F．研究発表

1．論文発表

論文執筆中。

2．学会発表

(1)大澤郁朗，太田成男：水素分子(H_2)による酸化ストレス細胞死の抑制．第 78 回日本生化学会学会，2005．10.

(2)石井徳恵，西槙貴代美，大澤郁朗，太田成男：トランスジェニック DAL マウスで惹起される酸化ストレスの飽和水素水飲用による抑制．第 78 回日本生化学会学会，2005．10.

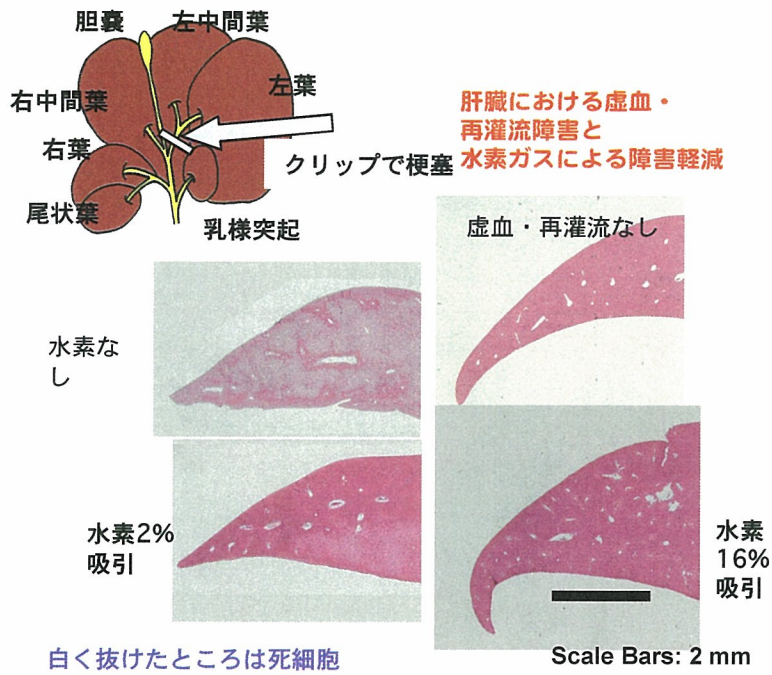
(3)福田慶一，麻生定光，大澤郁朗，山本保博，太田成男：水素ガスによる活性酸素フリーラジカルの除去—虚血再灌流傷害の軽減効果．第 78 回日本生化学会学会，2005．10.

(4)Shigeo Ohta：The Hydrogen Molecule as a Scavenger against Reactive Oxygen Species．THAI ASSOCIATION OF TISSUE BANKING, 2005．11.

G．知的財産の出願・登録状況

1．特許出願

太田成男・大澤郁朗：特願 2005-238572．生体内の有害なフリーラジカル除去剤及びその吸引装置



水素を吸引させると酸化ストレスによる障害が著しく軽減された。
虚血 90 分、再灌流 30 分。
(H&E 染色の結果)

1. 研究成果の刊行に関する一覧表

著者名	論文タイトル	発表誌名	巻号	ページ	出版年
Ohta S, and Ohsawa I,	Dysfunction of mitochondria and oxidative stress in the pathogenesis of Alzheimer's disease.	J. Alzheimer Disease		in press (review)	2006
大澤郁朗・太田成男	アルツハイマー病の危険因子である酵素活性欠損型アルデヒド脱水素酵素2遺伝子—その分子メカニズムとモデル動物の開発—	日本認知症学会誌	19(3) (通巻61)	284-295	2005
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太田成男	ミトコンドリア病を引き起こす変異tRNA:アンチコドンのタウリン修飾欠損	細胞工学(秀潤社)	24 (8)	814-819	2005
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太田成男	核コドンに変換したミトコンドリアDNA遺伝子の核への導入	蛋白質核酸酵素(共立出版)	50(14) :	1901-11903	2005 ;

Dysfunction of mitochondria and oxidative stress in the pathogenesis of Alzheimer's disease

------on defects in the cytochrome *c* oxidase complex and aldehyde detoxification

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Abstract: The mitochondrion is an organelle that plays a central role in energy production. It, at the same time, generates reactive oxygen species as by-products. Large-scale epidemiological case-control studies suggest the involvements of dihydrolipoamide succinyltransferase (DLST) of the mitochondrial Krebs cycle and mitochondrial aldehyde dehydrogenase-2 (ALDH2) in Alzheimer's disease (AD). The *DLST* gene has two gene-products, one of which, a novel gene product MIRTD, mediates the molecular assembly of the cytochrome *c* oxidase complex whose defect has been a candidate of the causes of AD. Since levels of MIRTD mRNA in the brains of AD patients were significantly low, a decrease in MIRTD could affect energy production. ALDH2, a matrix enzyme, was found to act as a protector against oxidative stress through oxidizing toxic aldehydes, such as 4-hydroxy-2-nonenal, that are spontaneously produced from lipid peroxides. Hence, a decrease in ALDH2 activity is proposed to contribute to AD. Indeed, transgenic mice with low activity of ALDH2 exhibited an age-dependent neurodegeneration accompanying memory loss. Since amyloid β peptide has been recently shown to be present in neuronal mitochondria to decline energy production and enhance ROS production, it has become possible to link AD more closely with roles of of mitochondria in the pathogenesis.

Key words: aldehyde; ALDH2; case-control study; cytochrome *c* oxidase; DLST

1. Introduction

The mitochondrion has been recognized as an organelle whose function is specific in energy metabolism. It oxidizes substrates such as carbohydrates, fatty acids, and amino acids through multiple steps and reduces NAD^+ and FADH. Using this reductive energy, it performs oxidation-reduction reactions through a series of the electron transfer system. The electrochemical potential across the mitochondrial inner membrane provides energy for ATP synthesis. At the same time, superoxide radicals are generated from oxygen molecules by accepting electrons that have been released from the electron transfer system. The other reactive oxygen species (ROS), hydrogen peroxide and hydroxyl radical were converted from superoxide radicals. Thus, mitochondria are the largest source of ROS. In addition to the role of energy metabolism, mitochondria have essential roles in apoptosis by storing the initiation signal factor, regulatory factors, and execution factors and releasing these factors during apoptosis. Moreover, mitochondria store calcium and regulate cell death by controlling calcium concentration. A decline in energy

production disturbs homeostasis in the cell and induces cell death due to necrosis. In these ways, mitochondria regulate living and death in a variety of manners [1].

