5. 痴呆性疾患における生物学的診断マーカー

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はじめに

痴呆性疾患は近年増加し，65歳以上の10人に1人は痴呆症という状況になってきている．その中でアルツハイマー型痴呆（AD）は近年本邦でも増加してきており，痴呆性疾患の約半数を占めている[1,2)]．また，近年塩酸ドネペジル（アリセプト®）が発売され治療が可能となり，有用性が報告されている[3)]．このことから，痴呆性疾患の中でも特にADの診断が早期にかつ的確にできるか否かが重要なポイントとなってくる．しかし，現在のAD診断は徹底した除外診断に基づいてなされており，より容易に誰でもできる診断マーカーの開発が望まれている．これまでに多くの診断マーカー開発のアプローチがなされているが，最も良い成績が出ているのはタウ蛋白関連のものである．そこで，タウ蛋白に関連した診断マーカー研究と新規マーカーであるWGA結合糖蛋白質について報告する．

■A．AD診断のためのマーカー

AD撲滅のために設立したレーガン研究所は，ADの生物学的診断マーカーの条件として，病態を良く反映し，患者への侵襲性が少なく，他の痴呆性疾患を区別して診断する精度が高いこと，つまりAD患者を検出する率（感度）と非患者を検出しない率（特異度）が共に80%を超えることを要求している．髄液中総タウ蛋白は上記基準をかなり満たすが，感度と特異度が共に80%以上は難しく，アミロイドβ蛋白と組み合わせることにより，AD index，AD unitという表現を用いているが，感度と特異度が共に80%以上という結果が得られている[4,5)]．総タウ蛋白で特に問題なのは，髄膜脳炎やクロイツフェルト・ヤコブ病（CJD）などで極端に高値を示すことである（図1）．そこで，単独でこの基準をクリアする方法はないかと考え，髄液中リン酸化タウ蛋白を検討した．AD患者脳にみられる神経原線維変化のタウ蛋白は高度にリン酸化されている．このため，リン酸化タウを選択的に測定できれば，総タウより良い結果が期待できる．そこで，我々のグループはセリン199のリン酸化部位に着目してリン酸化タウN末端断片を定量するサンドイッチELISAを開発した[6)]．その結果，総タウより良い結果が得られ，特に総タウで高値を示していた髄膜脳炎やクロイツフェルト・ヤコブ病が低値であった（図2）．このためROC解析でも明らかに改善し，単独で感度，特異度ともに80%以上を示すという結果が得られた（表1）[7)]．現

