDH2* family codes mitochondrial ALDH, which has a high affinity for acetaldehyde and plays an important role in its detoxification. The greater sensitivity of oriental populations to alcohol is associated with a genetic deficiency of ALDH2, due to the presence of a gene polymorphism of the allele *ALDH2*2* [6,25];
- the *ALDH3* family contains enzymes which have a specific role in fatty and peroxidic aldehyde metabolism and an high ALDH3 activity is present in the cornea, stomach, liver endoplasmic reticulum, as well as in some cancer cells [26];

- the *ALDH4* family codes for a mitochondrial enzyme with high activity in the oxidation of γ -semialdehydes [16];
- the *ALDH5* family contains enzymes metabolizing succinic semialdehyde. Succinic semialdehyde dehydrogenase (SSDH) is strongly expressed in the human brain [27], where it is involved in γ -aminobutyric acid degradation [18]. Genetic deficiency of SSDH alters the metabolism of succinic semialdehyde, causing it to accumulate, leading to a variety of moderate to severe phenotypic neurological disorders, including mental retardation, ataxia and seizures [28];
- the *ALDH6* family codes for the CoA-dependent methylmalonate-semialdehyde dehydrogenase (MMSDH). This enzyme participates in the degradation of valine and pyrimidines, causing the transformation of malonate and methylmalonate into acetyl- and propionyl-CoA, respectively [29];
- the *ALDH9* family codes for enzymes involved in the metabolism of 4-aminobutyraldehyde and aminoaldehydes derived from polyamines and choline. Although this gene is strongly expressed in the early stages of embryonic brain development, it is poorly expressed in the human adult brain. Conversely, in adults, this enzyme is present in the liver, kidney and muscle [16];
- the *ALDH16* family codes for an enzyme which is widely expressed in a variety of tissues, including bone marrow, heart, kidney and lung, even if its physiological significance and function still remain to be established [30];
- the *ALDH18* family codes for a bifunctional ATP- and NAD(P)H-dependent mitochondrial inner membrane enzyme with both gamma-glutamyl kinase and gamma-glutamyl phosphate reductase activities. Traditionally known as Δ^1 -pyrroline-5-carboxylate synthetase, ALDH18 catalyzes the reduction of L-glutamate to Δ^1 -pyrroline-5-carboxylate, a critical step in the de-novo biosynthesis of proline, ornithine and arginine. It is expressed at high levels in human pancreas, ovary, testis and kidney. Defects in ALDH18A1 lead to a variety of metabolic and neurologic abnormalities, including hypoprolinemia, hypoorithinemia, hypocitrullinemia, hypoargininemia and hyperammonemia with cataract formation, neurodegeneration and connective tissue anomalies [30].

Other families contain plant, protozoan or yeast genes [18]. The main characteristics of ALDH families and pathologies associated with altered expression are summarized in Table 1.

ALDH families in stem cells

Interestingly, it has been demonstrated that various ALDH families are strongly active in both normal and cancer stem cells. Therefore, ALDH may be considered a marker for these cells and may well play a functional role, in terms of self-protection, differentiation and/or expansion of the

stem cell populations. These observations have been facilitated by the possibility to assess functional ALDH activity in live cells by immunohistochemistry (Aldefluor). As ALDHs have a cross-reactivity and a relatively wide substrate spectrum, it has been possible to determine that the ALDH-positivity, evidenced in various types of stem cells, is not due to the expression of the same isoforms. Although ALDH1A1 was first indicated as a marker and characteristic feature in primitive human hematopoietic progenitor cells (HSCs) isolated from bone marrow and in neural stem cells [31,32], recent studies have reported that ALDHs (ALDH1A2, ALDH1A3, ALDH1A7, ALDH2*2, ALDH3A1, ALDH4A1, ALDH5A1, ALDH6A1 and ALDH9A1 [33,34]) are also involved, since ALDH1A1 deficiency does not alter Aldefluor positivity. It is believed that this heterogeneity indicates that the isoform responsible for Aldefluor activity in the case of normal cells depends on the type of cell and on the tissue of origin in the case of cancer cells. That is why most studies on ALDH and stem cells do not specify the ALDH isoform, but speak rather generically of ALDH. However, particularly ALDH1A1 has been identified as a marker for the isolation and identification of normal, or cancer stem cells, and, along with ALDH3A1, it plays a role in differentiation and cell proliferation [34].

The enzymatic activity of ALDH in HSCs is higher than in the more mature hematopoietic cells: lymphocytes express the lowest level, whilst immature erythroid cells express an intermediate level [35]. The high ALDH expression present in HSCs is associated with an enhanced repopulating function and cellular resistance to cytotoxic drugs. Indeed, the presence of high ALDH1A1 and ALDH3A1 activity makes HSCs resistant to alkylating agents, such as activated oxazaphosphorine (e.g. mafosfamide or 4-hydroperoxycyclophosphamide, 4-HC). By inhibiting ALDH activity with various inhibitors, HSCs become sensitive to these anticancer agents employed to purge resident tumour cells *ex vivo* and/or in the treatment of autologous bone marrow transplantation [36]. HSCs with high ALDH activity and the ability to produce long-term, multilineage hematopoietic colonies have been isolated, not only from bone marrow, but also from umbilical cord blood [37] and circulating cells [38]. In the light of their extreme brightness at ALDEFLUOR staining, cells with high ALDH activity have also been indicated as ALDH^{bri} cell populations. There are between 0.5% and 5% of ALDH^{bri} cells in human bone marrow, umbilical cord blood and peripheral blood stem cells. The most intriguing recent observation is that ALDH^{bri} populations isolated from bone marrow include haematopoietic, endothelial, mesenchymal and neural progenitor cells, which are crucial in repair protocols for various pathological conditions, such as ischemic diseases [37]. As do HSCs, several other types of normal stem cells display high levels of cytosolic ALDH expression: neural cells [39], myogenic cells [40, 41], mammary cells [42], prostate cells [43] and intestinal crypt cells [44]. ALDH^{bri} cells, isolated from dissociated murine brain tissue, are