Aging is the most common risk factor of the development of age-dependent neurodegenerative disorders including Alzheimer's disease (AD). However, there is no clear answer to the question of how aging becomes a risk factor of AD. Components that constitute cells including proteins, nucleic acids, and lipids are believed to degenerate with aging and their degeneration causes the degeneration of tissues and the senescence of individuals. Oxidative stress has been shown to be the primary cause for such degeneration. In eukaryotes, 90% of oxygen is used for energy metabolism primarily in mitochondria. In this process, ROS is generated as a by-product in energy metabolism. Therefore, the cell should be protected by some mechanisms to reduce this oxidative stress inside and outside mitochondria. If these protective mechanisms are exhausted, aging may be accelerated, thus increasing the risk of neurodegenerative disorders including AD.

In this review, we would like to discuss a specific decrease in activity of cytochrome *c* oxidase (COX), which is the terminal oxidase in the respiratory chain and interaction of COX with amyloid β peptide (Aβ) in AD. Moreover, we would like to emphasize the role of mitochondrial aldehyde dehydrogenase 2 (ALDH2), which has been believed to be involved in only alcohol metabolism, in the pathogenesis of AD.

2. Decrease in cytochrome *c* oxidase activity in AD patients

It has been well-known that glucose consumption is low in the brain of patients with AD, leading to a decline of energy production [2]. However, many investigators seem to have understood the decrease in energy metabolism as a secondary effect of neuronal changes toward death rather than a cause of AD. With regard to mitochondrial dysfunction, a specific decrease in COX activity has been suggested [3]. COX is an enzyme that functions in the terminal step of the electron transfer system and reduces oxygen molecules into water. It is a large complex composed of 10 subunits of nuclear gene-products and 3 subunits of mitochondrial gene-products. As compared with controls, mean protein concentration of four subunits, including mitochondrial gene- and nuclear gene-products, were significantly decreased in the brain of AD patients [4]. Since COX activity is reduced even in platelet mitochondria as well as brain mitochondria in AD patients, a decrease in COX activity cannot simply be explained as the secondary effect [5, 6, 7]. Additionally, symptoms resembling those of AD can be presented by a treatment with a COX inhibitor, azide [8].

2-1. Defect on the assembly of cytochrome *c* oxidase

We have long directed attention to dihydrolipoamide succinyltransferase (DLST), a component of α-ketoglutarate dehydrogenase complex in the mitochondrial Krebs cycle. Since the *DLST* gene is located in the region where a candidate gene for a familial AD is located [9], we paid attention to the *DLST* gene as a candidate gene responsible for the familial AD. As a result, the responsible gene of the familial AD was not DLST but was presenilin-1. However, since a frequency of a *DLST* haplotype in sporadic AD was significantly higher than that of controls, it is suggests that the haplotype is a risk factor for sporadic AD [10]. This correlation between the *DLST* haplotype and AD was also reported from the other groups [11, 12], but negative results have also been presented [13]. This discrepancy may be due to a weak risk for AD. By further analyzing the *DLST* gene, we clarified that the *DLST* gene encodes two gene products, one of which, named MIRTD (a mitochondrial respiratory complex assembler of truncated DLST), mediates the molecular assembly of the respiratory complexes including COX [14]. MIRTD mRNA is transcribed from intron 7 (Figure 1A). While

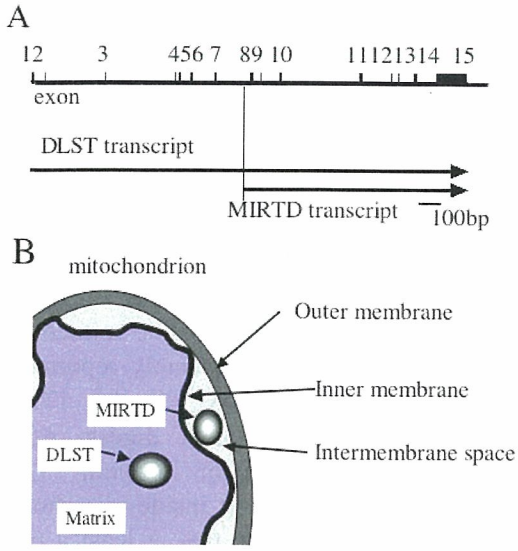

Figure 1: *The DLST gene bifucntionally encodes two gene-products.*
(A) Structure of the DLST gene composed by 15 exons. The novel gene product, named MIRTD, is transcribed from intron 7. (B) The Proteins are synthesized by the same reading frame. Full length DLST is present in the mitochondrial matrix, while MIRTD (exons 8-15) with about half the length on the C-terminal side, is present in the intermembrane space.

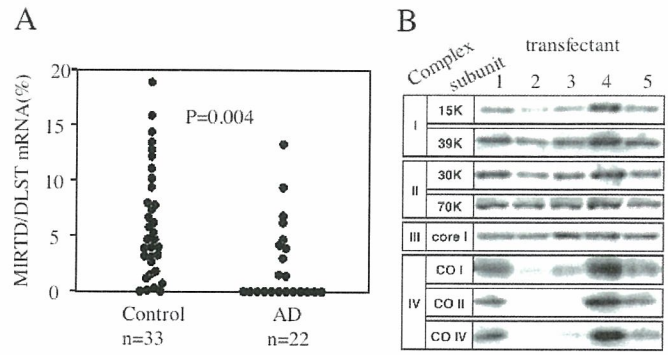

Figure 2: *Decrease in MIRTD expression in the brains of AD patients and decrease in MIRTD results in defect in the molecular assembly of the respiratory complexes.*
(A): Expression of MIRTD in the brains of AD patients and elderly individuals obtained by autopsy. The ratio between amounts of MIRTD mRNA and those of DLST mRNA was evaluated. The amounts of MIRTD mRNA shows a wide individual variation, but it was significantly lower in the brains of AD patients than those in the controls, and MIRTD mRNA could not be detected in the brains of half the patients
(B): Defect in the molecular assembly of complexes I and IV (cytochrome c oxidase) in cultured cells by the knocked-down expression of MIRTD. Clones 2 and 3 are cell lines that express less MIRTD. The others are control cell lines. The decrease in the subunits was apparent in complex IV (COX). COI and COII are mitochondrial gene products, and COXIV is a nuclear gene product. All were synthesized normally, suggesting a defect in molecular assembly.

