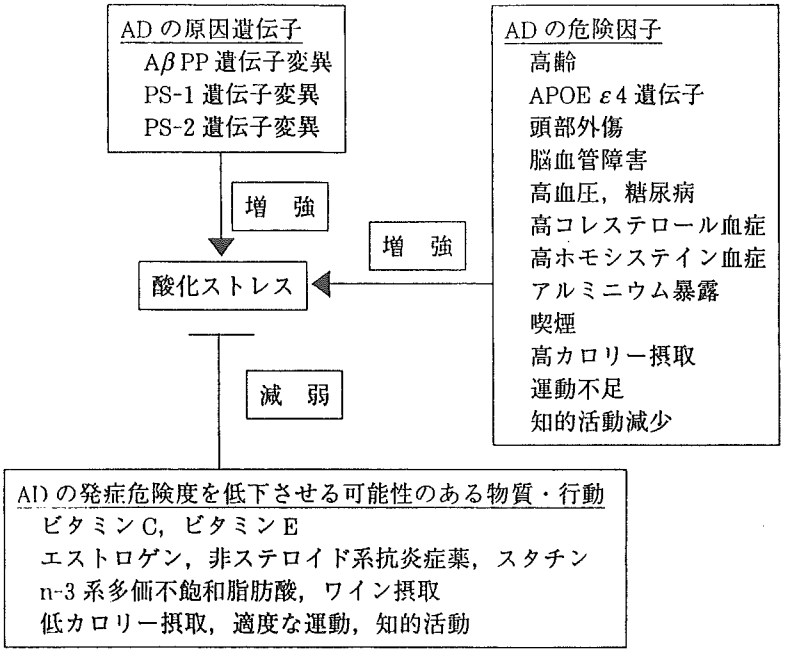

ADの原因遺伝子を導入した培養細胞や実験動物ではOSの増加やOSに対する脆弱性が観察されている．また，ADの種々の危険因子，すなわち，遺伝的，医学的，環境的，および生活習慣関連要因の多くはOS増加と関連している．逆に，ADの発症危険度を低下させることが報告されている種々の栄養素や薬物はOS抑制と関連している．さらに，AD発症危険度の低下と関連する低カロリー摂取，適度な運動，および知的活動は脳内の抗酸化酵素の産生を増加させる可能性が示唆されている．

図1　ADの原因遺伝子および危険因子と酸化ストレス（OS）

し，しかもその作用にはアイソフォーム依存性が認められ（E2＞E3＞E4），ADの危険因子であるAPOE4で最も抗酸化作用が弱いことが報告されている[23]．さらに，AD剖検脳において，脂質過酸化物の蓄積がAPOE ε4遺伝子と関連して増加することが明らかにされている[44]．その他のAD危険因子として，頭部外傷，脳血管障害，高血圧，糖尿病，高コレステロール血症，および高ホモシステイン血症などの医学的要因，アルミニウム暴露，喫煙，高カロリー摂取，運動不足，知的活動減少などの環境・生活習慣関連要因があげられる．興味深いことに，これらはいずれもOSの増加と関連している[33~35]．

実際に，ビタミンCおよびE，エストロゲン，非ステロイド系抗炎症薬，スタチン，n-3系多価不飽和脂肪酸，およびワインはADの発症危険度を低下させることが報告されているが，それぞれが抗酸化作用を有することも報告されていることは注目に値する[33~35]．また，実験動物において，カロリー制限，適度な運動，および知的活動がOS抑制を介して神経細胞の生存に寄与する可能性も示唆されている[20]．

以上のことから，OSは特定の遺伝的背景にかかわらず普遍的にADに関連しており，しかもその関連性はADの病理学的カスケードの上流に遡ることが推定される．

Ⅱ．OSはADの早期段階の変化である（表1）

筆者らは，AD剖検脳において，核酸，とくにRNAの酸化傷害の指標である8-ヒドロキシグアノシンが神経細胞内で増加しており，この指標とミトコンドリアDNAの欠失変異やニトロチロシン（タンパク質の酸化傷害の指標）の増加が同一

表1　酸化ストレスがADの病理学的カスケードの上流に位置することを示唆する報告

材料/対象	所　見
培養細胞モデル	・酸化ストレスは神経細胞内Aβ蓄積およびタウのリン酸化を誘導する[6, 22]
トランスジェニック動物モデル	・AβPP変異マウスモデルにおいて，過酸化脂質の増加が，Aβ沈着に先行する[38] ・AβPP変異マウスにマンガン含有SODノックアウトマウスを交配させることによってAβ沈着が増加する[14] ・AβPP変異マウスにビタミンEを投与することによって，Aβレベルおよび Aβ沈着が減少する[43] ・ヒトタウ過剰発現マウスにビタミンEを投与することによって，タウ不溶化およびタウ陽性封入体形成が抑制される[26] ・AβPP変異マウスに銅を投与することによって，脳内の銅・亜鉛含有SOD活性が安定化してAβ産生が減少する[2]
剖検脳（ダウン症候群）	・AD脳の病理学的モデルであるダウン症候群脳において，核酸やタンパク質の酸化傷害がAβ沈着に先行する[28]
剖検脳（AD）	・核酸やタンパク質の酸化傷害は，Aβ沈着が軽度の症例あるいは罹病期間が短い症例でより高度である[29] ・核酸の酸化傷害は，神経原線維変化を伴う神経細胞よりも神経原線維変化を伴わない神経細胞でより高度である[29] ・PS-1変異を有しながらAD発症前に死亡した症例で核酸の酸化傷害が認められる[30]
剖検脳（MCI）	・タンパク質および脂質の酸化傷害が増加している[11]
脳脊髄液（AD）	・核酸の酸化傷害は，罹病期間が短い症例あるいはMini-Mental State Examinationの得点が高い症例でより高度である[1]
脳脊髄液，血清，尿，および末梢血白血球（MCI）	・脳脊髄液，血清，および尿中の過酸化脂質が増加している[39] ・血清中の抗酸化物質（ビタミンA，C，E，カロテン，SODなど）が減少している[40] ・末梢血白血球において核酸の酸化傷害が増加している[21]