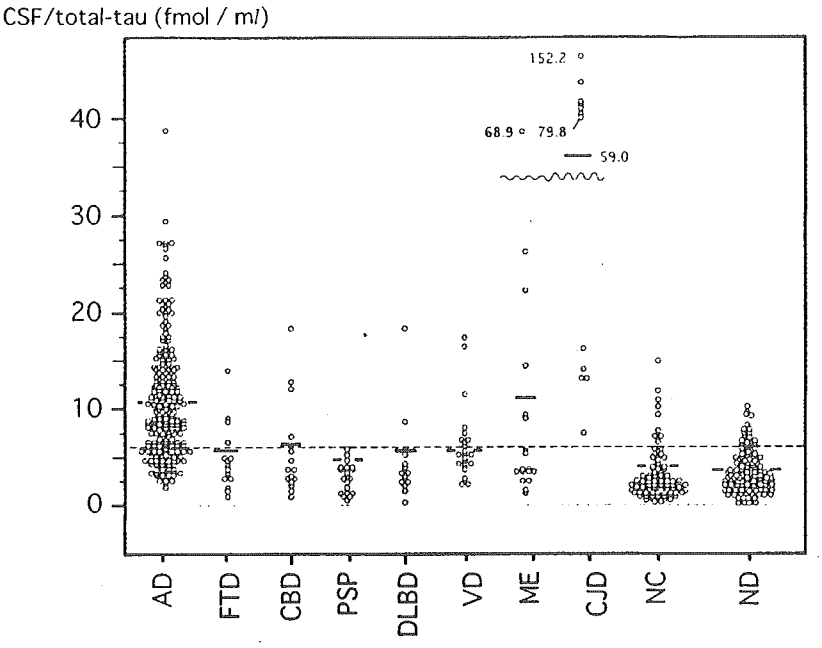

図1

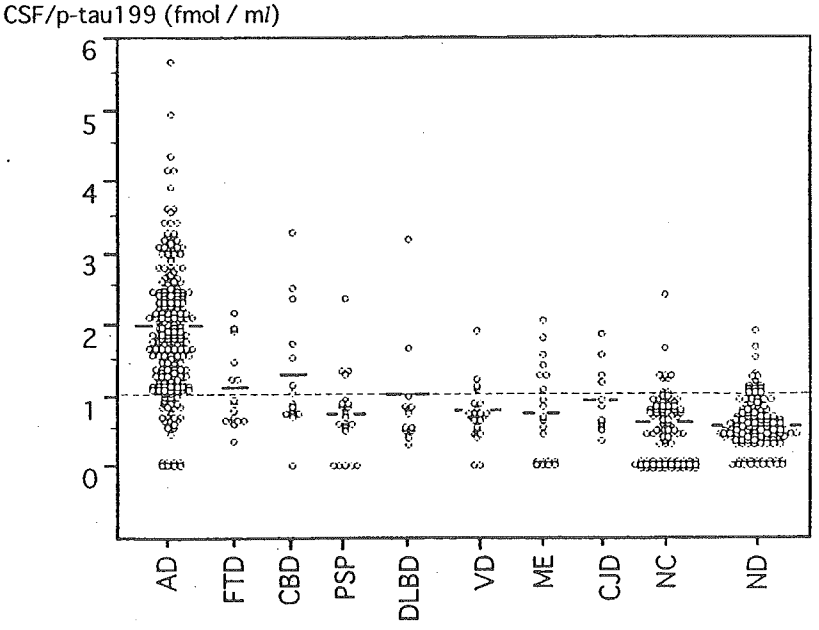

図2

在リン酸化タウは我々のグループが報告しているセリン 199 のリン酸化部位をみる方法以外に，スレオニン 181，スレオニン 231 のリン酸化部位をみる方法が報告されている．いずれも良い成績であり，現時点では髄液中リン酸化タウ測定が最も信頼性のおける診断マーカーと考えられる．さらに，診断精度を上げるためには，大脳皮質基底核変性症（CBD）や進行性核上性麻痺（PSP）などに代表されるタウオパチーとの鑑別力をあげていく試みが必要と思われる[8,9]

表1　ROC Analysis

AD vs NC+ND			
	Cut off level	Sensitivity	Specificity
total tau	4.8 fmol/ml	82.7%	82.0%
p-tau 199	0.96	87.3	87.4
AD vs Others			
	Cut off level	Sensitivity	Specificity
total tau	6.0 fmol/ml	77.1%	77.6%
p-tau 199	1.05	85.2	85.0

■B．WGA 結合糖蛋白測定の試み

WGA（小麦胚芽レクチン，Wheat Germ Agglutinin）は，特異的な糖鎖を認識して結合するレクチンの一種である．我々は，250 kDa の WGA 結合糖蛋白が AD の髄液中で減少しているという報告があり[10]，アルツハイマー型痴呆（AD）の髄液中診断マーカーとしての有用性を検討した．対象は，AD 58 例（男/女:19/39），AD 以外の痴呆: 脳血管性痴呆（VD），正常圧水頭症（NPH），レビー小体型痴呆（DLB），CJD，CBD，PSP の計 31 例（20/11），コントロールとして痴呆のない症例 50 例（28/22）の髄液において，レクチンブロット法を用いて検討を行った．総蛋白 5 μg にあたる髄液を 5〜20% gradient gel にアプライして SDS-PAGE で電気泳動して PVDF メンブレンにブロッティングし，一次抗体に WGA-Biotin，二次抗体はアルカリホスファターゼ標識 Streptavidin を反応させ，Western Blue で発色したバンドを検討した．リン酸化タウ蛋白（Tau pS199）の測定は，ヒトリン酸化タウ（pS199）ELISA キット（BioSource 社）を用いて測定した．