DLST (exons 1-15) is located in the mitochondrial matrix, MIRTD (exons 8-15) is located in the intermembrane space of mitochondria (Figure 1B). mRNA of MIRTD was significantly decreased in the brain of AD patients compared with age-matched controls (Figure 2A).

When MIRTD was knocked-down to evaluate the function of MIRTD, the steady state level of subunits of COX markedly decreased, accompanying a modest decrease in the respiratory complex I, leading to the decline of oxygen consumption. Since translation of these subunits was normal, the decrease of the MIRTD protein results in the defect of their assembly (Figure 2B). Thus, full-length DLST and MIRTD, which are both

gene products derived from the *DLST* gene, are both involved in mitochondrial energy metabolism, one (full length DLST; exons 1-15) as a rate-regulating enzyme of the Krebs cycle in the matrix, and the other (MIRTD; exons 8-15) by mediating predominantly the molecular assembly of COX in the intermembrane space [14]. Since the knocked-down of MIRTD predominantly decreased in all the subunits of COX including nuclear gene- and mitochondrial gene-products, this finding agrees with the results in

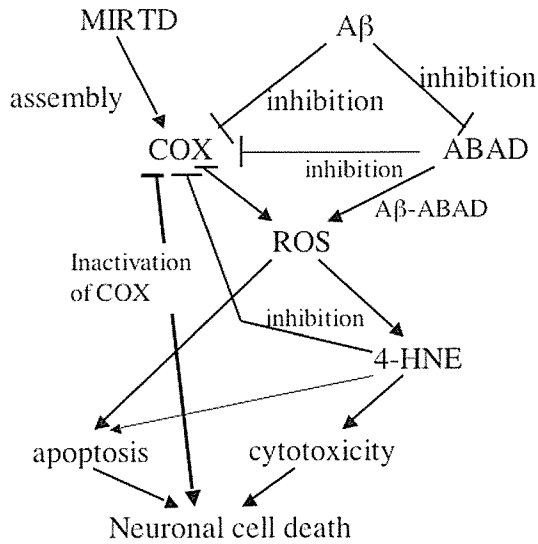

Figure 3: Interrelations among MIRTD, COX, Aβ, and ABAD in mitochondria.
Since MIRTD regulates the molecular assembly of COX, COX activity would be reduced by a decrease in MIRTD. Aβ inhibits COX activity and enhances ROS generation. Aβ also reduces the activity of ABAD and enhances the ROS generation. ROS produces toxic 4-HNE via lipid peroxides.

ref [4].

To summarize the above observations, *DLST* gene polymorphisms, observed more frequently in patients with AD, reduce the expression of MIRTD, mediate the molecular assembly of COX, and decline activity of the mitochondrial respiratory chain. In addition, as oxidative stress reduced the expression of MIRTD mRNA, the quantity of MIRTD is more likely to be regulated not only by the *DLST* polymorphisms but also by many internal and external environmental factors. In some patients, MIRTD was markedly reduced in their brain regardless of the *DLST* polymorphism. Particularly, no expression of MIRTD was observed in the brain of half the patients with AD. The decrease in COX activity in the brain of AD patients can be, at least in part, explained by the decrease in MIRTD [14].

2-2. Inhibition of COX activity by Aβ

The COX activity is a rate-limiting step of mitochondrial energy metabolism, and its decline reduces the ATP synthesis in the cell. Therefore, the decrease in COX activity is sufficient to induce cell death. However, it does not explain how the decrease in COX involves AD. COX is located in the mitochondrial inner membrane. Aβ has recently revealed to exist inside mitochondria and several groups have recently reported that Aβ inhibits COX activity [15, 16, 17]. In addition, γ-secretase, which digests Aβ out from amyloid precursor protein (APP), was also reported to exist inside mitochondria, indicating the possibility that at least a part of Aβ are generated in mitochondria [18]. This inhibition of COX activity by Aβ may induce the generation of ROS by not only reducing energy metabolism but also arresting the electron transfer system.

3. Aβ-binding alcohol dehydrogenase and Aβ

Recently, an alcohol dehydrogenase in mitochondria was revealed to play an important role in AD. Aβ-binding alcohol dehydrogenase (ABAD) was shown to directly link Aβ to mitochondrial dysfunction [19]. On crystal structure analysis of the ABAD-Aβ complex performed in the presence of NAD^+, the three-dimensional structure of the binding site in ABAD was markedly changed when it was bound to Aβ, and its binding activity with NAD^+ was abolished. In contrast, a peptide that is derived from ABAD specifically inhibited the ABAD-Aβ interaction and then suppressed Aβ-induced apoptosis and ROS generation. A mutant APP gene and the ABAD gene were introduced into transgenic mice to enhance the Aβ production. In the transgenic mice, an increase in oxidative stress in neurons was accompanied with memory loss [19]. These results indicate that Aβ affected mitochondrial function by binding to ABAD. Thus, the ABAD-Aβ interaction can be a therapeutic target of AD. In addition, a decrease in ATP production was accompanied with increases in ROS

generation and apoptosis in transgenic mice that overexpressed a mutant form of APP [20]. The increase in ROS correlated with a decrease in COX activity. When COX activity is inhibited, more ROS should be generated. Thus, the decrease in COX activity by ABAD bound to Aβ may promote the generation of ROS. ROS enhances the toxicity of Aβ [21]. Thus, the recent reports agree with the previous findings and linked mitochondrial function to Aβ in AD.

Correlations among the contributors are summarized in Figure 3.