SOD；スーパーオキシド・ジスムターゼ
肩付き番号は文末の文献番号を示す．

細胞内に認められることを観察した[9, 27]．さらに，AD脳における核酸やタンパク質の酸化傷害は，アミロイドβ（amyloid β；Aβ）沈着が軽度の症例あるいは罹病期間が短い症例でより高度であることを観察した[29]．また，加齢に伴ってAD脳と同一の病理学的変化を呈するダウン症候群脳では，核酸やタンパク質の酸化傷害はAβ沈着開始に先行していた[28]．以上のことから，AD脳では神経細胞変性過程の早期段階からOSが生じていることが推定される．この推定は，ADやその他の認知症の前段階と位置づけられている軽度認知障害（Mild Cognitive Impairment；MCI）例の剖検脳，髄液，血液，および尿を用いたOSの検討[11, 21, 39, 40]やAβ沈着を呈するトランスジェニック動物におけるOSの検討[38]からも支持される．また，培養細胞モデルでは，OSが神経細胞内Aβ蓄積やタウのリン酸化を誘導することも明らかにされている[6, 22]．

興味深いことに，ADのトランスジェニック動物モデルにおいて脳内Aβレベルあるいは Aβ沈着の減少効果が明らかにされている物質（ビタミンE，非ステロイド系抗炎症薬，金属キレート剤，n-3系多価不飽和脂肪酸，メラトニン，クルクミン）や行動的介入（カロリー制限，豊かな環境下での運動）の多くはOS抑制と関連している[3, 13, 15, 19, 47, 48]．このことは，OS抑制を介したADの予防・治療の可能性を強く示唆している．

Ⅲ．OS抑制を介したADの予防・治療
――ビタミンE臨床試験を中心に――

以上のように，OSはADの臨床的亜型を問わず，早期段階から病態に関与していることが推定され，OS抑制はADの予防や早期治療の戦略として注目される．生体のOS防御にかかわる物質は，スーパーオキシド・ジスムターゼ（superoxide dismutase；SOD），グルタチオン・ペルオキシダーゼ，金属キレート性タンパク質などのフリーラジカル発生防止型抗酸化物質，ビタミンC，ビタミンE，カロテノイド，フラボノイドなどのフリーラジカル捕捉型抗酸化物質，およびリパーゼ，プロテアーゼ，DNA修復酵素などの酸化傷害修復酵素に分けられる[35]．実際，金属キレート剤であるクリオキノールやビタミンCおよびビタミンEなどがADの発症危険度低下あるいは進行抑制に寄与することが報告されている[4,25,41,42,49]．種々の抗酸化物質のなかで，ADの予防・治療効果について最も精力的に検討されてきた抗酸化物質はビタミンEである[32]．ビタミンEは血液脳関門を通過し，脳内で脂質過酸化を抑制することが明らかにされている[45]．

認知症のない高齢者の認知機能と血清中の抗酸化栄養素濃度との関連性の検討では，4,809人の高齢者を対象にした研究で，ビタミンA，Cおよびβ-カロテンの濃度は認知機能検査の成績に関連せず，血清ビタミンE濃度のみが関連していたという[36]．高齢者の認知機能と抗酸化栄養素摂取との関連について，Chicago Health and Aging Project[21]では，平均74歳の高齢者2,889人を対象に平均3.2年間追跡し，食物およびサプリメントからのビタミンE，ビタミンC，およびカロテン摂取の影響を食品摂取頻度調査票から検討した．その結果，ビタミンEのみが，Mini-Mental State Examination（MMSE）などで評価される認知機能の加齢による衰退を遅延させることが報告された．また，Honolulu-Asia Aging Study[18]やNurses' Health Study[7]では，ビタミンEあるいはビタミンC，あるいはその両方のサプリメント摂取が高齢者における認知機能検査の好成績と関連することが報告されている．以上のように，高齢者の認知機能の衰退防止にビタミンEが役立つことが示唆されている．

血清中ビタミンA，ビタミンE，およびマロンジアルデヒド（脂質過酸化の指標）の濃度とAD発症危険度との関係を検討した前向きコホート研究（626人の高齢者を追跡して46人に認知症発症）では，ビタミンEの低濃度のみが認知症全体あるいはADの発症危険度の増加（オッズ比は順に3.12と3.06）と関連していたという[8]．したがって，抗酸化栄養素のうちでもとくにビタミンEのAD発症予防効果が期待される．近年，認知症のない高齢者を対象にした前向きコホート研究により抗酸化栄養素であるビタミンE，ビタミンC，β-カロテン，あるいはフラボノイドの摂取がADの発症を予防するかどうかを検討した結果が相次いで報告された（表2）．このうち，最も規模が大きく長期にわたり，かつ対象高齢者の平均年齢が最低であるEngelhartら[4]のRotterdam studyでは，食物から摂取されたビタミンEあるいはビタミンCにAD発症予防効果が認められた．食物由来のビタミンE摂取がAD発症危険度を低下させることはMorrisら[25]の研究からも示唆されたが，その効果が喫煙習慣やAPOE遺伝子型に左右されることには注意を要する．Luchsingerら[17]の報告は食物由来のビタミンEのAD発症予防効果を否定しているが，彼らの報告では，対象中にビタミンE摂取量が十分な例が少なかった可能性が指摘されている．MorrisらとLuchsingerらの研究に共通する結果として，サプリメント由来の場合，いずれの抗酸化栄養素にもAD発症予防効果が認められなかったことは注目すべきである．食物由来の抗酸化栄養素にAD発症予防効果が認められてサプリメント由来の場合にはそれが検出されない理由として，サプリメント使用者が元来健康上の問題をより多く有しているというバイアスの存在，サプリメント使用は限られた期間であるが食事習