AD で低値を示す WGA 結合糖蛋白は 3 種を候補としており，これらのうち多症例での検討によりさらに約 75 kDa と約 25 kDa の 2 種の蛋白（それぞれ a，c とする）が診断マーカーとして有用であることが示唆された．いずれの蛋白も，AD 群と non-AD 群と比較して検討すると，有意に（$p<0.001$）低値を示した．さらにリン酸化タウ蛋白を同じ症例で測定し，比較検討した．その結果 2 種の蛋白のうち，WGA 結合糖蛋白-a は，AD で減少し，リン酸化タウ蛋白は増加していたため，WGA 結合糖蛋白-a に対するリン酸化タウ蛋白の比を検討したところ，AD 群で non-AD 群よりも明らかに高値を示し（$p<0.001$），この指標は WGA 結合糖蛋白単独よりもさらに精度の高いマーカーとなる可能性が示唆された（図 3）．さらに DLB では，AD 群と比較して明らかに低値を示しており，この指標によりタウオパチーである DLB と AD との鑑別診断への有用性が期待できる．今後，これらの蛋白の同定と，その働きに注目する必要がある．

まとめ

現時点では，AD の診断マーカーとして髄液中リン酸化タウ測定が最も信頼性があり，有用であると考えられる．リン酸化タウで鑑別困難なタウオパチーの鑑別に髄液中 WGA と結合する髄

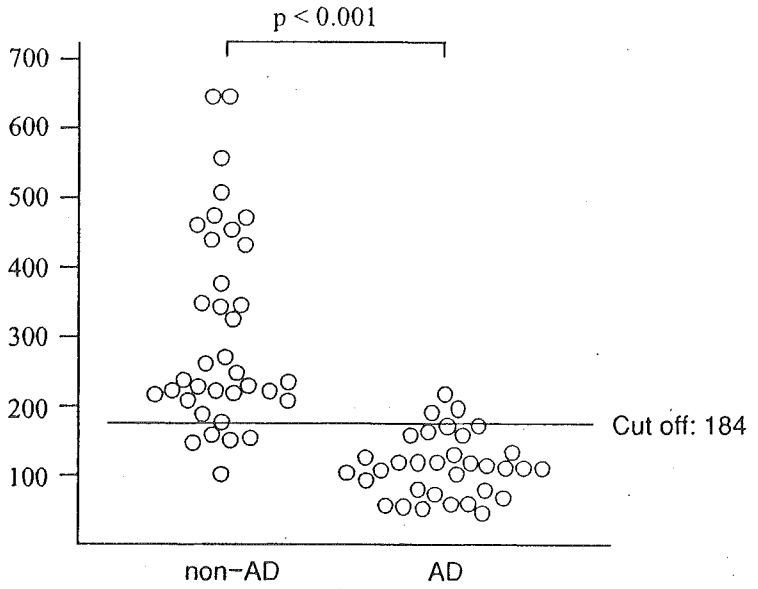

図3　WGA-binding-a/Tau pS199

液中糖蛋白が有用である可能性がされた.

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講演4

アルツハイマー病早期診断に役立つ生物学的診断マーカー

Early diagnostic marker for Alzheimer's disease

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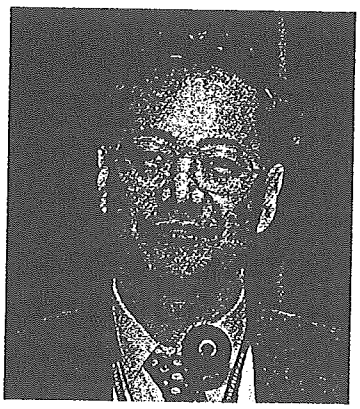