4. Contribution of a decrease in ALDH2 activity to onset of AD

Mitochondria are a major source of ROS generation as mentioned above. Superoxide radicals are converted rapidly to hydrogen peroxides by Mn-superoxide dismutase (Mn-SOD) then to water by catalase or glutathione peroxidase. Superoxide-produced in mitochondria does not have so strong oxidative activity and does not directly damage DNA or proteins. However, as knocking-out of Mn-SOD exerts serious effects primarily on the nervous system, superoxide is undoubtedly very toxic to neurons [22]. However, how these ROS cause cell death is poorly understood. Our study on ALDH2 provided clues to the clarification of the relationship between ROS and AD [23].

4-1. The dominant-negative ALDH2 by a genetic polymorphism

Aldehyde dehydrogenases belong to a large family consisting of at least 16 different genes in humans, and are involved in metabolic systems of various alcohols and aldehydes according to their expression distribution and substrate specificity [24]. Among them, the *ALDH2* gene is located on chromosome 12q24.2 and codes for an enzyme consisting of a tetramer localized in the mitochondrial matrix. ALDH2 has two genetic variants, *i.e.*, active ALDH2∗1 and inactive ALDH2∗2, and their structural difference is the replacement of

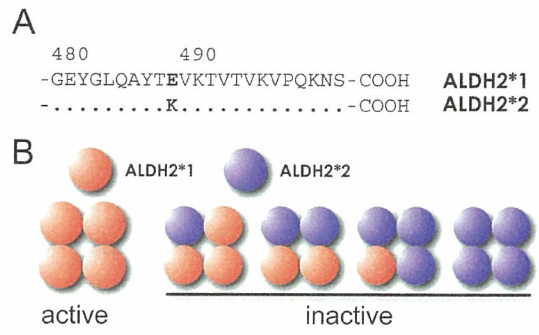

***Figure 4**: ALDH2 gene polymorphism*
A: C-terminal amino acid sequence of ALDH2. Single base substitution of the ALDH2 gene makes the enzyme active (ALDH2∗1) or inactive (ALDH2∗2). B: ALDH2 forms a tetramer consisting of the same subunit. Since the enzyme activity is lost if even one of the subunits is the inactive variant, ALDH2∗2 acts in a dominant-negative manner.

glutamate at the 487th position by lysine by a single nucleotide substitution [25]. When even one component of the tetramer of ALDH2∗1 is replaced by ALDH2∗2, its binding ability with NAD^+, a coenzyme, is reduced due to a structural change, resulting in loss of the enzyme activity [26]. Therefore, ALDH2∗2 acts in a dominant-negative manner, and if ALDH2∗1 and ALDH2∗2 are present at an equal ratio, the enzyme activity should be reduced to 1/16 (Figure 4). This ALDH2 catalyzes a low concentration of acetaldehyde as a substrate. When drinking ethanol, ALDH2 oxidizes acetaldehyde, generated by the oxidation of ethanol by alcohol dehydrogenase (ADH), into acetate. For this reason, if the ALDH2 activity is low, acetaldehyde accumulates in drinking and causes symptoms characteristic in those susceptible to the effects of alcohol such as facial flushing, nausea, and tachycardia. The presence of the inactive ALDH2∗2 allele is limited to East Asian races, the Mongoloids. In the Japanese, about 30% have heterozygous ALDH2∗2 allele with low ALDH2 activity, and about 10% are ALDH2∗2 homozygotes having no ALDH2 activity [27].

Table 1: Frequencies of ALDH2 genotypes in AD patients and controls

Subjects	Number of genotype [frequency]			
	1/1	*1/2*	*2/2*	*1/2 & 2/2*
Patients (n = 447)	232 [0.519]	183 [0.409]	32 [0.072]	215 [0.481] *
Controls (n = 447)	280 [0.626]	138 [0.309]	29 [0.065]	167 [0.374]

The frequencies of the *ALDH2*1* and *ALDH2*2* alleles were 0.724 and 0.276 in the AD patients but were 0.781 and 0.219 in the controls (p=0.005). *p=0.001, OR=1.6 (95% C.I. = 1.19-2.03)

4-2. *ALDH2*2* allele is a risk factor for late-onset Alzheimer's disease

We analyzed *ALDH2* gene polymorphisms in 472 AD patients whose onset was later than 65 years and 472 non-demented controls [28]. The frequencies of *ALDH2* gene polymorphisms vary widely among countries and even among regions in Japan. Additionally, the frequencies of gene polymorphisms related to gerontological disorders are expected to change with aging. In fact, the genotype frequency varied depending upon age [37]. Therefore, the controls were matched not only for gender and age but also for the region. Table 1 shows the results. The percentage of individuals having at least one *ALDH2*2* allele was 48.1% in the AD group but was 37.4% in the non-demented control group. The odds ratio was 1.6, and the *p* value was 0.001, indicating sufficient significance. The results were similar also when analysis was performed separately for males and females, and no gender difference was noted.

An allele of Apolipoprotein E (ApoE), ApoE-ε4 is widely accepted to be a risk factor for late-onset AD and the odds ratio of the onset of AD in individuals having the *APOE-ε4* allele is about 3.0. Figure 4 shows the results of cross comparison of APOE gene polymorphisms and ALDH2 gene polymorphisms. These results indicate that the coexistence of the *APOE-ε4* allele and

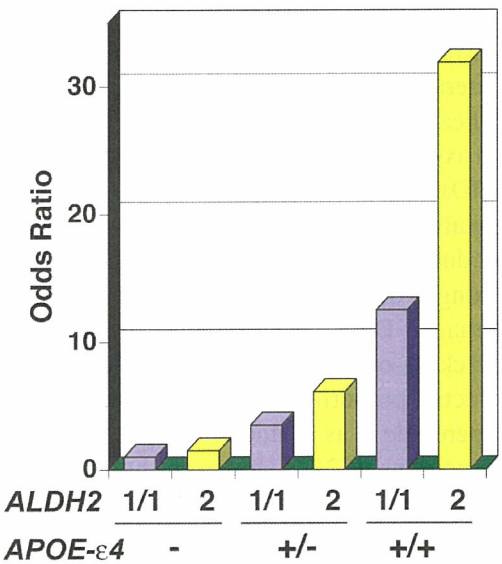

***Figure 5**: Synergism between ALDH2*2 and APOE-ε4 in the risk for the occurrence of sporadic AD*

*Concerning ALDH2, (1/1) means homozygotes of the ALDH2*1 allele, and (2) means homozygotes or heterozygotes having the ALDH2*2 allele. Concerning APOE-ε4, (-) means individuals having no ε4 allele, (+/-) means heterozygotes of the ε4 allele, and (+/+) means homozygotes of the ε4 allele. The odds ratio of the occurrence of AD is 31 times higher in individuals having the ALDH2*2 allele and being homozygous concerning the ε4 allele than in those having neither allele.*