multipotent, self-renewing and able to generate new neurospheres and neuroepithelial stem-like cells in culture, including neurons, astrocytes and glia cells. Consistent with the importance of ALDH in regulating proliferation, its expression is much stronger in neural embryonic tissues than in adult counterparts [39, 45]. A subpopulation of ALDH^{br}CD56⁺/CD34⁻ cells possess strong myogenic potential in vivo upon intramuscular transplantation in normal myoblast progenitor cells, even if there is some heterogeneity of ALDH expression level [41]. Another study has shown that ALDH^{br} muscle cells are resistant to the cytotoxic effect of oxidative stress, a crucial aspect during repair processes after ischemic injury. These characteristics appear to be peculiar to human myogenic precursors, since in mouse, rat, rabbit and non-human primate myoblasts, ALDH is expressed at very low levels [40]. In mammary epithelial cells, the positivity to Aldefluor is due, in particular, to the high ALDH1 expression and the positive cells, which correspond to 8% of the total population and are able, not only to self-renew, but also to generate both luminal and myoepithelial cells [46]. Recent reports on benign breast biopsies state that the ALDH1 activity associated to EZH2, a protein involved in stem cell renewal and carcinogenesis, is a prognostic marker for the risk of developing cancer [47].

High ALDH activity, due to a strong expression of both ALDH1a1 and ALDH1a7, has been evidenced in central-acinar /terminal duct cells from peripheral acinar duct units in studies carried out on murine pancreas [48]. ALDH1-positive cells (< 5%) are mainly located at the normal crypt bottom in the colon and stomach [44,49]. In normal murine prostate, a cell subset expresses high levels of ALDH activity associated with antigens typical of prostate basal epithelium. When cells with low ALDH were compared to prostatic ALDH1-positive cells, there was a 2.5-fold increase in the formation of prostatic tissue after in vivo transplantation [43].

As to cancer stem cells, increased ALDH activity was first reported in acute myeloid leukaemia [50], subsequently, similar observations have been reported for several types of stem cells in solid cancers, including breast, colon, prostate, lung, liver, pancreas and ovary [34]. Recently, a close correlation between ALDH and metastasis formation has been evidenced in breast cancer stem cells (ALDH1A) [51], human prostate cancer cell lines (ALDH7A1) [52] and adenoid cystic carcinoma cells [53]. Although there is a small ALDH^{br} population in both normal stem cells and cancer stem cells, the latter have been shown to have higher tumourigenic properties than cancer cells with low ALDH [34]. Indeed, a positive statistically significant correlation was reported between the presence of a high percentage of ALDH^{br} cells and a poor survival rate [54,55].

Since it has been hypothesised that, in cancer, only a small number of cells have the property of replication, there has been a growing interest in cancer stem cells. Whilst the other cells are differentiated with a reduced capacity of replication. This theory is in contrast with the view that all

cancer cells have the capacity to enhance tumour development. The lack of chemotherapy efficacy observed in some cancers might be due to the presence of stem cells with a high ALDH content, which contributes to drug resistance by inhibiting the beneficial effects of chemotherapy.

The use of ALDH as a marker for cancer stem cells is a particularly interesting possibility in those cancers where the normal tissues have a low ALDH^{bri} content. An enhanced understanding of the pathways associated with ALDH1, ALDH3 and other ALDH isoforms might well facilitate the development of drugs able to improve the outcomes of bone marrow transplantation, regenerative medicine and cancer treatment.

Among the various ALDH families involved in stem cells, only ALDH3A1, belonging to the ALDH3 family, will be discussed at length in this review, due to its important role in regulating drug resistance and cell proliferation.

The ALDH3 family

The ALDH3 family includes enzymes with the capacity to oxidize medium chain aliphatic and aromatic aldehydes, such as peroxidic and fatty aldehydes, and enzymes which have non-catalytic functions, including that of antioxidant functionalities [30]. Four major mammalian ALDH3 genes have been reported to date i.e.

- ALDH3A1, which encodes the cytosolic form highly expressed in some normal tissues e.g. the stomach, lung, keratinocytes and cornea, which is poorly expressed, if at all, in normal liver. It has a UV filter function in the cornea [56,57] and is highly expressed in some human and rat tumours (hepatoma, breast and lung cancer), as well as in stem cells [58-60];
- ALDH3A2, which encodes the liver microsomal fatty aldehyde dehydrogenase [61,62] and has been implicated in the Sjögren-Larsson syndrome [63];
- ALDH3B1, which encodes the human lung, prostate and kidney cytosolic ALDH [64,65]. Recent studies have linked a single nucleotide polymorphism at the ALDH3B1 locus to the development of paranoid schizophrenia [66,67];
- ALDH3B2, which encodes the human salivary gland and placental ALDH [68].

