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Research report

Histological distribution of class III alcohol dehydrogenase in human brain

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Abstract

The distributions of class III alcohol dehydrogenase (ADH), a glutathione-dependent formaldehyde dehydrogenase, and class I ADH in the human brain were examined immunohistochemically. The most intense immunostaining of class III ADH was observed in the dendrites and cytoplasm of cerebellar Purkinje cells. Scattered cerebral cortical neurons in layers IV and V, and some hippocampal pyramidal neurons were also immunopositive. The neuronal distribution of class III ADH resembled that of the vulnerable neurons in patients with hypoxic encephalopathy, which in view of the intense staining in the Purkinje cells, raises the possibility that this enzyme contributes to the hypoxia and cerebellar degeneration suffered by chronic alcoholics. Perivascular and subependymal astrocytes, which contribute to the maintenance of the cerebral cellular milieu and isolate the brain from the systemic circulation and cerebrospinal fluid, were also class III ADH positive. As the substrates of this enzyme include intrinsic toxic formaldehyde, inflammatory intermediate of 20-hydroxy-leukoteiene B4, and possibly ethanol, the distribution of class III ADH immunostaining indicates this enzyme contributes to the defence of the brain against degenerative processes. The finding that, unlike ependymal cells, subependymal astrocytes were class III ADH positive, suggests this enzyme may be useful for differentiating astrocytes and ependymal cells.

Keywords: Alcohol dehydrogenase; Class III; Class I; Immunohistochemistry; Distribution; Defence mechanism

1. Introduction

Alcohol dehydrogenase (ADH; alcohol: NAD + oxydoreductase, EC 1.1.1.1.) is distributed in a variety of organs, including the liver. ADH has been found to be a key enzyme that catalyses the oxidation of ethanol in mammals. The isozymes of ADH are grouped into several classes according to their substrate specificities and amino acid sequences [16]. Class I ADH is inhibited by pyrazoles, and has low K_m values for short chain alcohols with basic isometric points. Class III ADH is relatively resistant to pyrazoles, and has very high K_m values for short chain alcohols with acidic isometric points. The properties of class II and IV ADHs are intermediate between those of the class I and III enzymes [2,10,14,21].

Class III ADH is distributed in every mammalian organs [1,8], and its genes are characteristic of housekeeping gene [13]. Originally, ADH was hypothesized to play a role in cellular defence mechanisms [5]. Other than its role as a glutathione-dependent formaldehyde dehydrogenase, the role of class III ADH in vivo has not been elucidated [6,18]. However, recent evidence indicates that the substrates of class III ADH are not only long chain alcohols and formaldehyde but also 20-OH-leukotrien B4 [9], which are intermediates of inflammatory process. Taken together, the available evidence suggests that class III ADH contributes to cytoprotection against a variety of degenerative processes in the brain, because the only ADH present in the brain in significant concentrations is the class III isozyme [3].

Previous studies have shown that ADH is localized in the subependymal layer and perivascular zone of the human brain, both of which showed intense immunohistological staining with an anti-class III antibody as well as high amounts of ADH enzymic activity [20]. However, the specific cells that reacted with the anti-class III ADH

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antibody and their distribution patterns in the human brain have not been identified.

In this study, we carried out immunohistochemistry and immunofluorescent microscopy to find which cells express class III and class I ADH, using specific antibodies against class I and class III ADH. The physiological roles of these isozymes in the brain, based on their subcellular localizations and substrate specificities, are discussed.

2. Materials and methods

2.1. Evaluation of the specificity of the antibodies against mouse ADH isozymes

Polyclonal antibodies against ADH isozymes were raised in rabbits by injecting them with required purified mouse ADH isozyme. The cross-reactivity of each antibody was checked by carrying out zymography of the ADH isozymes using antibody-treated liver extracts, which demonstrated that each antiserum was monospecifc for the respective ADH isozyme, and the only human ADH isozyme with which the anti-mouse class III ADH antibody cross-reacted was class III ADH [10,11].