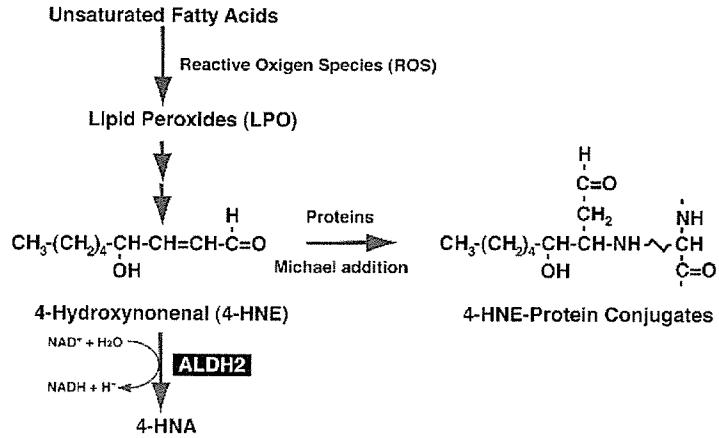

***Figure 6**: Mechanism of the formation of trans-4-hydroxy-2-nonenal (4-HNE) and its oxidation by ALDH2. 4-HNE is spontaneously generated from lipid peroxides and is oxidized by ALDH2. 4-HNE is highly cytotoxic, because it modifies proteins and nucleic acids and inactivates them.*

*ALDH2*2* allele synergistically increases the frequency of the onset of AD (Figure 5). Particularly, the frequency of the onset of AD was 31 times higher in individuals being *APOE-ε4* homozygous and having at least one *ALDH2*2* allele than in those having neither allele. About 0.6-1% of Japanese are estimated to belong to the group with the combination of these genotypes, and nearly all of them are expected to develop AD on the basis of calculation. The age at onset was also significantly accelerated by the synergy between *APOE-ε4* and *ALDH2*2* [28].

The reproducibility of the findings that the *ALDH2*2* allele is a risk factor for AD and that the risk is synergistically enhanced by its coexistence with *APOE-ε4* has been confirmed by the results of Tamaoka et al. of Tsukuba University using samples from patients after pathological definitive diagnoses (Tamaoka et al., 2003; Annual meeting in Japanese Society of Dementia Research). While *ALDH2* gene polymorphisms were also analyzed in Korea as a possible risk factor for AD, the cognitive ability was reported to have been unrelated to the ALDH activity [29]. However, the lack of statistical significance in this report, analyzing only 60 AD patients, is not persuasive because of an insufficient size.

4-3. *ALDH2*2* allele and increase in oxidative stress

Since the sensitivity to alcohol markedly depends upon *ALDH2* gene polymorphisms, the incidences of disorders due to excessive alcohol intake such as alcoholism and alcoholic hepatitis are low in individuals having the *ALDH2*2* allele [30]. It has also been reported that the *ALDH2*2* allele is a risk factor for polyneuropathy in diabetes mellitus, tumor, hypertension, and myocardial infarction [31, 32, 33, 34]. However, since the gene polymorphisms of ALDH2 are closely related to the lifestyle factor of drinking, it is difficult to distinguish the direct effect caused by gene polymorphisms from the secondary effects by ethanol consumption. This distinction is very important, because it is related to whether drinking should be recommended from the prophylactic viewpoint. We, therefore, strictly evaluated changes in individuals with the *ALDH2*2* allele by eliminating the effect of alcohol intake. In the large-scale epidemiological study by the Department of Epidemiology, National Institute of Longevity Science [35, 36], a medical check consisting of blood tests, urinalysis, and investigation of the lifestyle including ethanol consumption and clinical history was performed in about 2,300

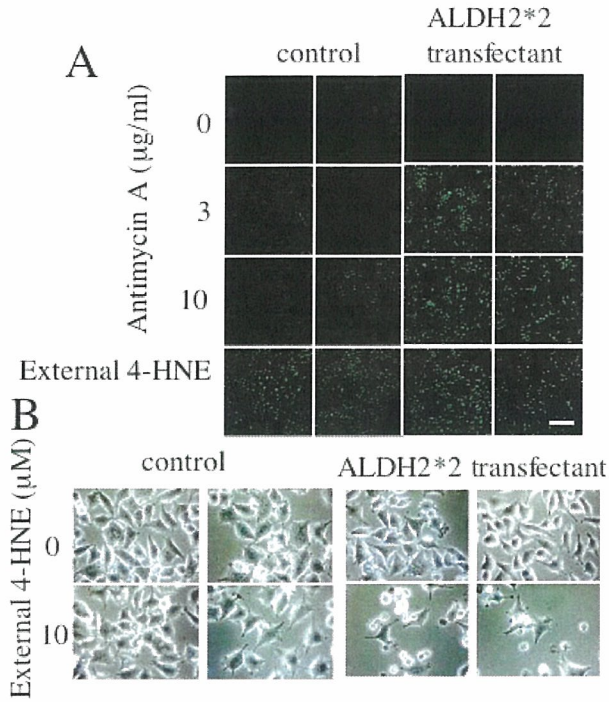

***Figure** 7: Transfection of inactive ALDH2*2 into PC12 accumulates 4-HNE after forced generation of ROS and made cells sensitive to 4-HNE (see ref.[44] for details).*