The *ALDH3A1* gene is located on chromosome 17 in human beings and on chromosomes 11 and 10 in the mouse and rat respectively. It belongs to the drug-metabolizing enzymes of the phase II group. In the liver, *ALDH3A1* is a member of the *Ah* gene battery, including the *Cytochrome P-450-1* family members, *Glutathione-S-TransferaseA2*, *NAD(P)H:quinone acceptor oxidoreductase* and *UDP-glucuronosyltransferase 1A6* (Figure 1). These genes are modulated by the presence of positive, or negative regulatory regions. Their activation takes place through the aryl hydrocarbon

(*Ah*) receptor, which is a transcription factor binding to the consensus sequence, *Ah* responsive element (*AhRE*) [69,70]. An important class of environmental pollutants that have a wide range of toxic and carcinogenic effects i.e. polycyclic aromatic hydrocarbons (PAH), or chlorinated compounds e.g. TCDD (2,3,7,8-Tetrachlorodibenzo-*p*-dioxin), which are ligands for the *Ah* receptor [71]. Two sequences that have significant similarity with the *AhRE* core have been observed in the 5'-flanking region of the *ALDH3A1* gene [72]. As well as positive regulation through *AhRE* sequences, the 5'-flanking region of *ALDH3A1* contains also at least two negative regulatory regions which repress promoter activity and the TCDD-response, respectively. These negative regions are bound by the Nuclear Factor 1 (NF-1)-like transcription factors and/or unique proteins. The 5' flanking region is responsible for the regulation of the constitutive expression of *ALDH3A1* in many tissues [72].

There is also activation of *ALDH3A1* by PAH in human MCF-10A mammary epithelial cells, when treated with benzo(a)pyrene (BaP), a known human carcinogen. BaP is metabolized by several pathways, some of which have bioactivation properties, whilst others have detoxification properties. BaP-quinones, formed via cytochrome P450 and peroxidase dependent pathways, have significant growth promoting and anti-apoptotic properties and activate phase 2 enzyme genes, including *ALDH3A1*. An increased *ALDH3A1* activity in human mammary epithelial cells is associated with increased cell growth and survival, which may play important roles in tumour promotion. [73]. Moreover, *ALDH3A1* levels are induced, not only by environmental contaminants, but also by polyphenol, e.g. catechol [74]. Since the alimentary tract and lungs are the major "ports of entry" for dietary and environment substances, it is feasible to presume that these substances may induce high levels of *ALDH3A1* at these sites.

ALDH3A1 in some non-tumoural and tumoural tissues

THE BREAST

It has been observed that *ALDH3* levels vary widely in normal, malignant primary and metastatic breast tissue samples. Although the average enzyme levels in the malignant breast tissue samples were significantly higher than those in normal breast tissue samples, the average *ALDH3* level in metastatic breast tumour samples were not significantly higher than those in primary breast tumour samples. Cellular levels of *ALDH3* in the malignant breast tissue samples appeared to be directly related to those present in the corresponding normal tissue [75]. A recent study has demonstrated that *ALDH3A1* is present in higher quantities in breast cancer fibroblasts (BCFs) than in normal mammary fibroblasts (NMFs) [76]. This study investigated the regulatory effects of BCFs vs NMFs on mammosphere formation and stem-cell-related gene expression, in MCF-7 breast cancer cells.

The cells were cultured in suspension to generate primary and secondary mammospheres. When MCF-7 cells were co-cultured with BCFs, there was a decrease in the mammosphere formation time, whilst the volume of the mammospheres increased with a higher mammosphere-forming efficiency than was observed in presence of NMFs. Both the BCF and NMF groups showed enhanced gene expression of *ALDH3A1* (fold-change, 5.23 and 1.85, respectively). Cancer fibroblast cells have the capacity to enhance mammosphere-forming efficiency and up-regulate stem cell-related gene expression in breast cancer cells [76]. *ALDH3A1* is upregulated in cultured breast cancer cells by exposure to *Ah* receptor ligands, e.g. 3-methylcholanthrene, or to electrophiles, such as catechol. The 3-methylcholanthrene-mediated upregulation, via transient transactivation of xenobiotic responsive element (XRE), is apparently estrogen receptor (ER)-dependent. Whilst, conversely, catechol-mediated up-regulation takes place via transient transactivation of an electrophile responsive element and is ER-independent [77].