2.2. Immunohistochemical microscopy

The brains of four autopsied patients (less than 8 h post mortem, 67.3 ± 3.4 years old, three females and one male) with neither neurological, hepatic nor alcoholic disease were studied. Five blocks (10 × 10 × 5 mm) from each brain, including the frontal (F), temporal (T), hippocampal (H), and parietal (P) cortices with subcortical white matter, and the cerebellum (C), were evaluated. These fresh blocks were embedded in O.C.T. compound (Tissue-Tek) and then immediately frozen by immersion in dry-ice/alcohol. Tissue sections (4–5 μm thick) were cut using a cryostat at −25°C, mounted on silane-coated glass slides, dried at room temperature for 30 min, fixed with acetone (at −20°C) for 10 min and washed with phosphate buffered saline (PBS, 10 mM, pH 7.2) for 10 min at room temperature. Then sections were incubated with anti-class III ADH or anti-class I ADH antiserum, diluted 1:300 and 1:200, respectively. Preimmune and normal sera were used to assess the specificity of each antiserum. Antigen-antibody complexes were detected with biotinylated secondary antibodies directed against rabbit IgG and with horseradish peroxidase and visualized by incubating the sections with diaminobenzidine. Double staining with alkaline phosphatase labeled streptavidin ABC complex (DAKO K0391) and BCIP/NBT (5-bromo-4-chrolo-3-indolylphosphate/nitroblue tetrazolium chloride) was performed using an LSAB kit (Dako). Secondary fluorescently labeled antibodies of goat anti-mouse or goat anti-rat secondary antibodies labeled with tetramethyl rhodamine isomer R (TRITC, Dako, code no. R0156) or fluorescein isothiocyanate (FITC, Dako, code no. F0479) were used at dilutions of 1:500. Double-immunostained sections were prepared by incubating the sections with both primary antibodies simultaneously, and after the appropriate washes, incubating again simultaneously with both secondary antibodies (Table 1).

Table 1
Antibodies used in the study

Antibody against	Type	Dilution	Source
Class I ADH	mouse polyclonal	1:200	Haseba
Class III ADH	mouse polyclonal	1:300	Haseba
GFAP	bovine polyclonal	1:100	Dako Z334
MAP2	rat monoclonal	1:300	Chemicon MAB364

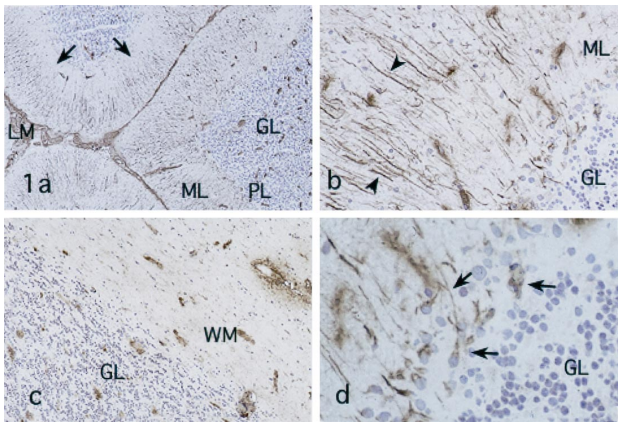

Fig. 1. Class III ADH-positive sites in the cerebellum. Fine linear staining perpendicular to the leptomeninges can be seen. The Purkinje cellular dendrites in the distal half are intensely stained (arrows) (a), (arrowheads) (b). The leptomeninges and small vessels are also stained (a). The granule cell layer and white matter, except for their vessels, are not class III ADH-positive (c). At higher magnification, not only the dendritic processes of Purkinje cells, but also the cytoplasm of some Purkinje cells were stained (arrows) (d). (a) (c): ×20, (b): ×100, (d): ×400. LM: leptomeninges, ML: molecular layer, PL: Purkinje cell layer, GL: granule cell layer, WM: white matter.

2.3. Western blotting analysis of normal human brain extracts

Brain tissues were homogenized in an extraction buffer [500 μM NAD, 650 μM DTT (dithiothreitol), 5 mM Tris–HCl, pH 8.5], centrifuged at 105,000 × *g* for 1 h at 4°C, and the resultant supernatants (brain extracts) were analyzed. The protein concentrations were determined by Bradford's method [4]. The brain extracts were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) using a 10% w/v gel, and then electroblotted onto a polyvinyl difluoride membrane (Immobilone, Millipore, Bedford, MA). Each blot was treated with the anti-class III ADH antibody, followed by an alkaline phosphatase-conjugated secondary antibody, detected by staining with BCIP/NBT and their reaction products were quantified using NIH Image software.