*(A) Accumulation of 4-HNE after treatment with antimycin A was imaged by confocal scanning laser microscopy. PC12 cells were stably transfected with the ALDH2*2 gene or an empty vector. Transfectants were treated with the indicated concentration of antimycin A to induce ROS or 1 μM of external 4-HNE, and incubated for 24 h. After fixation, cells were stained with anti-4-HNE antibody. Scale bar; 200 μm. Marked accumulation of 4-HNE was observed in transfectants in which ALDH2 activity was suppressed.*

*(B): 4-HNE caused marked cell death in PC 12 stable transfectants in which ALDH2 activity was suppressed in a dominant-negative manner by introducing the ALDH2*2 gene at concentrations that caused no death in the parent and control transfectants retaining ALDH2 activity. PC12 or each transfectant was treated with 10 μM 4-HNE or ethanol (1/1000 volume of medium) as a control (0 μM). One day after treatment, cells were observed under a phase-contrast microscope (x 200). Scale bar; 50 μm.*

healthy individuals aged in their 40s to 70s randomly selected from local residents. We performed a genetic analysis on the *ALDH2* polymorphisms and searched phenotypes specific in individuals with *ALDH2*2* allele in this cohort. Many phenotypes specific to the carriage of *ALDH2*2* were found in such serum levels of lipoproteins, but these phenotypes were correlated with ethanol intake, thus due to the drinking habit, but not to the direct genetic effect. As a result, the serum level of lipid peroxides (LPO) was significantly increased in significantly increased in females having the *ALDH2*2* allele even after normalizing with ethanol consumption to exclude the effect of alcohol intake. This result suggests the possibility that reduced ALDH2 activity increases oxidative stress independently of alcohol intake and that the *ALDH2*2* allele may be a risk factor for many age-associated diseases [37]. No significant difference was observed in males, but it was probably because the effect of alcohol intake on the accumulation of LPO was excessive. The

next question is what effect *ALDH2* gene polymorphisms have in AD.

4-4. Molecular mechanism of the promotion of the onset of AD by ALDH2*2

As observed above, epidemiological investigations have demonstrated that the presence of the *ALDH2*2* allele increases oxidative stress and is a risk factor for AD. Since the increase in oxidative stress is independent of alcohol metabolism, ALDH2 is considered to suppress oxidative stress by metabolizing substrates other than acetaldehyde. Additionally, as ALDH2 is localized in mitochondria, it is considered to metabolize aldehydes generated in mitochondria. Then, the next question is what is the aldehyde derivative.

LPO is generated by peroxidation of unsaturated fatty acids with ROS. From these LPO, aldehydes such as marondialdehyde (MDA), which is a marker of oxidative stress, and highly toxic 4-hydroxy-2-nonenal (4-HNE) are spontaneously generated, where MAD and 4-HNE are aldehyde derivatives. Particularly, 4-HNE causes protein denaturation by readily binding with lysine, histidine, serine, and cysteine residues [38]. In fact, 4-HNE reduces Na^+,K^+-ATPase activity [39]. It has also been shown *in vitro* to promote neuronal death [40]. Moreover, the accumulation of LPO and 4-HNE has been reported in neurodegenerative disorders including AD and Parkinson's disease [41, 42, 43].

Thus, we hypothesized that ALDH2 is involved in the detoxification of 4-HNE generated by oxidative stress of mitochondria and that defects in the ALDH2 activity cause neuronal death by stimulating the accumulation of 4-HNE due to oxidative stress. The hypothesis is summarized as follows: (a) ALDH2 detoxifies 4-HNE by oxidizing its aldehyde group; (b) in individuals with reduced ALDH2 activity, 4-HNE accumulates because of insufficient detoxification; (c) mitochondrial respiratory chain enzyme activities are inhibited by the accumulation of 4-HNE, and then the frequency of the generation of ROS increases; (d) ROS produces LPO and 4-HNE is generate by spontaneous reaction (Figure 6).

To verify this hypothesis, we prepared a mouse/rat-version *ALDH2*2* gene and introduced it into rat PC12 cells. As a result in a dominant negative manner, ALDH2 activity was suppressed in the cells into which the *ALDH2*2* gene was introduced, and cell death was induced readily by 4-HNE [44] (Figure 7B). The death of cells with defective in ALDH2 activity was promoted when ROS production was forcibly induced with antimycin A, which is an inhibitor of complex III of the mitochondrial respiratory chain. In this experiment, marked accumulation of 4-HNE was observed in the cells with reduced ALDH2 activity [44] (Figure 7A). It is possible to interpret that other enzymes could increase to compensate for the loss of ALDH2. However, since 4-HNE is an aldehyde derivative, it is reasonable that ALDH2 oxidizes 4-HNE. These results support the above hypothesis and indicate the role of ALDH2 protective against the mitochondrial oxidative stress [44].

ALDH2 has been discussed conventionally in relation only to ethanol drinking or its metabolism. However, as animals that do not drink also have the same gene, ALDH2 should be considered to have an intrinsic function other than ethanol-acetaldehyde metabolism. On the basis of the results we have obtained, it appears reasonable to think that ALDH2 is one of the protective mechanisms against oxidative stress [23].

4-5. Age-dependent degeneration of the central nervous system in ALDH2-suppressed transgenic mice

Construction of model animals by genetic manipulations is one of the best methods for analysis of the involvement of particular genes in the defense against oxidative stress at the animal level. In fact, in mice defective in Mn-SOD, oxidative stress accumulates, and mitochondrial dysfunction and subsequent cell

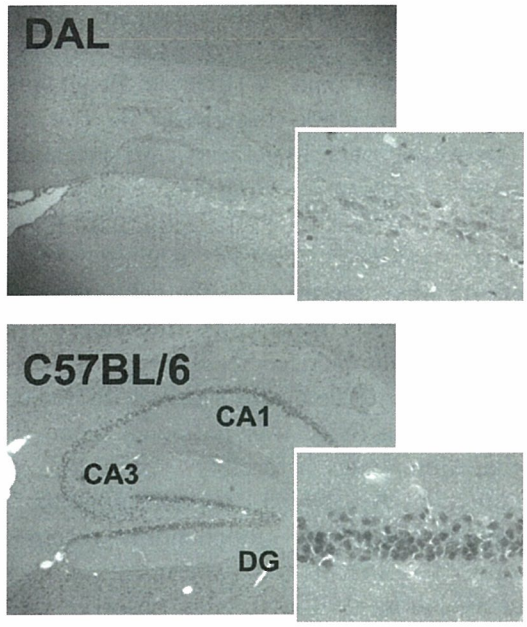

***Figure** 8: Hippocampal atrophy and pyramidal cell degeneration in DAL mice.*
In DAL mice (females), marked hippocampal atrophy and pyramidal cell degeneration were observed at the age of 18 months by H & E stain. Inserts are expansions of the CA1 region.