THE CORNEA

The mammalian cornea, located at the anterior surface of the eye, is an avascular tissue that has the function of a protective barrier between the environment and the internal ocular structures. Indeed, one of the primary sources of environmental stress for the cornea is solar radiation, in particular, those in the ultraviolet (UV)-range. The concomitant exposure to UV-radiation and molecular oxygen trigger an excessive production of reactive oxygen species (ROS), which, in turn, lead to substantial oxidative stress and subsequent corneal damage [78]. The cornea has developed several antioxidants and repair systems so as to defend itself against this dangerous condition [79]. It has been clearly shown that *ALDH3A1* plays a crucial role in corneal defence [80]. As its steady-state concentration in the cornea is over and above what would be simply needed for metabolism, it has been hypothesized that the enzyme has additional protective roles: 1) direct absorption of UV-radiation; 2) an antioxidant function, either directly by the scavenging of free radicals, or, indirectly, by the production of NADPH, 3) maintaining the refractive and transparent properties of the cornea, as a corneal crystalline, and 4) a chaperone-like activity. The hypothesis that direct absorption of UV-radiation by *ALDH3A1* protects corneal proteins and, consequently, leads to the inactivation and formation of soluble aggregates of *ALDH3A1*, has been further supported by experimental evidence. In C57BL/6J inbred mice, UV-radiation exposure caused an 85% reduction in *ALDH3A1* activity, whereas other metabolic enzyme activities remained intact [81]. Similarly, a large excess of *ALDH3A1 in vitro* reduced the UV-induced inactivation of glucose-6-phosphate dehydrogenase [82]. The inactivation of *ALDH3A1* by UV to preserve other corneal elements has been termed a “suicide response” [80,83].

Since ALDH3A1 is the primary enzyme responsible for the detoxification of aldehydes in the cornea [57], it might be reasonable to suppose that the loss of ALDH3A1 activity may severely impair the ability this tissue has to reduce and eliminate aldehydes, especially under conditions of oxidative stress. However, this is not the case, as the cornea shows an abundant expression of ALDH3A1 [84,85], which is combined with the enzyme's broad range of substrate specificity [86]. It is likely that both these factors make for the induction of aldehyde metabolisms, also in the presence of substantial ALDH3A1 inactivation. Therefore, it may be that, even in the presence of inactivation of ALDH3A1 by UV and other stresses, the cornea is not completely defenceless against toxic aldehydes [87].

THE LIVER

Cytosolic ALDH3A1 is poorly expressed, if at all, in liver cells, unlike the cytosolic ALDH1, which, in normal human liver, contributes to the detoxification of various exogenous and endogenous, aliphatic and aromatic aldehydes, with various chain lengths. ALDH3A1 is induced by PAH, or chlorinated compounds in normal rat liver [71] and its expression increases in direct correlation with the degree of deviation in hepatoma cells, or chemically-induced hepatoma [88,89].

Table 2 shows the levels of ALDH3A1 activity: there is a higher activity in hepatoma cells than in hepatocytes, a higher activity in hepatoma compared to neoplastic nodules during chemically-induced hepatocarcinogenesis and a higher activity in the more deviated JM2 and HTC hepatoma cell lines compared to the less deviated 7777 and MH1C1 hepatoma cell lines [88].

ALDH3A1 activity is higher in the more deviated SK-HEP-1 hepatoma cells in human beings, compared to less deviated HepG2 (Table 2). Examination of hepatocellular carcinoma tissues from patients with this form of cancer showed that approximately 50% had a high ALDH3 expression as a result of the expression, or activation, of nuclear proteins reacting to the ALDH3 promoter region. Two of these nuclear proteins, along with 35 and 16 kDa, have been determined in ALDH3-positive hepatoma cell lines and cancerous liver, but not in ALDH3-negative normal liver tissues. DNaseI footprint analysis identified two protective regions within the ALDH3 promoter. The first protected region has one putative CCAAT-box and one putative Sp1-site. The second protected region contains a putative HiNF-A binding sequence [90].

THE LUNG

ALDH3A1, as is ALDH1A1, is important in both alveolar pneumocyte physiology and lung cancers. In the lung, ALDH3A1 has the function of metabolizing aldehydes, which may occur naturally in the environment as a breakdown product of xenobiotics, or may derive from an

endogenous metabolism. It has been shown that cigarette smoking alone suffices to make the *ALDH3A1* expression rise in normal pneumocytes. Indeed, its expression was higher in the normal pneumocytes of smokers than in those of non-smokers. Carcinogenic compounds found in cigarette smoke, which include aldehydes, upregulate ALDH isozymes in lung tissue, as reported by a study on lung cancer samples taken from patients with, or without, a history of cigarette smoking [4]. There is a high *ALDH3A1* expression in some non-small cell lung cancer cell lines, such as A549 cells, as well as in specimens from lung cancer patients [4,10,91,92]. The expression of the enzyme gradually increases during the transition from normal to atypical pneumocytes, carcinoma in situ, and then adenocarcinoma. These observations show that an elevated expression of the enzyme is related to the malignant transformation. Immunohistochemical staining for ALDH3A1 on specimens from control patients without lung cancer and from patients with one of the primary lung cancers, i.e. squamous cell cancer (SCCA), adenocarcinoma, or small-cell lung cancer (SCLC), indicated a significantly higher level of ALDH3A1 expression in SCCA and adenocarcinoma than in SCLC [4]. A similar pattern was also evidenced for ALDH1A1 expression [4]. The fact that SCCA and adenocarcinoma express ALDH3A1 takes on importance in the treatment phase, as the presence of this enzyme has been associated with resistance to oxazaphosphorines, such as cyclophosphamide [93]. Moreover, it has been shown that selectively knocking down RNA expression of ALDH3A1 in A549 cells leads to an increased susceptibility to cyclophosphamide [94]. Indeed, decreasing ALDH3A1 with arachidonic acid, A549 cells become more susceptible to the cytostatic effect of lipid peroxidation aldehydes [10].