浦上克哉 先生

アルツハイマー病早期診断に役立つ生物学的診断マーカー

Early diagnostic marker for Alzheimer's disease

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はじめに

アルツハイマー病（Alzheimer's disease: AD）の診断において，髄液（cerebrospinal fluid: CSF）中の総タウ蛋白は有用な診断マーカーであるが，AD患者と健常対照群を明確に鑑別できない[1,2]。そこで，酵素免疫抗体法（enzyme immunoassay: EIA）を用いたリン酸化タウ蛋白の測定法について検討した。

従来，CSF中の総タウ蛋白の定量にはInnogenetics社製のサンドイッチEIA[3]が用いられているが，われわれは三菱化学との共同開発により，199番目のリン酸化部位を検出できるリン酸化タウ蛋白の測定方法を確立した[4,5]ので，その有用性を比較検討した。次に，リン酸化タウ蛋白199では鑑別が困難であったADとタウオパチーの鑑別診断に役立つ診断マーカーを検討するために，WGA（wheat germ agglutinin）結合糖蛋白について検討した[6]ので報告する。

タウ蛋白測定に関する検討

対象は，対照（NC）群95名，認知症を有さない神経疾患（疾患対照：ND）群122例，AD群235例，前頭側頭型認知症（frontotemporal dementia: FTD）群16例，進行性核上性麻痺（progressive supranuclear palsy: PSP）群21例，大脳皮質基底核変性症（corticobasal degeneration: CBD）群15例，レビー小体型認知症（dementia with Lewy bodies: DLB）群13例，脳血管性認知症（vascular

dementia: VaD) 群 23 例，髄膜脳炎 (meningoencephalitis: ME) 群 18 例，クロイツフェルト-ヤコブ病 (Creutzfeldt-Jakob disease: CJD) 群 11 例であった。

従来法による CSF 中の総タウ蛋白測定では，NC 群や他の疾患対照群に比較して AD 群で有意に高値を示したが，ME 群，CJD 群でも同様に高値を示し，他群と重複領域が観察され，さらにタウオパチーとの鑑別は困難であった。一方，CSF 中のリン酸化タウ蛋白 199 の測定では，AD 群では NC 群や他の疾患対照群に比較して有意に高値を示し，重複領域の減少がみられた。重要な改善点としては，ME 群や CJD 群では低値であり，AD 群と明瞭に区別できたことである。しかし，タウオパチーについては総タウ蛋白と同様に特異性を示せなかった。

ROC 分析 (receiver operating characteristic analysis) を用いた認知症を有さない対照 (NC＋ND) 群と AD 群の比較分析においては，カットオフ値を総タウ蛋白で 4.8 fmol/mL，リン酸化タウ蛋白 199 で 0.96 fmol/mL に設定すると，感度と特異度は総タウ蛋白でそれぞれ 82.7%，82.0%，リン酸化タウ蛋白 199 では 87.3%，87.4% であった。AD 群以外のすべての群との比較では，カットオフ値を総タウ蛋白で 6.0 fmol/mL，リン酸化タウ蛋白 199 で 1.05 fmol/mL に設定すると，感度と特異度は総タウ蛋白でそれぞれ 77.1%，77.6%，リン酸化タウ蛋白 199 では 85.2%，85.0% であり，感度と特異度がともに 85% を超えた初めての報告となった (表 1)[5]。

一方，軽度認知障害 (mild cognitive impairment: MCI) は，AD と正常の中間の状態である。筆者らは，Petersen の診断基準[7] を用いて診断した MCI 患者を対象に，リン酸化タウ蛋白の測定を実施した。その結果，MCI 発症後 2

表 1　ROC 分析による総タウ蛋白ならびにリン酸化タウ蛋白 199 の感度と特異度

AD 群 vs (NC + ND) 群			
	カットオフ値	感度	特異度
総タウ蛋白	4.8 fmol/mL	82.7%	82.0%
リン酸化タウ蛋白 199	0.96 fmol/mL	87.3%	87.4%
AD 群 vs その他			
	カットオフ値	感度	特異度
総タウ蛋白	6.0 fmol/mL	77.1%	77.6%
リン酸化タウ蛋白 199	1.05 fmol/mL	85.2%	85.0%