表2　抗酸化栄養素摂取とAD発症予防効果（前向きコホート研究）

報告者（報告年）	研究計画名称	追跡対象者数（年齢）追跡期間	ビタミンE	ビタミンC	β-カロテンあるいはカロテノイド	フラボノイド
Engelhartら（2002）[4]	Rotterdam Study	5,395人（55歳以上 平均67.7歳）平均6.0年	**食物からの摂取で効果あり** *	**食物からの摂取で効果あり** *	**食物からの摂取で喫煙者にのみ効果あり**	**食物からの摂取で喫煙者にのみ効果あり**
Morrisら（2002）[25]	Chicago Health and Aging Project	815人（65歳以上 平均73.3歳）平均3.9年	**食物からの摂取でAPOE ε4非保有者にのみ効果あり** サプリメントの効果なし	食物，サプリメントともに効果なし	食物，サプリメントともに効果なし	N/E
Luchsingerら（2003）[17]	Washington Heights-Inwood Columbia Aging Project	980人（65歳以上 平均75.3歳）平均4.0年	食物，サプリメントともに効果なし	食物，サプリメントともに効果なし	食物，サプリメントともに効果なし	N/E
Zandiら（2004）[49]	Cache Country Study	4,740人（65歳以上 平均74.0歳）平均3.0年	サプリメント **ビタミンEとCの併用で効果あり** ビタミンE，Cそれぞれ単独では効果なし		N/E	N/E

N/E；not examined
* 喫煙者でより顕著な効果あり．サプリメント使用者を除いても影響なく，APOE遺伝子型の影響もない．
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慣は調査期間を超えて長期にわたるため長期効果が反映される可能性，食物に含まれるその他の栄養素がビタミンの吸収や効果発現を助長する可能性，などが考えられる．Zandiら[49]は，サプリメント由来であってもビタミンEとビタミンCを併用することによってAD発症予防効果が得られると報告しているが，Masakiら[18]は，両ビタミンの併用は血管性痴呆の予防には有効であるがADの予防には有意な効果が認められなかったと報告している．

最近，ビタミンE投与によりMCIからADへの移行を予防できるかどうかに関して，アメリカの多施設共同研究の結果が報告された[37]．高用量のビタミンE投与（2,000 IU/日）の効果を3年間追跡した二重盲検比較で，ビタミンEにMCIからADへの移行を予防する効果は認められなかった．これは，サプリメント由来の単独のビタミンにはAD発症予防効果は認められないという従来のコホート研究結果[17,25,49]と合致している．

一方，1997年にアメリカの多施設共同研究によって2,000 IU/日のビタミンEが中等度の重症度のAD患者に対して進行抑制作用を示すという臨床試験結果が報告された[42]．この研究では，Clinical Dementia Rating（CDR）で「2」段階の重症度と判定されたAD患者を対象に，患者があるend pointに至るまでの日数を二重盲検比較した．ここでいうend pointとは，死亡，入院あるいは施設入所，基本的日常生活能力の喪失，あるいはCDRで「3」段階への進行のいずれかを指している．2年間の調査の結果，ビタミンE投与（中央値：670日）は，対照（440日）よりも有意にend pointに至るまでの期間を延長させ，ADの進行抑制作用を有すると報告された．また，この研究では，選択的モノアミンオキシダーゼB阻害薬であるセレギリン投与やセレギリンとビタミンEの併用投与でもビタミンE単独投与と同様のAD進行予防効果が得られている．副作用としては，各治療群で転倒と失神の出現率および歯科受

療率が若干増加したのみであった．しかし，治療群においてMMSEやAlzheimer's Disease Assessment Scale-cognitive subscale（ADAS-cog.）上の改善は認められず，最終結果は抗酸化物質一般に認められる非特異的な健康増進作用（たとえば，心血管系機能や免疫機能の増強作用）を介している可能性があることも指摘されている．近年作成された認知症疾患の治療ガイドライン，すなわち，アメリカ精神医学会のPractice Guideline for the Treatment of Patients with Alzheimer's Disease and other Dementias of Late Life（1997），アメリカ神経学会のPractice Parameter : Management of Dementia（2001），およびわが国で日本神経学会を中心に作成された「痴呆疾患の治療ガイドライン」（2003）では，いずれも以上の臨床試験を根拠の中心として，軽度あるいは中等度のADに対するビタミンEの投与を推奨している．毒性が低く，薬物間相互作用の少ないビタミンEがセレギリンよりも好ましいとされる．ところで，上記の臨床試験で検討されたビタミンEの用量は2,000 IU/日で，わが国の臨床で使用される酢酸トコフェロールあるいはニコチン酸トコフェロールの常用量（ビタミンE換算でそれぞれ100～300 IU/日，255～510 IU/日）と比べるとかなり高用量である．高用量のビタミンEを用いた場合にビタミンK欠乏患者で血液凝固障害を悪化させることに注意を要する．また，近年，高用量（400 IU/日以上）のビタミンE投与の安全性に疑問を投げかける報告があることにも注意しなければならない[36]．同様に高用量のビタミンCやβ-カロテンについても有害作用の報告がある[35]．

Ⅳ．OS抑制を介したADの予防・治療の将来

以上のように，抗酸化ビタミンの摂取1つをとってみても，ADの予防・治療上，安全性と有効性を満足する摂取方法の確立は容易ではない．単に高用量のサプリメントを長期間継続すればよいというものではなさそうである．とくにADの予防上，食物からの抗酸化ビタミン摂取がサプリメントからの摂取よりも有効であるという指摘は示唆に富むものであろう．複数の抗酸化ビタミンを他の栄養素とバランスよく摂取することにより，それらの吸収や活性に有利に働く可能性がある．*In vitro*の実験系では個々に顕著な抗酸化作用を示す物質であっても，生体内においてその効力がどの程度発揮されるかは予測困難である．また，*in vitro*でもある条件下では，ビタミンC，ビタミンE，カロテノイド，およびフラボノイドは，それらの抗酸化活性を失うのみならず，プロオキシダントとしてOS促進的に働くことも起こりうる[35]．生体における複雑かつ精妙なレドックス制御機構を考慮すれば，抗酸化栄養素が豊富に含まれる食物の適量を摂取することに加えて，本来生体に備わっている抗酸化システムを活性化する方法を探ることが重要であろう．すなわち，外来性および内在性，両方のOS防御機構を同時に高める試みが必要と考えられる．