OTHER NORMAL TISSUES AND TUMOURS

Very few papers were found on the expression of ALDH3A1 in animal and human skin cells. One reported that *ALDH3A1* was constitutively expressed in the skin of mouse and rat, but not in that of the guinea-pig. It also reported that immunohistochemistry staining showed the localization of ALDH3A1 predominantly in the epidermis, sebaceous glands and hair follicles of mouse and rat skin [95]. As to human skin, one paper reports that ALDH3 enzyme is expressed, constitutively, in three different anatomical regions of human skin (foreskin, breast, abdomen) and may play a significant role in the metabolism of endogenous or xenobiotic aldehydes [96]. Another paper reported that the human keratinocytes NCTC 2544 display a high ALDH3A1 expression [97]. High ALDH3A1 levels have been observed in certain other human tissues and cells e.g. normal stomach mucosa, parotid gland tumours, colon cancer cell lines and in some, but not all, colonic adenocarcinoma specimens [98]. The two DNA binding proteins, discovered in liver cancers and responsible for increased *ALDH3A1* expression, are not found in normal stomach tissues, or in

stomach carcinoma KATO III cells [90]. The high ALDH3A1 levels in gastrointestinal cancers may be one reason why cyclophosphamide has poor clinical efficacy in this type of cancer [99].

ALDH3A1 as cytoprotective enzyme

The effect the presence of high ALDH3A1 activity has on non tumoural cells differs from the effect it has on tumoural cells. Indeed high ALDH3A1 activity protects healthy cells from the effect of some carcinogens, whilst it contributes to the onset of drug resistance in tumoural cells.

PROTECTION FROM CARCINOGEN EFFECT

It has recently been suggested that ALDH3A1, as other detoxifying and antioxidant enzymes, can also be used as biomarkers, to determine whether the dose of chemopreventive agent administered reaches the target cells and upregulates genes and proteins so as to protect cells from endogenous and exogenous carcinogens. Among natural chemopreventive agents, sulforaphane, an isothiocyanate found in cruciferous vegetables, with particularly high levels in broccoli sprouts is known to protect cells from damage, by inducing detoxication and antioxidant enzymes. That is why it is considered to be an attractive chemopreventive agent, because sulforaphane is safe and is easily extracted from broccoli sprouts. Treating MCF10A and MCF12A cells with sulforaphane induced marked upregulation of cytoprotective enzymes, including ALDH3A1 [100].

DRUG RESISTANCE

The increased ALDH expression observed in tumour cells is one of reasons why they are resistant to drug toxicity and their consequent poor response to antitumoural drugs. In particular, ALDH is responsible for tumour resistance against alkylating agents, such as oxazaphosphorines (eg. 4-HC) and to lipid peroxidation products. Some anti-neoplastic agents have been shown to produce oxidative stress in tumours during cancer chemotherapy, with consequent lipid peroxidation and generation of aldehydes. The direct correlation between ALDH3A1 activity and resistance to oxazaphosphorines has been evidenced *in vitro*: culturing human tumour cells in the presence of high concentrations of these agents, within a single period of 30-60 minutes, increased ALDH3A1 activity, making the cells stably insensitive to oxazaphosphorines [101].

This correlation has been also confirmed transfecting rat ALDH3 into human MCF-7 cells, conferring oxazaphosphorines-specific resistance. The resistance was reversed by pretreatment with the ALDH3 inhibitor diethylaminobenzaldehyde [75]. Analysis of the metabolites formed by ALDH3 from cyclophosphamide has provided new and full evidence to support the hypothesis that

aldophosphamide oxidation is the primary mechanism of oxazaphosphorine-specific resistance conferred by this isoenzyme.[102].

In human beings, it has been reported that interindividual variation in ALDH3 and ALDH1 activity in breast cancer could contribute to the extremely varied clinical resistance to 4-HC, which is a mainstay of chemotherapeutic regimens used to treat breast cancers [75,103]. Given the wide range of ALDH3 levels found in malignant breast tissues, the measurement of ALDH level in normal breast tissue and/or primary breast malignancies, prior to the initiation of chemotherapy, may well be of value in predicting the therapeutic potential, or lack thereof, of 4-HC in treating metastatic breast cancer, thus providing a rational basis for the design of individualized therapeutic regimens when treating this disease.

Since ALDH3A1 is crucial in determining sensitivity to oxazaphosphorines, it is possible to pursue clinical strategies that raise the sensitivity of tumour cells to oxazaphosphorines, by inhibiting its synthesis, or decreasing the sensitivity of vulnerable and essential normal cells to these drugs, e.g., pluripotent haematopoietic cells, by transferring the *ALDH3A1* gene [101]. Moreover, *ALDH3A1* is one of the downstream genes of the metastasis gene *Metadherin (MTDH)* involved in multi-drug chemoresistance. MTDH is a downstream mediator of the transforming activity of Ha-Ras and c-Myc and, as it regulates several crucial aspects of tumour progression, it has an important role in the carcinogenesis process including transformation, evasion of apoptosis, invasion, metastasis and chemoresistance. MTDH is overexpressed in more than 40% of breast-cancer patients and is associated with poor clinical outcomes [104].