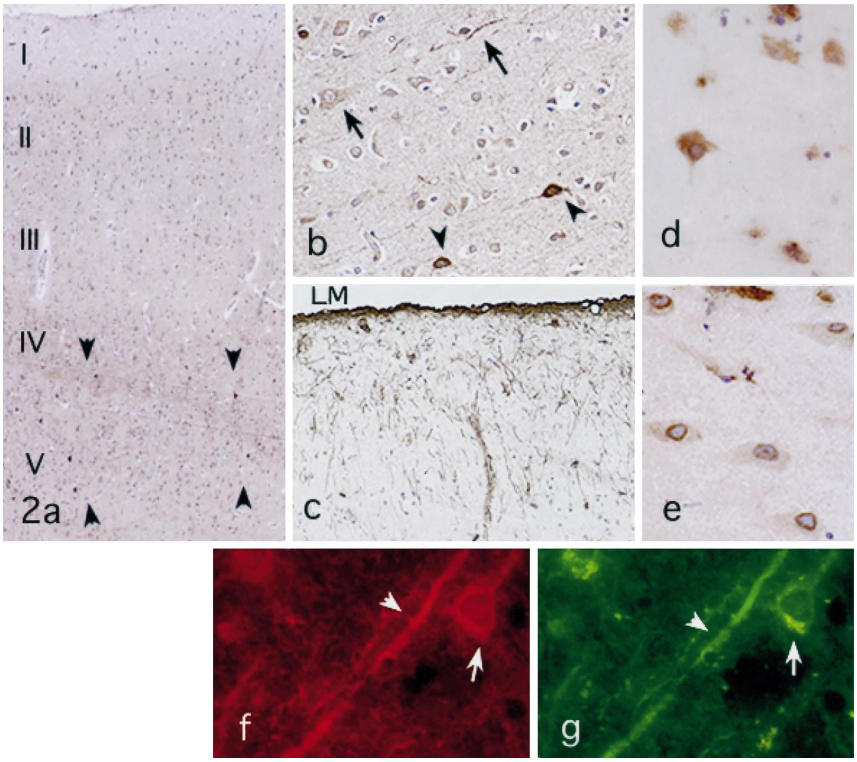

Fig. 2. Class III ADH-positive neurons and dendrites in the cerebral cortex. The positive neurons are scattered in layers IV and V (between arrowheads) (a). At high power view, dense class III positive neurons look shrunken (arrowheads) (b). A normal-looking neuron is not immunopositive, but its dendrite shows fine positive staining (arrows) (b). Dense class III positive neurons look shrunken (arrowheads) (b). The layer I of frontal cortex is class III ADH positive and fine linear staining of superficially located dendrites is visible mainly in layers II and III of the cerebral cortex (c). Some hippocampal pyramidal neurons show positive staining at the nuclear periphery (e) and both the perinuclear portions and cytoplasm of some are positive (d). Red-colored class III ADH-positive dendrites and neurons (f), co-exist with green colored microtubule associated protein 2 (MAP 2)-positive dendrite and neurons (g), in almost the same portion of the same section (arrow head: dendrite, arrow: neuron). (a) (c): ×20, (b): ×200, (d) (e): ×400, (f) (g): ×600. LM: leptomeninges.