実験動物においてカロリー制限が種々の加齢性変化の進行を遅延させることはよく知られており，そのメカニズムとしてOS抑制が推定されている[32～35]．近年，APOE ε4遺伝子を有する高齢者では，高カロリー摂取によってAD発症危険度が高まることが報告された[16]．食事以外の生活習慣関連要因では，AD症例では健常対照例に比べて20～60歳の間の身体運動や知的活動が少ないという報告があり[5]，身体運動やレジャー活動がAD発症危険度の低下と関連するという報告もある[12,46]．Mattsonら[20]は，カロリー制限，適度な身体運動および知的活動はいずれも軽度の代謝的および心理的ストレス反応を惹起し，神経細胞の応答として生じる神経栄養因子やストレス・タンパク質の産生がOS抑制にかかわるSOD，グルタチオン・ペルオキシダーゼ，カタラーゼなどの発現増加をもたらす可能性を指摘している．したがって，内在性の抗酸化システムの活性化を介したAD予防戦略として，低カロリー摂取，適度な身体運動，および知的活動の推奨など生活習慣への

介入が有望である．

結　語

ADでは，病理学的カスケードの上流においてOSが関与しており，OS抑制はADの予防・治療上，有望な戦略のひとつと考えられる．現在，複数のAD治療ガイドラインにおいてビタミンEの使用が推奨されているが，予防効果の面からは，食物からの抗酸化栄養素の摂取が重視されている．今後，ADの予防・早期治療において，外来性の抗酸化物質摂取のみならず，内在性OS防御機構の活性化を介した戦略が重要と考えられる．低カロリー摂取，適度な身体運動，および知的活動の推奨など生活習慣への介入が有効な手段であるかもしれない．

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Oxidative Stress in Alzheimer Disease: The Earliest Cytological and Biochemical Feature

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Alzheimer Disease and Its Pathological Hallmarks

Alzheimer disease (AD) is defined pathologically by amyloid-β (Aβ) senile plaques and neurofibrillary tangles (NFTs) composed of tau. From the time of their original description, nearly a century ago, a major focus has been to understand the role that these lesions play in the pathogenesis of AD. Although senile plaques and NFTs are pathological hallmarks of AD, it is still questionable whether these pathological alterations cause associated neurodegeneration or behavioral and cognitive deficits that accompany the disease. Senile plaques and NFTs are present in a considerable percentage of brains of cognitively normal elderly subjects. Surprisingly, a study investigating autopsied subjects aged between 69 and 100 who were cognitively normal revealed that 49% of those normal subjects met the Khachaturian criteria for AD based on senile plaque density, 25% met the CERAD criteria based on senile plaque density, and 24% were in stages IV–VI of the Braak and Braak staging of AD based on NFT density [1]. Furthermore, it is well known that there is no correlation or a poor correlation between neuronal loss and senile plaque density as well as between disease severity and senile plaque density in AD [2]. By contrast, neuronal loss and clinical severity correlate with NFT density; however, the amount of neuronal loss largely exceeds the amount of

NFTs [3]. Indeed, neurons with NFTs are estimated to be able to survive for decades [4], which suggests that NFTs themselves are not obligatory for neuronal death in AD.

Therefore, senile plaques and NFTs may not be indispensable for death of vulnerable neurons in AD. We cannot exclude the possibility that the processes of senile plaques and NFTs formation are involved in compensatory changes of the aging brain against the pathogenesis of AD [5].

Aging, Oxidative Stress, and Alzheimer Disease

AD is a disease with a prevalence that increases exponentially throughout aging, with about half of the population afflicted by the age of 95 [6], which strongly supports an association between advancing age and AD. As in other organ systems, cells in the brain encounter a cumulative burden of oxidative and metabolic stress that may be a universal feature of the aging process as well as a major causal factor of senescence. 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 [7, 8].

One gene that appears to have an influence on aging in general as well as on the risk of AD is apolipoprotein E (APOE). Individuals with an APOE4 allele have a reduced life span [9] and are at risk of AD [10]. Interestingly, APOE shows allele-specific antioxidant activity, with APOE2 being the most effective and APOE4 being the least effective, which suggests a link between oxidative stress and AD [11]. Actually, in autopsied brains with AD, there are increases in lipid peroxidation, protein oxidation, and DNA oxidation. The representatives of these oxidized products demonstrated in AD are 4-hydroxynonenal, protein carbonyls, and 8-hydroxydeoxyguanosine, respectively [12]. In 1999, we reported increased RNA oxidation in AD by in situ demonstration of an oxidized nucleoside derived from RNA, 8-hydroxyguanosine (8OHG) in vulnerable neurons of AD [13]. A significant increase in 8OHG in vulnerable neurons has also been demonstrated in Parkinson disease and Lewy body dementia, where increases in oxidized products of lipid, protein, and DNA have been shown as well [14, 15]. Certainly, oxidative stress is not a specific feature of AD but a common feature of age-associated neurodegeneration. One would expect that this oxidative damage is a common final product resulting from various pathways of neurodegeneration, but this is not the case, at least in AD.