death are observed [22]. Since the model mice die about 1 week after birth, and analysis of age-dependent changes is impossible, this type is too sever to investigate age-dependent degeneration. Recently, prolongation of life was reported in mice into which the catalase gene equipped with a target sequence of mitochondria was introduced [45]. This finding clearly indicates the importance of the control of oxidative stress in aging.

We constructed ALDH2-deficient mice by introducing a mutant *ALDH2*2* gene. As mentioned above, ALDH2 belongs to a large family of aldehyde dehydrogenases. For aldehydes including 4-HNE, which are detoxified by ALDH2, there are multiple detoxification systems such as glutathione in addition to ALDH2. Knocking-out one such gene is likely to cause little change in aldehyde metabolism, because the knocked-out enzyme would be complemented with other members of the gene family. In fact, in ALDH2 knock-out mice, methoxyacetaldehyde (MALD) metabolism is reduced markedly, but no abnormality is observed in the development process or physical functions [46]. In humans, on the other hand, the suppression of ALDH2 activity by ALDH2*2 causes various disorders presumably due to increased oxidative stress. For example, the risk of the occurrence of AD is higher in individuals with the *ALDH2*2* allele. Therefore, we expected the development of model animals closer to humans by introducing the *ALDH2*2* gene and dominant-negatively suppressing the ALDH2 activity.

We first prepared transgenic mice by introducing the mouse-version of the *ALDH2*2* gene under a strong promoter which enhances ubiquitous expression. We named the resultant mice DAL (Dominant negative of ALDH2) mice. These mice showed no abnormality in the developmental process even when maintained as homozygotes. Females exhibited no particular abnormality on physical examinations compared with C57BL/6 mice when observed until 24 months after birth. A DAL line exhibited specific expression of the ALDH2*2 gene in hippocampus and cortex, nevertheless we used a promoter which enhances ubiquitous expression. Therefore, whether central neurons were vulnerable to 4-HNE similarly to PC12 cells was also evaluated. The cerebral cortex was removed from DAL mice at embryonic day 16, and 4-HNE was added to its primary culture. Neuronal death was promoted in DAL mice, suggesting an increase in oxidative stress in the brain (manuscript in preparation). Females, which showed no apparently different phenotype compared with C57BL/6 mice, were particularly analyzed. Autopsy of the brain was performed in 6-month-old mice, but no difference compared with the brain of C57BL/6 mice was noted. However, in 18-month-old DAL mice, signs of neurodegeneration such as atrophy of the hippocampus and associated loss of pyramidal neurons and activation of glial cells were observed (Figure 8). These changes began to be observed sporadically at the age of 12 months and increased with aging. However, no marked difference was observed

no marked difference was observed in motor functions or sensory functions between DAL mice and control C57BL/6 mice. Therefore, the mice were tested using the water maze task, which is widely used as a test of spatial cognitive ability, which is related to the hippocampus. DAL mice exhibited a decrease in spatial cognitive ability at the age of 6 months and a marked decrease at the age of 18 months. Such brain degeneration and decrease in spatial cognitive ability are considered to be due to reduced resistance to oxidative stress similar to the neurons in the primary culture. We are analyzing age-dependent changes in oxidative stress markers such as 4-HNE. Actually, in DAL mice expressing ALDH2*2 in a muscle-specific manner, signs of muscle atrophy and associated mitochondrial abnormalities and accumulation of 4-HNE were noted (manuscript in preparation).

4-6. 4-HNE metabolized by ALDH2 and its contribution to AD

From the above observations, we propose that the *ALDH2*2* allele is a risk of AD, because highly toxic aldehydes such as 4-HNE accumulate in the brain due to age-dependent increases in oxidative stress, and suppression of ALDH2 activity promotes the onset of AD (Figure 9). This leads us to two questions. First, do aldehydes such as 4-HNE accumulate before the onset of AD? Recently, marked increases in 4-HNE were reported in the hippocampus and superior and middle temporal gyrus of patients with mild cognitive impairment (MCI) and those with early AD compared with healthy individuals [47]. These results, which are in agreement with the results of analysis of LPO in cerebrospinal fluid [48], suggest that accumulation of oxidative stress, typically represented by 4-HNE, occurs before the onset of AD. Secondly, does the accumulation of aldehydes such as 4-HNE cause symptoms characteristic of AD? 4-HNE not only induces neuronal death but also causes synapse dysfunction due to mechanisms such as reducing the Na^+,K^+-

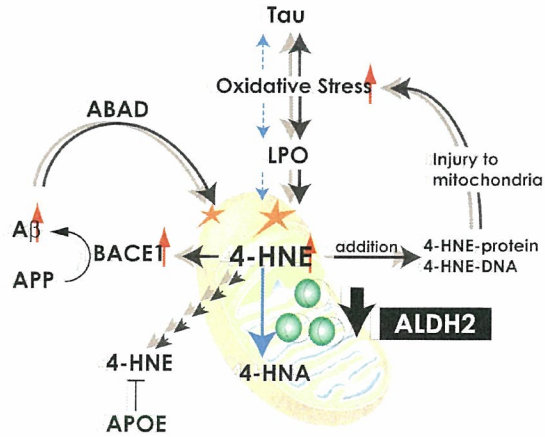

***Figure 9**: A model of accumulation of 4-HNE by the suppression of ALDH2 activity and its effects. In mitochondria, a decrease in ALDH2 activity promotes the accumulation of 4-HNE caused by oxidative stress, and binding of 4-HNE with proteins and DNA induces mitochondrial disorders. Mitochondrial disorders further enhance oxidative stress. Since this process is accelerated by aging, a decrease in ALDH2 activity is a risk factor of neurodegenerative disorders including AD. Oxidative stress including 4-HNE causes phosphorylation and structural changes of tau, and promotes NFT formation. 4-HNE also increases the expression of BACE1 and promotes the accumulation of Aβ. Furthermore, Aβ binds with ABAD and damages mitochondria. On the other hand, APOE promotes the elimination of 4-HNE.*