It has been reported that *MTDH* knockdown represses *ALDH3A1* expression and induces chemosensitization. To further investigate the functional importance of ALDH3A1 in MTDH-mediated chemoresistance, LM2 cell line was engineered to knockdown ALDH3A1. This resulted in LM2 cells being more sensitive to the chemotherapeutic agents, paclitaxel, doxorubicin and 4-HC. Whilst restoring ALDH3A1 expression made LM2 cells resistant to these agents again [104]. Therefore, ALDH3A1 plays a role in broad-spectrum chemoresistance [104]. Moreover, it may also contribute to increasing the chemoresistance of cancer stem cells [46,105].

ALDH3A1 and cell proliferation

The content and activity of ALDH3A1 is directly correlated to cell proliferation. This may be demonstrated in two ways: 1) by inhibiting or 2) by activating ALDH3A1. These approaches, not only show the direct correlation between ALDH3A1 and proliferation, but may also be useful in decreasing the cell proliferation of tumour cells, stimulating cell proliferation in normal cells during tissue regeneration, or in increasing cornea protection from UV-radiation damage. The close

relationship between ALDH3A1 and proliferation has also been confirmed by studies that detected high expression levels in both normal and cancer stem cells, including the aforementioned studies.

1) INHIBITION:

ALDH3A1 can be directly inhibited by the administration of synthetic specific inhibitors, antisense oligonucleotides, or siRNA to the cells. Moreover, indirect inhibition can be obtained through the activation of the peroxisome proliferator-activated receptor (PPAR) γ by administering synthetic, or natural ligands (clofibrate or polyunsaturated fatty acids), or via PPAR γ transfection. **Inhibiting ALDH3A1 with specific inhibitors, such as tetraethylthiuram disulfide (disulfiram), diethylaminobenzaldehyde, or 4-amino-4-methyl-pent-2-ynthioic acid, S-methyl ester (ampal thiolester) and some of its derivatives, including dimethyl ampal thiolester (DIMATE) and morpholino ampal thiolester (MATE), has a significant inhibitory effect on cancer cell proliferation. These specific inhibitors have been tested on several cancer cell lines, such as A549 and H522 lung cancer cells, glioblastoma U251 cells, HeLa cells, L1210 cells, hepatoma JM2 and 7777 cells [92,106-109]. Importantly, inhibition of enzyme activity preceded the inhibition of cell growth. Human prostate epithelial normal cells (HPENC) and human prostate epithelial cancer cells (DU145) were also used to investigate DIMATE and MATE more thoroughly for selectivity and growth-inhibitory activity. Unequivocal evidence was obtained, showing that both compounds are reversible growth inhibitors of HPENC, but irreversible growth inhibitors of DU145. ALDH3A1 is inhibited to the same extent by both compounds. Growth inhibition of DU145 takes place through the induction of apoptosis. [110]. The use of antisense oligonucleotides against ALDH3A1 completely inhibits ALDH3A1 expression and consequently decreases cell proliferation, without, however, affecting cell viability [107].**

The knock-down of ALDH3A1 in lung H522 and A549 cancer cell lines by siRNA, abolishes up to 95% of ALDH3A1 activity, leading to a significant reducing in both the growth rate and cell motility and migration [97]. The indirect inhibition of ALDH3A1 and, consequently, the inhibition of cell proliferation, can be achieved via increased PPAR expression: administration of PPAR ligands and PPAR transfection both reduce ALDH3A1 content as well as inhibiting cell proliferation.

PPARs are transcription factors belonging to the family of ligand-inducible nuclear receptors. They form heterodimers with the retinoid X receptors and bind to a DNA sequence named "PPRE" (Peroxisome Proliferator Response Element), two short hexameric sequence motifs corresponding to, or closely related to, 5'-aggctca-3', with a space of one nucleotide. Their transcriptional activity is modulated by co-repressors and co-activators. The activation of PPAR γ seems to reduce the

expression of ALDH3A1 through the inhibition of NF κ B or AP-1 binding activity [10,91], or by binding to the putative PPRE present in the introns 1 and 11 of *ALDH3A1* (data submitted for publication).

When JM2 rat hepatoma cells were treated with clofibrate, a hypolipidemic drug and synthetic PPAR γ ligand, induced PPAR γ , decreased ALDH3A1 expression and inhibited cell growth. The blocking of PPAR γ with antisense oligonucleotides prevented the inhibitory effect of clofibrate on cell proliferation and ALDH3A1 expression [111]. Similar results have been obtained treating rat hepatoma cells and human lung tumour cells with polyunsaturated fatty acids, such as arachidonic acid and docosaenoic acid, natural ligands of PPARs [10,91,112]. The inhibition of cell proliferation by polyunsaturated fatty acids is inversely related to the ALDH3A1 content, as evidenced in Figure 2: as 7777 hepatoma cells have a low ALDH3A1 content, they are more susceptible to the arachidonic acid effect than are JM2 hepatoma cells, which have a high content of this enzyme. PPAR γ transfection in human lung cancer cells and normal keratinocytes also confirmed the correlation with this receptor, ALDH3A1 and cell proliferation: high levels of PPAR γ mRNA and protein due to PPAR γ transfection decreased ALDH3A1 and inhibited cell proliferation (Figure 3) [97].

2) ACTIVATION:

ALDH3A1 can be activated by inhibiting PPAR γ with specific antagonists, antisense oligonucleotides (AS-ODN), siRNA, or by using medical device for tissue regeneration (composite polypropylene prosthesis for hernia repair). The antagonist GW9662 prevents the arachidonic acid-mediated reduction of ALDH3A1 expression as it prevents the growth inhibition of A549 cells [10]. Two other approaches to increase PPAR γ -dependent ALDH expression in rat hepatoma and human normal keratinocytes are AS-ODN and siRNA against PPAR γ . Both techniques decrease PPAR γ , whilst, at the same time, increasing both ALDH3A1 and cell proliferation [97,111].