年後からADに移行した群（AD移行群）と，MCIからADに移行しなかった群（非AD移行群），および健常群でリン酸化タウ蛋白199を用いて比較した結果，AD移行群で有意に高値であることが示された。

本研究で，CSF中のリン酸化タウ蛋白199は早期AD診断に活用できることが示唆された。

WGA結合糖蛋白の検討

リン酸化タウ蛋白199では鑑別が困難であったADとタウオパチーの鑑別診断に役立つ診断マーカーを検討するために，新規蛋白であるWGA結合糖蛋白について検討を行った。WGA結合糖蛋白はシアル酸に特異的に結合し，とくにO型やN型グルコース，そのなかでも高マンノース型や混合型，複合型を検出することが知られている。また，大脳皮質に高濃度に存在し，ADでは側頭・頭頂葉皮質での減少が報告されている[8]。

われわれは，ウエスタンブロット法を用いて非AD群とAD群のWGA結合糖蛋白におけるband A，band Bおよびband Cを検出し，比較検討を行った。Band CはAD群と非AD群で有意差は認められなかったが，band Aとband BではAD群で有意差（$p<0.05$）を認めた。さらに，band Aにおいてリン酸化タウ蛋白199/WGA結合糖蛋白比を検証した結果，AD群（40例）と非

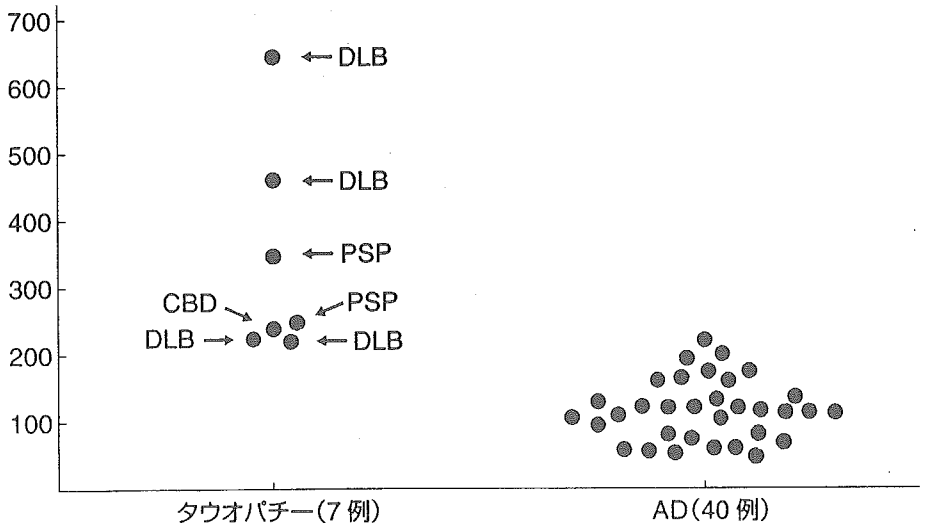

図1　タウオパチーとADにおけるリン酸化タウ蛋白199/WGA結合糖蛋白比
DLB: dementia with Lewy bodies（レビー小体型認知症），PSP: progressive supranuclear palsy（進行性核上性麻痺），CBD: corticobasal degeneration（大脳皮質基底核変性症）

AD群（36例）で有意差（$p<0.001$）が認められた。AD群（40例）とタウオパチー群（7例）におけるリン酸化タウ蛋白199/WGA結合糖蛋白比の検討の結果，両者を鑑別できる可能性が示唆された（図1）[6]。今後，さらに例数を増やして検討を行う予定である。

まとめ

CSF中のリン酸化タウ蛋白199は，ADの早期診断マーカーとして有用であり，MCIの検出にも活用できると考えられた。また，CSF中のリン酸化タウ蛋白199/WGA結合糖蛋白比は，ADとタウオパチーの鑑別に有用であることが期待された。

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Oxidative Stress and Age-Related Neurodegeneration