ATPase activity [49] and markedly inhibits microtubule formation and neurite outgrowth [50]. Furthermore, there have been a number of reports on the relationship between neurofibrillary tangle (NFT), which is a pathological feature characteristic of AD, and oxidative stress [51]. Concerning 4-HNE, in particular, it has been reported to induce structural changes in phosphorylated tau by modifying it and to make tau a structure in NFT [52, 53], so that 4-HNE is considered to play an important role in NFT formation. Concerning senile plaques, there have been many reports on increases in oxidative stress due to Aβ but few reports on the relationship between 4-HNE and the mechanism of Aβ production. Recently,

production. Recently, however, an increase in the quantity of BACE1 expression associated with the activation of stress response pathways by 4-HNE was reported, and the possibility that 4-HNE increases Aβ production was suggested [54]. Also, in transgenic mice, which are a model of Aβ deposition, accumulation of LPO was reported to precede accumulation of Aβ [55].

According to our epidemiological investigation, the *ALDH2*2* allele and *APOE-ε4* allele synergistically increased the risk of AD. Concerning this association between APOE and 4-HNE, the cytoplasm of pyramidal cells was reported to be positive for 4-HNE only in individuals with the *APOE-ε4* allele on immunostaining of the brain of AD patients using anti-4-HNE antibody [56]. Moreover, the strength of binding between APOE and 4-HNE was ε2>ε3>ε4, and this order was in agreement with the preventive effect of APOE against cell death due to 4-HNE [57]. From these observations, it is considered that APOE eliminates free 4-HNE in the body and that the possession of APOE-ε4, the APOE with the weakest 4-HNE elimination ability, leads to the accumulation of 4-HNE in neurons and an increase in oxidative stress. Accumulation of 4-HNE is considered to be further intensified if the ALDH2 activity is also reduced, resulting in an increase in the risk of AD.

5. Diversity of 4-HNE elimination mechanisms

Since highly toxic aldehydes such as 4-HNE are generated spontaneously by lipid peroxidation, there are a variety of mechanisms for their elimination including oxidation by ALDH2, etc., reduction by aldose reductase, etc. [58], and binding with glutathione [59]. We recently discovered that ADH polymorphism is a risk factor for cerebral infarction [60]. The possible involvement of ADH in the reduction of 4-HNE has been suggested by a study using hepatocytes [61], and this study must be extended to the nervous system. Also, multiple aldehyde dehydrogenases are considered to oxidize 4-HNE, and the report that ALDH5A present in mitochondria, as is ALDH2, plays an important role in the detoxification of 4-HNE in the central nervous system is interesting [62].

DAL mice gradually develop neurodegeneration with aging after the growth period. Analysis of these mice may clarify the relationship between lesions characteristic of AD and oxidative stress. Also, the development of appropriate methods for the prevention of lesions occurring in these mice is considered to provide clues to the development of prophylactic and therapeutic methods against diseases including AD. Figure 9 summarizes our conclusion.

6. Concluding remarks

Reports of the inhibition of COX activity by Aβ and generation of ROS by its binding with an ADH in mitochondria showed strong evidence that mitochondria play a direct role in the pathogenesis of AD. Also, our results that amounts of MIRTD, which mediates the molecular assembly of the respiratory complexes including COX, was low in the brain of AD patients were in agreement with these previous reports. The findings that ALDH2 is a risk factor for AD and that it acts as a protective mechanism against oxidative stress contributed to the clarification of the role of mitochondria from a novel view. Since transgenic mice with the declined ALDH2 activity showed age-dependent neurodegeneration accompanying memory-loss, analysis of these mice is expected to clarify the relationship between lesions characteristic of AD and oxidative stress.

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総説

アルツハイマー病の危険因子である酵素活性欠損型アルデヒド脱水素酵素2遺伝子

——その分子メカニズムとモデル動物の開発——

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はじめに

アルツハイマー病（AD）をはじめとする加齢に伴う神経変性疾患の発症に対してもっとも普遍的な危険因子は老化である．しかし，老化がなぜ危険因子となるのかについての明確な答えはない．細胞を構成する蛋白質，核酸，脂質などの物質が年数を経るにつれ変性し，それが細胞及び個体の変性，すなわち老化を引き起こすとされている．その変性を引き起こす主体となるものが酸化ストレスであることが明らかとなってきた．生命体は，その維持と活動に必要な代謝エネルギーを効率良く生み出すために27億年の昔から酸素を利用するようになった．真核細胞においては，主にミトコンドリアで85～90%の酸素がエネルギー代謝に用いられる．しかし，その過程では常に電子の漏れが生じ，その電子によって酸素が還元されて活性酸素種（ROS）となり，細胞に酸化ストレスを与える．その為，ミトコンドリアの内外でこの酸化ストレスを除去する機構が存在し，常に細胞を防御している．この防御機構が疲弊すると老化は促進される．従って，ADなどの神経変性疾患が発症する危険性が増大することが予想される．

本稿では，アルコール代謝に関わっているとされてきたミトコンドリアのアルデヒド脱水素酵素2（ALDH2）が，酸化ストレスによる脂質の過酸化で派生する毒性の高いアルデヒド類を除去する酵素であることを概説し，ALDH2酵素活性欠損型遺伝子の保持がADの危険因子である原因を分子レベルで論じる．さらにALDH2活性を抑制したトランスジェニック・マウスでは加齢に伴う神経変性と空間的学習能力の低下が認められたので，その一部について紹介する．

The deficiency in a mitochondrial aldehyde dehydrogenase as a risk factor of Alzheimer's disease: Its molecular mechanism and a model animal
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