To further examine the physiological roles of PPAR γ and ALDH3A1 in cell proliferation, changes in their expression were examined in a model of tissue regeneration. To this aim, human keratinocytes were grown on a composite polypropylene prosthesis for hernia repair. The prosthesis acts as a scaffold where seeded cells can migrate and grow [113,114]. There was a higher percentage of cell growth on the prosthesis than when the same number of cells was grown without the prosthesis. At 6 post-cell seeding days, there was an 8.2 increase in the number of cells counted at 3 days on the prosthesis, whereas, without the support of the prosthesis, there was only a 3.1 increase. At the same time, ALDH3A1 protein content increased during the experiment, while

PPAR γ protein decreased (Figure 4). Further experiments are ongoing to investigate whether this protocol may be suitable for clinical application to improve tissue repair and regeneration.

How does ALDH3A1 inhibition reduce cell proliferation?

Although the mechanisms through which ALDH3A1 influences cell proliferation have not yet been fully understood, it is likely that they include the modulation of the genes involved in regulating transcription, cell growth, differentiation and apoptosis and/or the effects of the products of lipid peroxidation, which have the ability to modulate the gene expression.

Gene modulation

Since ALDH3A1 is highly expressed in pathological specimens from patients with primary non-small-cell lung carcinoma (NSCLC) [85], studies have been carried out on lung cancer A549 cells to investigate into the mechanisms that make ALDH activity so important in cell homeostasis and cancer transformation. Both cell growth and proliferation are significantly affected when ALDH3A1 activity is reduced by siRNA, or inhibitors, possibly due to the effect they have on a wide spectrum of genes that have different biological roles in cells. These genes include *CCL20*, *GPR37*, *DDX3Y*, *ID4*, *GPC6*, *RPS4Y1*, *EIF1AY*, and *HMGGA2*, and are involved in transcription regulation, cell growth, differentiation and apoptosis [92,115,116]. Microarray results show a high *RPS4Y1* and *CCL20* expression in A549 cells, whilst there is a weaker expression of the other 5 genes. *HMGGA2* is reported to be overexpressed in lung cancer and to have an inverse correlation with survival [116]. It is located on chromosome 12q13-15 and clinically-relevant chromosome 12 abnormalities have been reported among the frequent chromosomal abnormalities described in NSCLC [117,118]. The largest cluster of the genes affected by ALDH knock-down is on chromosome 12 and three of them are located on the Y chromosome. Deletion of the Y chromosome is one of the frequent abnormalities reported in NSCLC and is associated with malignant transformation and the development of lung cancer [119,120]. Therefore, these genes are good candidates for further investigation with the aim of defining which gene networks in the ALDH isozymes play important roles in cancer biology [92].

Lipid peroxidation

ALDH3A1 also has a fundamental role in the metabolism of aldehydes derived from lipid peroxidation, such as toxic medium-chain alkanals, alkenals and 4-hydroxyalkenals. Lipid peroxidation is a consequence of oxidative stress exerted on polyunsaturated fatty acids and leads to the formation of reactive by-products such as lipid hydroperoxides and aldehydes. There are several

factors able to induce oxidative stress, including the metabolism of xenobiotics, exposure to UV-radiation, or exposure to pro-oxidant agents e.g. ADP plus iron, or ascorbate plus iron [121,122]. Human ALDH3A1 demonstrated high substrate specificity for medium-chain (6 carbons and more) saturated and unsaturated aldehydes, including 4-hydroxy-2-nonenal (HNE), 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. HNE is the most reactive and cytotoxic of the aldehydeic byproducts of lipid peroxidation [123]. This aldehyde has been shown to have a variety of effects on biological systems, more often than not resulting in the inhibition of cell proliferation and/or cytotoxicity. For example, it induces glutathione depletion [124], inhibits DNA and RNA synthesis [125] and mitochondrial respiration [126] and induces morphological changes [127]. The effects of HNE, as those of other aldehydes, are correlated, not only with the extent of lipid peroxidation, but also with the enzyme metabolizing aldehyde activity. These include ALDH3A1, which, as aforementioned, is highly expressed in some normal and tumoural cells [5,88,89,128,129]. An ALDH3A1 increase makes both normal and tumour cells resistant to lipid peroxidation byproducts [88]. JM2 rat hepatoma cells with high ALDH3A1 content are more resistant to the cytostatic and cytotoxic effects of HNE than are 7777 rat hepatoma cells, as shown by the colony formation efficiency and viability after HNE treatment. The lower susceptibility of JM2 cells, due to their high ALDH3A1 activity, is confirmed by the marked capability these cells have in removing aldehyde.