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21 Temporal Primacy of Oxidative Stress in the Pathological Cascade of Alzheimer Disease

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ABSTRACT

Most of the known mutations in specific genes, and genetic, medical, environmental, and lifestyle-related risk factors for Alzheimer disease (AD) are associated with an increase in oxidative stress. In contrast, several agents, nutrients, and behavior that reduce the risk of AD are associated with a protection against oxidative stress. This evidence strongly suggests that oxidative stress is universally involved in the pathogenesis of full-scale AD, especially in the upstream of the pathological cascade. An early involvement of oxidative stress in the pathogenesis of AD is more directly demonstrated by recent studies. Indeed, oxidative stress

induces intracellular amyloid-β (Aβ) accumulation and tau phosphorylation in cell cultures, while vitamin E, a radical scavenging antioxidant, reduces Aβ and tau lesions in the transgenic animals. Oxidative damage precedes Aβ deposition in Down syndrome patients and transgenic animal models of AD. Furthermore, individuals with mild cognitive impairment (MCI), who, at least in part, represent the prodromal stage of AD, show increased oxidative damage.

21.1 INTRODUCTION

Alzheimer disease (AD) is a disease with a prevalence that increases exponentially with age, with about half of the population afflicted by the age of 95.[1] This strongly supports an association between old age and AD. As in other organ systems, cells in the brain encounter a cumulative burden of oxidative and metabolic stress, which may be a universal feature of the aging process as well as a major causal factor of senesence. Each of the macromolecules, including nucleic acids, proteins, and lipids, is oxidatively modified during aging. Indeed, the brain is especially vulnerable to free radical damage because of its high oxygen consumption rate, abundant lipid content, and relative paucity of antioxidant enzymes compared with other organs.[2,3]

Both the aging process as well as the genetic, medical, environmental, and lifestyle-related risk factors for AD are associated with oxidative stress. This chapter focuses on the early involvement of oxidative stress in the pathological cascade of AD.

21.2 GENES IN AUTOSOMAL-DOMINANT FAMILIAL ALZHEIMER DISEASE AND OXIDATIVE STRESS

Recently, an increasing number of *in vitro* and *in vivo* studies have suggested that oxidative stress is involved in the pathogenesis of AD and autosomal-dominant familial AD with amyloid β protein precursor (AβPP), presenilin-1 (PS-1), or presenilin-2 (PS-2) gene mutations. Indeed, increased oxidative stress, elevated vulnerability to oxidative stress-induced cell death, and reduced antioxidant defenses have been demonstrated in (1) cell lines expressing mutant human AβPP, PS-1, or PS-2;[4–7] (2) transgenic mice expressing mutant human AβPP and PS-1 as well as knock-in mice expressing mutant human PS-1;[8–14] (3) fibroblasts and lymphoblasts from familial AD patients with AβPP or PS-1 gene mutation;[15] and (4) cerebral cortex of autopsied brain samples from patients with AβPP or PS-1 gene mutations.[16,17] These mutations, however, account for only a small proportion of patients with AD.

21.3 RISK FACTORS FOR ALZHEIMER DISEASE AND OXIDATIVE STRESS

There are several known risk factors for AD other than advanced age, namely, genetic, medical, environmental, and lifestyle-related factors. The major genetic risk

factor for early- to late-onset sporadic and familial AD is the possession of one or both of the apolipoprotein E4 (ApoE4) alleles. *In vitro*, ApoE shows allele-specific antioxidant activity, with ApoE2 being the most effective, and ApoE4 the least effective.[18] Indeed, oxidative damage in ApoE genotype-dependent manner has been demonstrated in autopsy brain samples of AD patients.[19–21]

Medical risk factors for AD include traumatic brain injury, cerebral infarcts, diabetes mellitus, hypercholesterolemia, and hyperhomocysteinemia. Environmental and lifestyle-related risk factors include aluminum exposure, smoking, high calorie intake, and lack of exercise and intellectual activities.[3,22–24] Indeed, all these AD risk factors are associated with an increase in oxidative stress.[3,25–30]