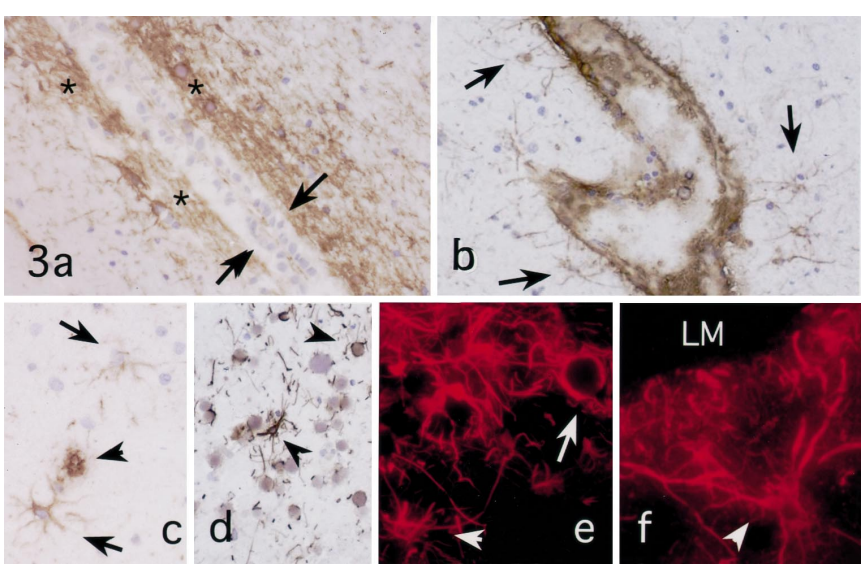

Fig. 3. Distribution of class III ADH-immunopositive astrocytes. Class III ADH is present along the subependymal astrocytic layers (asterisks). The ependymal cells (along the temporal horn of the lateral ventricle, between the arrows) are not stained (a). The perivascular astrocytic fibers and cytoplasm are stained (arrows) (b). An astrocyte (arrow) adjacent to a capillary (arrowhead) is class III ADH positive (c), as are the surfaces and fibrillary processes of corpora amylacea (arrowhead) (d). Immunofluorescent microscopy of specimens treated with TRITC (Dako, code no. R0156)-conjugated immunoglobulin revealed red-colored class III ADH-positive corpora amylacea, the core of which are not positive (arrow) (e). A fiber rich astrocyte is also class III ADH-positive (arrowhead) (e) (f).

3. Results

The most striking observation was the presence of class III ADH positive dendrites in the molecular layer of the cerebellum (Fig. 1a, b and d), in contrast to the lack of class I ADH staining in this area. Neither the cerebellar granule cells nor white matter were class I (not shown) or class III ADH positive (Fig. 1c). The Purkinje cellular cytoplasm also showed positive class III ADH staining (Fig. 1d). Fixation with buffered formalin for 10 min revealed some class III ADH positive neurons scattered in layers IV and V of the cerebral cortices (Fig. 2a). These neurons looked shrunken (Fig. 2b arrowheads). A normal looking neuron was not stained, but its dendrite was class III ADH positive (Fig. 2b arrows), whereas the astrocyte was negative. Examination of frozen sections, revealed that superficially located apical dendrites and layer I of the cerebral cortex were stained intensely with the anti-class III ADH antibody (Fig. 2c). The cytoplasm of large neurons in the hippocampus was also class III ADH positive (Fig. 2d), and some hippocampal neurons showed perinuclear positive staining without cytoplasmic staining (Fig. 2e). Double staining of class III ADH (Fig. 2f) and microtubule associated protein 2 (MAP 2) (Fig. 2g) with rhodamine and FITC, respectively, showed these two proteins co-existed in the cerebral cortical dendrites and neurons.

Intense immunohistochemical staining of class III ADH was also observed along the subependymal glial layer (Fig. 3a, asterisks), whereas the ependymal cells were class III negative (Fig. 3a, between arrows). A similar layered staining pattern was observed in the 1st layer of the cerebral cortex subjacent to the leptomeninges, which is composed of astrocytes (Fig. 2c). As well as these areas which isolate the brain from the cerebrospinal fluid, the perivascular areas throughout the brain were stained with the anti-class III ADH antibody. Class III ADH positive fine astrocytic processes extended towards arterioles, venules and capillaries (Fig. 3b and c) and many of the corpora amylacea were stained along their peripheries due to the presence of such astrocytic processes (Fig. 3d). This was confirmed by immunofluorescent microscopy of rhodamine stained specimen (Fig. 3e and f). Western blottings of the SDS–PAGE separated extracts of different brain regions showed relatively high amounts of class III ADH with a molecular weight of 38 kDa in the cerebellum and hippocampus, a finding consistent with the immunohistochemical pattern of ADH distribution (Fig. 4). The immunostaining results are summarized in Table 2.

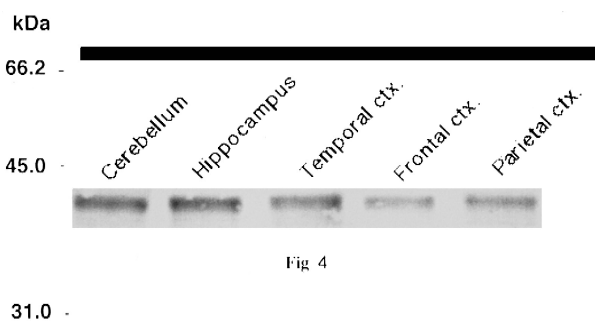

Fig. 4

Fig. 4. Western-blotting analysis showed 38 kDa of class III ADH in a variety of brain regions, including the cerebellum and hippocampus, in which the most dense aggregates were detected.