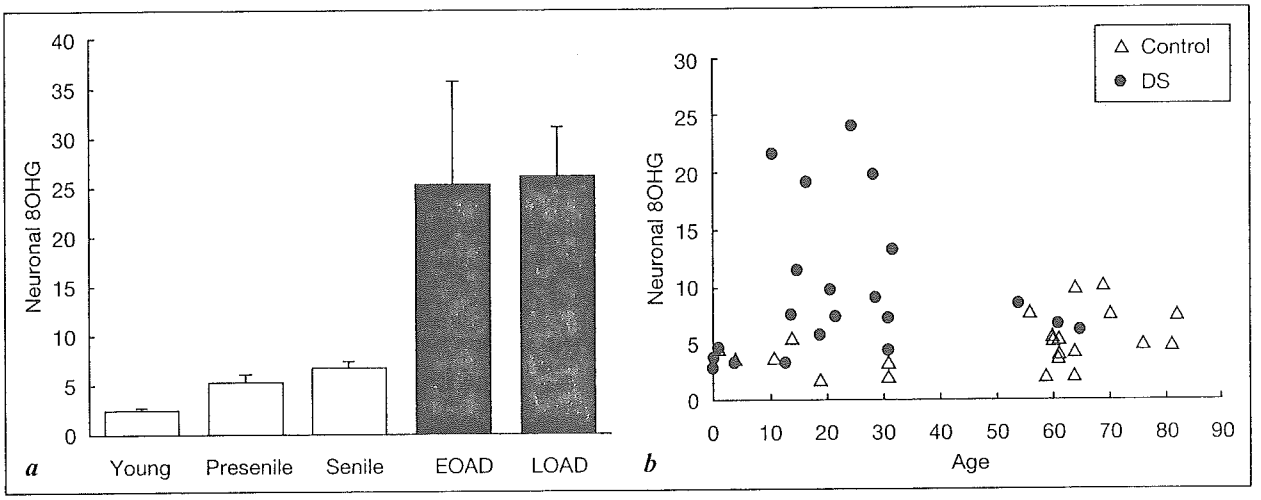

Fig. 1. Relative scale of 8OHG immunoreactivity with 1F7 antibody in AD and DS. Details of the methods for semiquantitative image analysis were described previously [13]. ***a*** The hippocampal subiculum neurons of 27 controls (4 young controls, 10 presenile controls, and 13 senile controls) and 16 cases with AD, i.e. 4 cases of early-onset AD (EOAD) and 12 cases of late-onset AD (LOAD), were examined. Values shown are the means with SE. The difference among all groups is significant by ANOVA ($p < 0.0001$) with post hoc analysis showing significant differences between each control group and EOAD as well as between each control group and LOAD. ***b*** Neurons in layer III of the occipitotemporal gyrus of 23 controls and 22 cases with DS were examined. Individuals with DS show elevation in neuronal 8OHG in their teens and twenties, while controls maintain low levels of neuronal 8OHG between the ages of 4 months and 82 years. In cases with DS, ANOVA followed by post hoc analysis reveals significantly higher 8OHG levels in age groups of the second and third decades compared with the first decade ($p < 0.01$).

Oxidized RNA Nucleoside: A Marker for Steady-State Levels of Oxidative Stress

We selected an in situ approach to identify the oxidized nucleoside 8OHG in AD brains. Immunocytochemically, neurons exhibiting marked immunoreactivity with 8OHG in the cytoplasm were widely distributed in the hippocampal region and cerebral neocortex, whereas neuronal cytoplasm was immunolabeled only faintly in controls. Relative intensity measurements of neuronal 8OHG immunoreactivity showed that there was a significant increase in nucleic acid oxidation in AD compared with controls (fig. 1a). Treatment with nuclease (DNase or RNase) before the immunostaining demonstrated that RNA was a major site of nucleic acid oxidation in AD [13], which was further supported by immunolabeling with ribosomal structures in immunoelectron microscopy for

8OHG [16]. Importantly, we found completely overlapping distributions of neurons showing increased 8OHG and other oxidative stress markers, such as mitochondrial DNA deletion detected with in situ hybridization as well as nitrotyrosine, a protein modification, detected with immunocytochemistry [16]. Because there is no evidence that 8OHG and nitrotyrosine, a non-crosslink protein adduct, are accumulated in cells, the levels of these oxidative markers are expected to reflect steady-state balance rather than a history of oxidative damage [17]. Therefore, an evaluation of the relative levels of these oxidative markers in vulnerable neurons in cases of AD with histopathological alterations of various severity enables us to investigate the relationship between oxidative stress and histological alterations.

Oxidative Stress: The Earliest Event in Alzheimer Disease

To determine whether oxidative stress is an early- or end-stage event in the process of neurodegeneration in AD, we investigated the relationship between the levels of oxidative damage and the extent of Aβ plaques and NFT formation, as well as the duration of dementia. Immunocytochemistry revealed that the levels of neuronal 8OHG and nitrotyrosine, markers of RNA and protein oxidation, were parallel in AD cases [16]. When we measured the area covered by Aβ deposition by image analysis, surprisingly, we found that increases in Aβ deposition were associated with decreased oxidative damage in neurons. Furthermore, a similar pattern of decrease in neuronal 8OHG was noted with increasing disease duration [16]. Moreover, when we investigated the effect of NFTs on the relative amount of 8OHG in neurons, we found that neurons with NFTs showed a 40–60% decrease in relative 8OHG levels compared with neurons free of NFTs [16]. These observations indicate that increased RNA oxidation and protein oxidation are early events in AD.

The early involvement of oxidative stress in the pathological cascade of AD is further supported by an investigation of a series of Down syndrome (DS) brains, in which an AD-like neuropathology is observed as an invariable feature starting in early adulthood. Double immunostaining with 8OHG and Aβ in cases of DS of various ages revealed that strong immunoreaction with 8OHG in neurons was observed in their teens and twenties, whereas the 8OHG immunoreactivity actually decreases with increasing Aβ deposition [18]. Semiquantitative analysis of neuronal 8OHG immunoreactivity and extent of Aβ burden demonstrated that, as a function of age, individuals with DS showed significant elevations in 8OHG in their teens and twenties but showed decreased 8OHG after 30 (fig. 1b), coincident with the accumulation of Aβ. These findings suggest that increased RNA oxidation occurs prior to the onset of Aβ deposition.

In concordance with our data from postmortem brain samples, cerebrospinal fluid from patients with AD showed not only significantly increased levels of 8OHG compared with controls but also greater elevation of 8OHG with shorter disease duration [19]. Furthermore, a recent study using a transgenic mouse model of AD supported the temporal primacy of oxidative stress versus Aβ deposition; in this model, increased lipid peroxidation preceded Aβ plaque formation in a transgenic mouse overexpressing a human AβPP transgene with a double Swedish mutation [20]. Biochemical analyses revealed that levels of urinary and plasma isoprostane, a product of lipid peroxidation were significantly higher in the transgenic than in the wild-type animals as early as 8 months of age. Increased levels of isoprastane were found as well from the age of 8 months in brain homogenates of the total brain cortex or hippocampus but not of the cerebellum. In contrast, pathologically, Aβ plaque formation occurred at 12 months of age in this animal model. The same research group investigated the levels of isoprostane in urine, plasma, and cerebrospinal fluid samples from subjects with AD and mild cognitive impairment (MCI) [21]. In all kinds of samples, levels of isoprostane were significantly higher in MCI than in controls, and significantly higher in AD than in MCI. The rate of progression from MCI to AD is estimated at approximately 12% per year, supporting the concept that MCI, at least in part, represents the prodromal stage of AD. Therefore, the oxidative stress is involved very early or in the preclinical stage of the pathological cascade of AD.