Human lung tumour A549 cells, which express high levels of ALDH3A1 protein, were significantly less susceptible to the antiproliferative effects of HNE than were human hepatoma HepG2 or SK-HEP-1 cells which have lower ALDH3A1 expression (Figure 5) [10]. Expression of ALDH3A1, by stable transfection in V79 hamster fibroblasts, conferred a high level of protection against growth inhibition by the medium-chain length aldehyde substrates, including hexanal, trans-2-hexenal, trans-2-octenal, trans-2-nonenal, and HNE. This was reflected in the parallel ability ALDH3A1 has to prevent these aldehydes causing the depletion of glutathione. The potent HNE induction of apoptosis was completely blocked by ALDH3A1 expression in both V79 cells and RAW 264.7 murine macrophages, consistent with the observed total prevention of HNE-protein adduct formation [130].

One of the proposed functions for ALDH3A1 is the detoxification of aldehydes formed during UV-induced lipid peroxidation, such as in the human cornea, where immunohistochemistry revealed ALDH3A1 expression in epithelial cells and stromal keratocytes [131]. This protective role ALDH3A1 plays against HNE was demonstrated by stably transfecting human ALDH3A1 in a human corneal epithelial cell line lacking endogenous enzyme. Cells transfected with ALDH3A1

were more resistant to HNE-induced apoptosis than were mock-transfected cells. It was also reported that ALDH3A1 expression prevented 4-HNE-induced protein adduct formation in the cornea [132]. As a whole, the data suggest that ALDH3A1 is a regulatory element of the cellular defence system that plays a protective role against HNE, which arises from oxidative stress induced by several factors, such as UV radiation and PUFAs.

Conclusions

Not only has the close correlation between ALDH expression and cell proliferation been evidenced in both normal and cancer cells, but it has more recently been confirmed by studies on normal and cancer stem cells. Among the human ALDH isoenzymes, ALDH3A1 plays an important role in metabolizing the aldehydes derived from lipid peroxidation, favouring both a reduction in the cytostatic effects these compounds have and cell proliferation. Moreover, its marked expression in cancer cells, like that of ALDH1A1, confers resistance against antitumoural drugs. In the light of the fact that ALDH controls several cell functions, it seems important to stimulate a positive, or negative modulation of its expression, so as to provide “tailor made” clinical strategies for several pathological conditions that require the induction (tissue regeneration), or inhibition (cancer) of cell proliferation. These clinical strategies could be achieved by the use of specific inhibitors, antisense oligonucleotides and siRNA, which cause negative ALDH regulation, or either by activating, or inhibiting the nuclear receptor PPAR γ , which determines negative, or positive ALDH modulation. Its expression is indirectly regulated by PPAR γ through the inhibition of NF κ B and AP-1 binding activity. Moreover, putative PPRE sequences have been found in the *ALDH* gene. The modulation of ALDH3 may play a key role in the regulation of the growth and differentiation of both normal and cancer cells, also influencing some aspects of the cancer phenotype i.e. drug resistance and lipid peroxidation products.

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FIGURE LEGENDS

Figure 1.

Ah gene bacterium.

Figure 2.

The effect of arachidonic acid enrichment has on growth in rat hepatoma cell lines.

Hepatoma cell lines (7777 and JM2) were incubated in the presence of 60 μM arachidonic acid and exposed to 4 doses of ascorbate/ FeSO_4 (500 μM /100 μM) after 24 hours, administered at 12-hour intervals. The cells were harvested at the following time intervals:

day 1: 24 hours after arachidonic acid enrichment;

day 3: 12 hours after the fourth dose of ascorbate/ FeSO_4 .

Data are the averages \pm SD of 6 experiments. For each cell line, averages with different letters differ statistically ($p < 0.05$) from one another as determined by variance analysis followed by *post hoc* Newman–Keuls analysis.

C, control cells; ARA, cells treated with arachidonic acid and exposed to 4 doses of ascorbate/ FeSO_4 .

Figure 3.

The effect of transfecting cells with pSG5 vector containing PPAR γ has on cell proliferation and PPAR γ and ALDH3A1 protein content.

Human lung tumour cells (A549) and normal keratinocytes (NCTC 2544) were transfected with 3, or 5 μg pSG5 vector containing PPAR γ ($\gamma 3$, $\gamma 5$, respectively), or pSV- β -galactosidase (C) and harvested at 24, or 48 hours for cell count and Western blot analysis.

Panel A: data are averages \pm S.D. from 3 experiments. For each type of cell and for 24, or 48 h, averages with different letters have a statistically significant difference from one another ($p < 0.05$) as determined by analysis of variance followed by *post hoc* Newman–Keuls analysis.

Panel B: the polyclonal antibody used recognized both isoforms PPAR $\gamma 1$ and $\gamma 2$. The densitometry value given for each protein is referred to the corresponding β -actin value and expressed by arbitrarily normalizing the control value as 100.

Figure 4

ALDH3A1 and PPAR γ protein content in human normal keratinocytes (NCTC 2544 cells) seeded on a composite prosthesis, a model for tissue repair.

The cells were then harvested at 3 and 6 days after cell seeding. For each protein, the data are densitometry values normalized to the corresponding β -actin value and referred to their respective control values, taken as 100.

C, cells seeded without prosthesis; prosthesis, cells seeded on the prosthesis.

Figure 5

The effect exogenously added HNE has on cell proliferation.

Human lung (A549) and liver (SK-HEP-1, HepG2) tumour cells were exposed for 48 hours to different concentrations of HNE.

Data are averages \pm S.D. from 3 experiments. For each cell line, averages with different letters have a statistically significant difference ($p < 0.05$) from one another, as determined by variance analysis followed by *post hoc* Newman–Keuls analysis.