With this notion, it is not surprising that agents or nutrients inhibiting free radical formation reduce the incidence of AD. Indeed, agents or nutrients such as vitamins E and C, as well as estrogen, nonsteroidal anti-inflammatory drugs, statins, n−3 polyunsaturated fatty acids, and wine have been proven to have an antioxidant activity and reduce the incidence of AD.[22,24,31–35] Furthermore, calorie restriction, exercise, and intellectual activity have been proven to promote neuronal survival through decreased oxidative stress in experimental animals.[3,23]

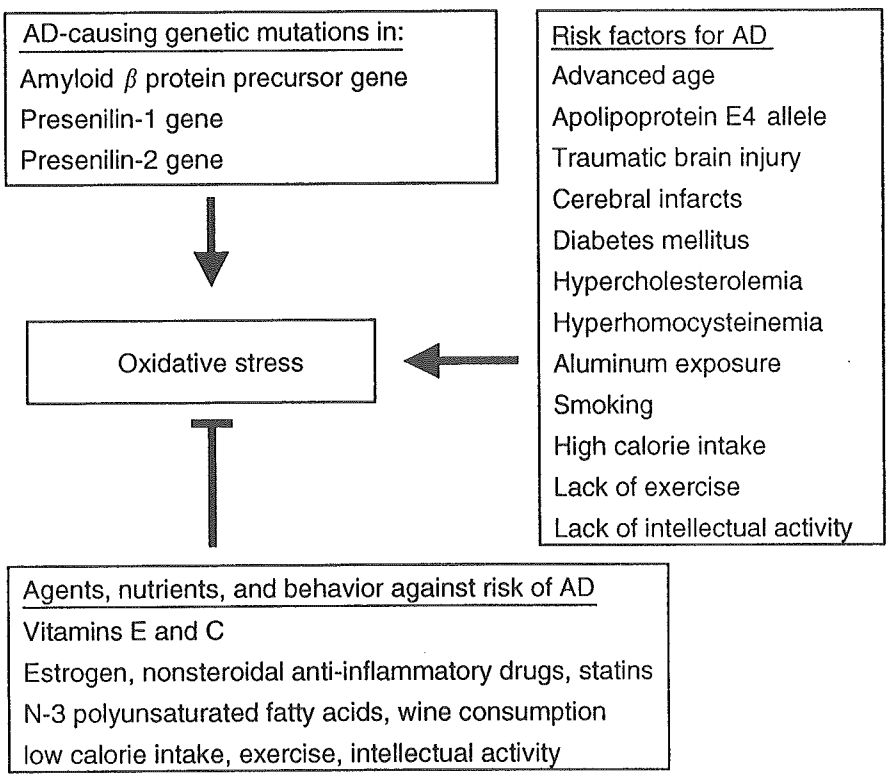

FIGURE 21.1 Genetic, medical, environmental, and lifestyle-related factors for AD: relation to oxidative stress. Most of the known genetic mutations and risk factors for AD are associated with an increase in oxidative stress. In contrast, several agents and nutrients that are known to reduce the incidence of AD have antioxidant properties *per se* or help to prevent/reduce free radical generation/propagation. A low-calorie diet as well as physical and intellectual activities are suggested to enhance the production of antioxidant enzymes in the brain.

TABLE 21.1
Evidence for Temporal Primacy of Oxidative Stress in the Pathological Cascade of AD