Oxidative Stress: An Attractive Therapeutic Target of Alzheimer Disease

With the notion that increased oxidative stress is one of the earliest changes in the pathogenesis of AD, it is not surprising that agents inhibiting free radical formation reduce both the incidence and the progression of AD. Agents such as vitamin E, selegiline, *Gingko biloba*, estrogen, and nonsteroidal anti-inflammatory drugs have been proven to have an antioxidant activity and to reduce the incidence and/or the rate of progression of AD [22]. According to two recent prospective epidemiological cohort studies [23, 24], higher dietary intake of antioxidants, especially vitamin E, was associated with a lower risk of AD. However, when we consider the complicated system of the fine regulation of cellular redox balance in human body, it is no wonder that extrinsic in vitro antioxidants may show only limited effects on the reduction of oxidative damage in biological systems. In fact, well-known dietary antioxidants such as carotenoids as well as flavonoids can act as prooxidants in certain experimental conditions [25, 26]. Therefore, we should find ways of activating

our intrinsic system in order to reduce oxidative damage, which might effectively slow down disease progression, at least in the subclinical and early stage of AD.

It is well known that experimental animals on dietary restriction that lowers steady-state levels of oxidative stress show various signs of retarded aging [27]. Recently, the association between caloric intake and the risk of AD was reported [28]. Compared with individuals in the lowest quartile of total calorie or fat intake, those in the highest quartile had an increased risk of AD. This increased risk was significant only among individuals carrying the APOE4 allele. The hazard ratios of AD for total calorie intake and fat intake are 2.27 and 2.31, respectively. A life-style-related factor other than diet, i.e. lack of exercise is considered to be a risk factor for AD. Friedland et al. [29] evaluated passive, intellectual, and physical activities by using a scale in terms of 'diversity', expressed in total number of activities, and in terms of 'intensity', expressed in hours per month. In patients with AD, the scores were significantly lower in passive diversity, intellectual diversity, and physical diversity as well as in intellectual intensity in early and middle adulthood. Mattson et al. [8] suggest that dietary restriction, intellectual activity, and exercise promote neuronal survival through decreased oxidative stress. They argue that each of them induces mild cellular stress responses, and consequently neurons respond to these stresses by activating on signaling pathways that produce growth factors and protein chaperones. These aspects may be important to prevent AD or at least delay its onset, especially in subjects at high risk of developing AD, such as APOE4 carriers.

Conclusion

The early involvement of oxidative stress in the pathogenesis of AD is supported by cytological and biochemical analyses of human brain samples of AD, DS and MCI as well as a transgenic animal model of AD. Therefore, reducing oxidative stress is an attractive therapeutic target, especially in subjects at high risk of developing AD, such as APOE4 carriers. Not only intake of antioxidants but also calorie restriction as well as maintaining intellectual and physical activities are possible strategies against oxidative stress.

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Neuronal RNA oxidation is a prominent feature of familial Alzheimer's disease

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Neuronal RNA oxidation is a prominent feature of familial Alzheimer's disease

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An in situ approach was used to identify the oxidized RNA nucleoside 8-hydroxyguanosine (8OHG) in the frontal cortex of familial Alzheimer's disease (FAD) with a mutation in presenilin-1 (PS-1) or amyloid β protein precursor (AβPP) gene ($n = 13$, age 47–81 years). Neurons with marked 8OHG immunoreaction in the cytoplasm were widely distributed in the superior/middle frontal gyrus of FAD. Relative intensity measurements of neuronal 8OHG immunoreactivity showed that there was a significant increase in FAD compared with controls ($n = 15$, age 59–81 years), while there was no difference in relative 8OHG between the PS-1 and the AβPP FAD. Interestingly, a presymptomatic case carrying a PS-1 mutation showed a considerable level of relative 8OHG, and the increased levels of neuronal 8OHG in FAD were more prominent in cases with a lower percentage area of Aβ42 burden. These results suggest that oxidative stress is an early event involved in the pathological cascade of FAD.

Keywords: Amyloid β protein precursor; Familial Alzheimer's disease; 8-Hydroxyguanosine; Oxidative stress; Presenilin; RNA

Introduction

Numerous studies have now established the association of neuronal oxidative stress with major neurodegenerative disorders such as Alzheimer's disease (AD) (reviewed in Markesbery and Carney, 1999; Perry et al., 1998; Smith et al., 2000) and Parkinson disease (reviewed in Jenner, 1998; Zhang et al., 2000). We have reported RNA oxidation in vulnerable neurons of sporadic type of AD (Nunomura et al., 1999, 2001), Parkinson disease (Zhang et al., 1999), dementia with Lewy bodies (Nunomura et al., 2002), as well as Down syndrome (Nunomura et al., 2000), which suggests links between oxidative stress and not only age-associated degenerative diseases but also neurodegeneration due to genetic factors.

In this study, we used an in situ approach to identify the oxidized RNA nucleoside 8-hydroxyguanosine (8OHG) in the cerebral cortex of familial AD (FAD) with a mutation in presenilin-1 (PS-1) or amyloid β protein precursor (AβPP) gene. Neurons with marked 8OHG immunoreaction in the cytoplasm were widely distributed in the cerebral cortex of FAD. Importantly, a presymptomatic case carrying a PS-1 mutation (Lippa et al., 1998) showed a considerable level of neuronal 8OHG. Moreover, semiquantitative analysis showed that increased levels of neuronal 8OHG in FAD were more prominent in cases with a lower amount of amyloid β (Aβ), which was immunolabeled with an end-specific antibody for the Aβ1–42 (Aβ42), as we showed in individuals with Down syndrome (Nunomura et al., 2000). These results suggest that oxidative stress is involved in the pathological cascade of FAD especially as an early stage event of the cascade.