Table 2
Summary of immunohistochemical staining of class I and class III ADH

	Class I ADH	Class III ADH
Cortical neuron (layers IV and V, hippocampus)	−	+
Dendrite in cortex	−	+
Purkinje cell	−	+
Granule cell	−	−
Axon	−	−
Myelin	−	−
Astrocyte	−	+
Vessel	+	+

4. Discussion

Among the subclasses of mammalian ADH, class III ADH shows the smallest species variations of the enzyme protein [13]. The amino acid sequences of human and mouse class III ADHs are very similar; 93% are identical, whereas 83% of those of their class I ADHs are identical [7]. The similarities of amino acid sequences among the ADH classes are around 60% to each other [16]. Therefore, the antibody against anti-mouse class III ADH antibody reacts specifically with both mouse class III ADH and human class III ADH, but does not cross-react with other classes of ADHs [10,11].

The immunoreactive cells that were stained by the anti-class III ADH antibody were identified as neurons and astrocytes. The distribution patterns of the immunopositive neurons was reminiscent of that of the vulnerable portion of the brains of patients with hypoxic encephalopathy who show laminar necrosis in the cerebral cortex and eosinophilic degeneration of neurons. The influence of agonal hypoxia to the autopsied specimen is more or less inevitable. Actually, some scattered cortical neurons and pyramidal cells in the hippocampus showed mild eosinophilic degeneration after routine hematoxylin and eosin (HE) staining. In addition, the class III ADH-positive neurons looked shrunken, which we presume suggests they were undergoing a degenerative process.

In view of their major distribution sites, the intense class III ADH positive staining in the dendrites and cytoplasm of the cerebellar Purkinje cells we observed may be associated with the dorsal cerebellar degeneration and atrophy in chronic alcoholics. The cerebellar ataxia, suppressed diffuse neocortical activity and confusion shown by subjects with acute ethanol poisoning suggest the Purkinje cells and some cerebral cortical neurons are vulnerable to ethanol. The class III ADH enzyme is the only ADH

isozyme present in significant levels in the brain, although its metabolic activity toward ethanol is much lower than that of other ADH isozymes. However, a study carried out by our collaborators [15] showed that the activity of class III ADH in the livers of chronic alcoholics was higher than in the livers of normal subjects and was parallel the total amount of alcohol intake. Wickramasinghe [22] reported that several cultured glial cell lines showed a substantial capacity for ethanol metabolism, which was virtually insensitive to pyrazole. His inhibition studies suggested that half of the metabolic capacity was due to cytochrome P-450, not catalase. Of the mammalian ADH isozymes, class III ADH is the most insensitive to pyrazole. Therefore, at least half of the capacity for ethanol metabolism in glial cells may be attributed to class III ADH. Under hydrophobic conditions, the metabolic activity of class III ADH toward ethanol is activated markedly, in spite of its very high K_m for ethanol metabolism under hydrophilic conditions [12,19]. Therefore, class III ADH may metabolize ethanol under some micro-cellular conditions and produce acetaldehyde in vulnerable regions of the brain.

The astrocytic fibrillary processes and cytoplasm were stained with the anti-class III ADH antibody. Astrocytes aligned along the borders of the brain limit neuronal contact with the cerebrospinal fluid and the circulating blood, and act as a barrier to a variety of agents that attack the brain. There is evidence that class III ADH metabolizes formaldehyde (in a glutathione-dependent manner) [18], 20-OH-leukotriene B4 [9], which indicates that class III ADH-positive cells may protect the brain from toxic products and prolonged inflammation. This enzyme may also maintain the cellular milieu of the brain because of its contribution to the shunt pathway of cholesterol metabolism [17]. Therefore, class III ADH is probably associated with a variety of degenerative states mediated by unknown mechanisms in the brain.

Interestingly, unlike the astrocytes just beneath the ependymal layer, the ependymal cells lining the ventricular surface showed no staining with the anti-class III ADH antibody, in spite of their contribution to the cerebrospinal fluid (CSF)–brain barrier. This finding suggest that class III ADH may be a new astrocytic marker that will be useful for differentiating astrocytes and ependymal cells. We believe it is vital to investigate the roles of this enzyme in order to elucidate whether it is associated with a variety of neurological diseases.

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