Materials/Subjects	Findings	References
Cell culture models	Oxidative stress induces intracellular accumulation of Aβ and phosphorylation of tau	(42,43)
Transgenic animal models	1. Increased lipid peroxidation or protein oxidation precedes Aβ plaque deposition or Aβ fibril formation in transgenic mouse or *C.elegans* model of AD amyloidosis	(12,46)
	2. Vitamin E supplementation reduces Aβ levels and Aβ plaque deposition in young but not aged AβPP transgenic mice	(44)
	3. Vitamin E supplementation suppresses the development of tau pathology in transgenic mice overexpressing human tau	(45)
	4. Dietary copper stabilizes brain copper/zinc SOD activity and reduces Aβ production in AβPP transgenic mice	(47)
	5. AβPP mutant mice crossed with manganese SOD heterozygous knockout mice show increased Aβ plaque deposition in brain	(48)
Postmortem brains from patients with Down syndrome	Oxidative damages to nucleic acid and protein precede Aβ plaque deposition in a series of Down syndrome brains, a model of AD neuropathology.	(37)
Postmortem brains from patients with AD	1. Oxidative damages to nucleic acid and protein are more prominent in AD patients with smaller amounts of Aβ plaque deposition or shorter disease duration	(36)
	2. Oxidative damage to nucleic acid is more prominent in hippocampal neurons free of NFTs compared with neurons with NFTs	(36)
	3. Oxidative damage to nucleic acid is increased in a presymptomatic case with PS1 gene mutation	(17)
CSF from patients with AD	Oxidative damage to nucleic acid is more prominent in AD patients with shorter disease duration or higher scores in mini-mental state examination	(38)
CSF, plasma, urine, and peripheral leukocytes from subjects with MCI	1. Lipid peroxidation in CSF, plasma, and urine is increased.	(39)
	2. Plasma antioxidants (vitamins A, C, E, carotenoids, SOD, etc.) are depleted	(41)
	3. Oxidative damage to DNA in peripheral leukocytes is increased	(40)

Note: CSF, Cerebrospinal fluid; MCI, mild cognitive impairment; NFTs, neurofibrillary tangles; SOD, superoxide dismutase

Known genetic mutations and risk factors for AD that cause or promote oxidative damage as well as agents, nutrients, and behavior that prevent or attenuate oxidative damage are summarized in Figure 21.1. This evidence strongly suggests that oxidative stress is universally involved in the pathogenesis of full-scale AD, especially in the upstream of the pathological cascade.

21.4 TEMPORAL PRIMACY OF OXIDATIVE STRESS IN THE PATHOLOGICAL CASCADE OF ALZHEIMER DISEASE

The early involvement of oxidative stress in the pathogenesis of AD is demonstrated more directly by recent studies on cell culture models, transgenic animal models, postmortem brains from patients with AD and Down syndrome, and biological fluids from patients with AD and subjects with MCI (Table 21.1).

We selected an *in situ* approach to identify markers of nucleic acid oxidation and protein oxidation in postmortem brain samples. Surprisingly, the oxidative damage is not only more prominent in AD cases with smaller amounts of Aβ deposition or shorter disease duration,[36] but also precedes Aβ deposition in a series of Down syndrome brains, a model of AD neuropathology.[37] Our observation corresponds with the results of increased nucleic acid oxidation in cerebrospinal fluid from AD cases, in which the shorter the disease duration, the greater the oxidative damage.[38] Moreover, individuals with MCI who, at least in part, represent the prodromal stage of AD show significantly increased levels of lipid peroxidation and nucleic acid oxidation in peripheral samples[39,40] as well as decreased levels of plasma antioxidants.[41]

These data obtained from human subjects clearly indicate an early involvement of oxidative stress in AD pathogenesis, which is supported by the experimental studies using cell culture models and transgenic animal models of AD. Indeed, oxidative stress induces intracellular Aβ accumulation and tau phosphorylation in cell cultures,[42,43] and vitamin E reduces Aβ and tau lesions in transgenic animals.[44,45]

21.5 CONCLUSION

As we have reviewed here, a growing body of evidence supports the hypothesis that oxidative stress plays a primary role of oxidative stress in the pathogenesis of AD. This notion increases the importance of further development and testing of antioxidants as a strategy for the prevention and treatment of AD (reviewed by Moreira et al. in this book). Moreover, the induction of intracellular Aβ accumulation and tau phosphorylation with oxidative stress has led us to hypothesize a compensatory role for the Aβ and tau lesions in AD against oxidative stress (reviewed by Castellani et al. in this book).

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－現状と可能性、課題を考える－

酸化ストレス抑制を介したアルツハイマー病治療アプローチの現状と可能性

布村　明彦