Materials and methods

Tissue

Brain tissue was obtained at autopsy from 13 clinically and pathologically confirmed cases of FAD according to the CERAD criteria (2 males and 11 females; ages 47–81 years, average 59). Eleven females of the FAD group (ages 47–81 years, average 59) were members of families possessing a PS-1 gene mutation and the other two males of the FAD group (ages 57 and 58 years) were members of families possessing an AβPP gene mutation. Mutations in PS-1 gene in these subjects were found on M146L ($n = 1$), A246E ($n = 4$), L286V ($n = 1$), and C410Y ($n = 5$), and mutations in AβPP gene were found on KM670, 671NL (Swedish mutation; $n = 1$), and A692G (Flemish mutation; $n = 1$). All these FAD subjects died from pneumonia except for a case whose information about cause of death was not available. Another 51-year-old male

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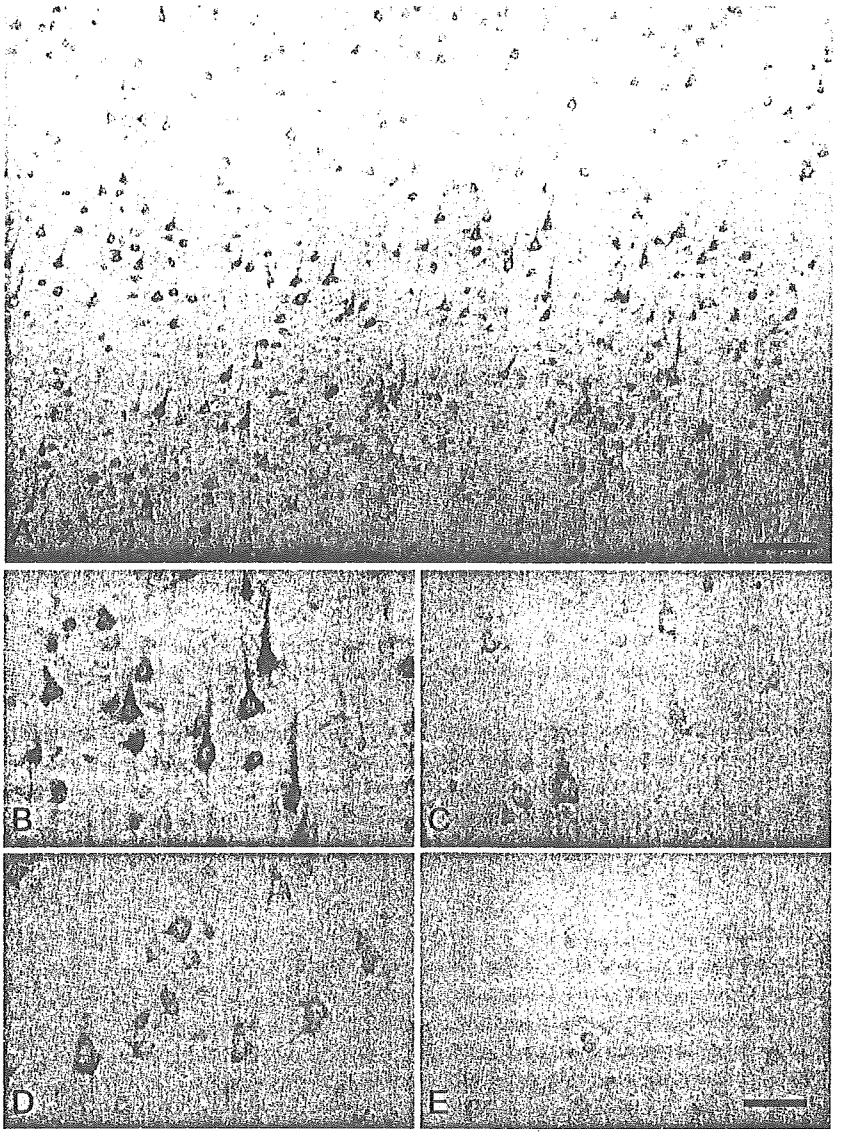

Fig. 1. Oxidized nucleoside 8OHG is abundant in vulnerable neurons in FAD. Neuronal 8OHG immunoreactivity showing cytoplasmic predominance is prominent in the frontal cortex from a case of FAD with a PS-1 gene mutation (C410Y, 55 years old) (A and B). In a case of FAD with an AβPP gene mutation (A692G, 57 years old) (C) and in a presymptomatic case with a PS-1 gene mutation (A264E, 51 years old) (D), moderately positive 8OHG immunoreactivity is observed in neurons of the frontal cortex. Whereas, in a control case (64 years old), the neuronal 8OHG immunoreactivity is faint in the frontal cortex (E). Scale bars, A = 100 μm, B–E = 50 μm.

was a member of a family with a PS-1 gene mutation (A246E) who died from myocardial infarction and had shown no clinical symptoms of dementia immediately before death (Lippa et al., 1998). As for controls, we investigated a consecutive series of 15 subjects without dementia (seven males and eight females; ages 59–81 years, average 66). Causes of death of these controls were internal malignancy (n = 9), leukemia (n = 2), cardiac failure (n = 3), and unknown (n = 1). Postmortem intervals before fixation were 2–19 h (average 8) in the FAD group, 14 h in the presymptomatic case with PS-1 gene mutation, and 3–20 h (average 9) in controls. Duration of dementia was known from clinical records in 12 cases of FAD as 5–25 years (average 11). Slices of the frontal cortex (the superior/middle frontal gyrus) or the temporal cortex (the inferior temporal/occipitotemporal gyrus) from all the subjects were fixed in neutral formalin, dehydrated through graded ethanol followed by xylene, and embedded in paraffin. Six-micron-thick sections were cut and mounted on Silane® (Sigma, St. Louis, MO)-coated glass slides.

Immunocytochemistry and antibodies

Following deparaffinization with xylene, sections were hydrated through graded ethanol. Endogenous peroxidase activity in the tissue was eliminated by a 30-min incubation with 3% H_2O_2 in methanol and nonspecific binding sites were blocked in a 30-min incubation with 10% normal goat serum in Tris-buffered saline (150 mM Tris–HCl, 150 mM NaCl, pH 7.6). To detect oxidized nucleosides, we used a mouse monoclonal antibody against 8OHG, 1F7 (Yin et al., 1995) (1:30; Trevigen, Gaithersburg, MD), after treatment with 10 μg/ml proteinase K (Boehringer Mannheim, Indianapolis, IN) in phosphate-buffered saline (pH7.4) for 40 min at 37°C. Immunostaining was developed by

the peroxidase–antiperoxidase procedure (Sternberger, 1986) by using 0.75 mg/ml 3,3′ -diaminobenzidine co-substrate in 0.015% H_2O_2, 50 mM Tris–HCl, pH 7.6 for exactly 10 min. The specificity of 1F7 to 8OHG was confirmed by primary antibody omission or by absorption with purified 8OHG (Cayman Chemical, Ann Arbor, MI) (Nunomura et al., 1999). Although 1F7 recognizes RNA-derived 8OHG as well as DNA-derived 8-hydroxydeoxyguanosine with similar binding affinities (Yin et al., 1995), we have confirmed that 1F7 immunolabeling in neurons in sporadic AD is predominantly in RNA the by pretreatment with DNase or RNase (Nunomura et al., 1999) as well as by immunoelectronmicroscopy, which showed most 8OHG is present in the endoplasmic reticulum (Nunomura et al., 2001). For FAD cases, additional sections were pretreated with RNase-free DNase I (10 U/μl for 2 h at 37°C; Roche, Mannheim, Germany) or DNase-free RNase (0.5 μg/μl for 2 h at 37°C; Boehringer Mannheim) after the proteinase-K treatment. For the detection of Aβ deposition in FAD cases, we used either of mouse monoclonal antibody, BC05 (1:1000; gift of Fukumoto, H., Takeda Chemical Industries, Osaka, Japan) specific for the carboxyl terminus of Aβ1–42 (Aβ42), or BA27 (1:3500; gift of Fukumoto, H.) specific for the carboxyl terminus of Aβ1–40 (Aβ40), with a 5-min pretreatment of 70% formic acid.

Relative scale of 8OHG and Aβ deposition

All measurements were performed in layer III of the cerebral cortex (the superior/middle frontal gyrus or the inferior temporal/occipitotemporal gyrus) using a Q500IW-EX Image Processing and Analysis System (Leica) linked to a SONY CCD Camera (XC-75CE) mounted on a Nikon MICROPHOT-FX microscope. The intensity of immunoreaction with 1F7 was evaluated by measuring the average optical density in an area comprising the cytoplasm and nucleus, as we described previously (Nunomura et al., 1999). Three adjacent fields (each field = 460 × 428 μm) were selected, and in each field of the video camera, five pyramidal neurons sectioned near their equator, based on a section plane that included the nucleolus, were selected and outlined manually so that of the area of the nucleus to cytoplasm was rather constant. The nucleus was included because damage to RNA was nuclear as well as cytoplasmic. The average optical density measurement was obtained for each of the three fields and averaged. Finally, the optical density value was corrected for background by subtracting the optical density of the white matter on the same section. The superior/middle frontal gyrus was available in all 13 FAD cases, while the inferior temporal/occipitotemporal gyrus was available in all 19 controls. Both brain regions were available in two controls and an additional centenarian without dementia, in which levels of the relative 8OHG were similar in both brain regions. In these cases, the ratio of relative 8OHG in the frontal cortex to that in the temporal cortex was 0.78, 0.84, and 1.13 (average 0.92), which meant that regional differences in relative neuronal 8OHG between the frontal and temporal cortices were virtually negligible in controls. Therefore, we used data from the superior/middle frontal gyrus of FAD cases and the inferior temporal/occipitotemporal gyrus of controls for comparison.

For the measurement of the extent of Aβ42 or Aβ40 deposition in FAD cases, three adjacent fields (each field = 624 × 580 μm) were selected to include the same area used to measure 1F7 immunoreactivity in an adjacent serial section. The area of Aβ42 or Aβ40 deposits was determined with gray scale thresholding according to the methods described previously (Hyman et al., 1993). The sum of the areas of Aβ42 or Aβ40 deposits was divided by the total area to give the percentage Aβ42 or percentage Aβ40 burden.

All measurements were done under the same optical and light conditions as well as using an electronic shading correction to compensate for any unevenness that might be present in the illumination. Statistical analysis was performed with Mann–Whitney *U* test and linear regression analysis using StatView 5.0 program (Abacus Concepts, Berkeley, CA).

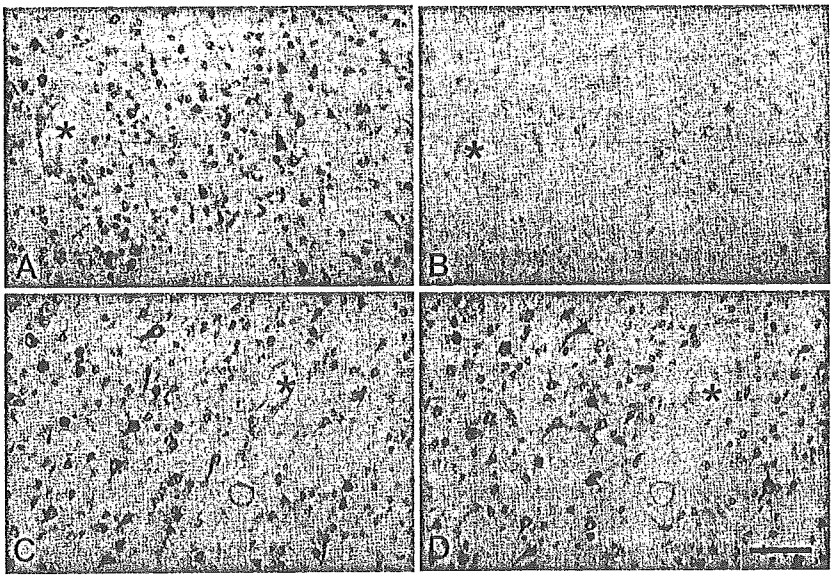

Fig. 2. RNA is a major site of nucleic acid oxidation in FAD. Immunoreaction with 1F7 antibody in FAD (A and C) is greatly diminished by treatment with DNase-free RNase (B), but only slightly by the treatment with RNase-free DNase (D). (A and B) are adjacent serial sections, so are (C and D). *indicates landmark blood vessel. The frontal cortex from a case of FAD with a PS-1 gene mutation (C410Y, 47 years old). Scale bar, 100 μm.