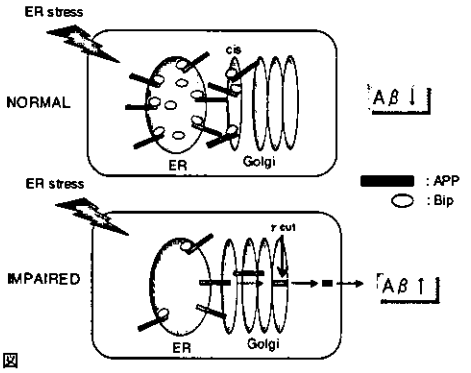
D. 考察

今回の免疫組織化学および細胞小器官分画の結果は、ER ストレスにより APP は ER やシスゴルジなどの early compartment に局在を変えることが示された。結合している ER シャペロン BiP の KDEL により unfolded 蛋白は認識され、逆行輸送されるので (Yamamoto,K., Fujii,R., Toyofuku,Y., Saito,T., Koseki,H., Hsu,V.W., and Aoe T. (2001) *EMBO J.* 20,3082-3091)、ER ストレス下で BiP は APP と結合し、APP を early compartment に逆行輸送し、止めると考えられる。一方、BN/PAGE の検討によれば、



図

γ complex 形成は ER ストレスにより障害されず、γ-secretase 活性は変化がないものと考えられる。γ-secretase 活性は細胞膜やエンドゾームなどの late compartment に存在するので (Cupers,P., Bentahir,M., Craessaerts,K., Orlans,I., Vanderstichele,H., Saftig,P., De Strooper,B., and Annaert W.(2001) *J. Cell Biol.* 154, 731-740) (Annaert,W.G., Levesque,L., Craessaerts,K., Dierinck,I., Snellings,G., Westaway,D., George-Hyslop, P.S., Cordell,B., Fraser,P., and De Strooper,B. (1999) *J.Cell Biol.* 147, 277-294)、early compartment に止まる ER ストレス下の APP はγ切断を受けず、その結果 Aβ産生が低下する示唆された (図)。

これまで家族性 AD の原因となる PS1 の変異は多数報告されてきたが、全ての変異が凝集性の高い Aβを上昇させる。また、我々は以前 PS1 の変異は ER ストレス反応を障害すると報告している (Katayama, T., Imaizumi, K., Sato, N., Miyoshi, K., Kudo, T., Hitomi, J., Morihara, T., Yoneda, T., Gomi, F., Mori, Y., Nakano, Y., Takeda, J., Tsuda, T., Itoyama, Y., Murayama, O., Takashima, A., George-Hyslop, P. S., Takeda, M., and Tohyama, M. (1999) *Nat. Cell Biolog.* 8, 479-485) (Katayama, T., Imaizumi, K., Honda, A., Yoneda, T., Kudo, T., Takeda, M., Mori, K., Rozmahel, R., Fraser, P., George-Hyslop, P. S., and Tohyama, M. (2001) *J Biol Chem.* 276, 43446-43454) (Yasuda, Y., Kudo, T., Katayama, T., Imaizumi, K., Yatera, M., Okochi, M., Yamamori, H., Matsumoto, N., Kida, T., Fukumori, A., Okumura, M., Tohyama, M., and Takeda, M. (2002) *Biochem. Biophys. Res. Commun.* 296, 313-318) (Kudo, T., Katayama, T., Imaizumi, K., Yasuda, Y., Yatera, M., Okochi, M., Tohyama, M., and Takeda, M. (2002) *Ann. N. Y. Acad. Sci.* 977, 349-355)。従って、ERストレス反応はAβ産生機構に何らかの関与を持つ可能性は大きいと考えられる。今回の結果は、通常状態では ER ストレスによって Aβ産生減少がみられるが、ER ストレス反応が障害された状態では、ER シャペロン BiP の誘導が十分でないためにストレス下でも BiP 崖都合した APP が少なく APP の early

compartment へのシフトが起こらず、Aβ産生抑制も起こらない。このため、ERストレス障害細胞では、相対的にAβの蓄積が起こることが示唆される（図）。

E. 結論

ERストレスによるシャペロン誘導はAβ産生調節機構のひとつとして働き、ERストレス反応が障害されるとこの機構が働かないために、相対的にAβ産生上昇が生じる可能性が示唆された。

F. 健康危険情報

特になし。

G. 研究発表

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H. 知的財産権の出願・登録状況

特になし。

厚生労働科学研究費補助金(こころの健康科学研究事業)
分担研究報告書

Aβ抗体を用いた免疫電顕による早期細胞内アミロイド分布の検討

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研究協力者　五十嵐善男、長澤大輔

研究要旨　アルツハイマー病の治療の可能性を探るため、細胞内にも存在するβアミロイドの存在様式を検討する目的で、老人斑アミロイドを主に構成する難溶性のAβ42の抗体のみでなく、易溶性で善玉と言われるAβ40の抗体も用いて、胞体内の染色性を検討した。神経細胞、astrocyte、血管壁の細胞胞体内に微細顆粒状にβアミロイド特にAβ40が認められた。これらβアミロイドは細胞内の微細構造であるendosome-lysosome系、或はER-Golgi系に対応していると考えられた。

A. 研究目的

近年、老人斑の構成要素であるアミロイドが細胞外の組織間隙のみでなく、細胞内にも存在する可能性が生化学的、免疫組織化学的に報告された。プレセニリン1(PS1)トランスジェニックマウス及び人アルツハイマー病の大脳を抗βアミロイド42(Abeta42)抗体で蛍光染色して共焦点顕微鏡で観察した研究(崔ら)では、大脳皮質の一部の神経細胞がAbeta42に陽性である。

βアミロイド蛋白には、細胞の膜成分に存在するアミロイド前駆体蛋白(amyloid precursor protein; APP)からβ,γ secretaseにより切り出されたアミノ酸残基40と42からなるmonomerがある。Aβ42は難溶性でpolymerとなり、老人斑のアミロイド繊維を形成する。一方Aβ40は易溶性で善玉と言われている。

我々はアルツハイマー病の細胞内に存在するβアミロイドの存在様式を検討する目的で、Aβ42抗体のみでなくAβ40抗体も用いて、胞体内の染色性を検討した。

B. 研究方法

① 4%paraformaldehydeに固定し、sucrose bufferに保存しておいた人アルツハイマー病の大脳側頭葉皮質を使用して、パラフィン包埋光顕切片を作成し、免疫染色を行った。用いた抗βアミロイド抗体は、Aβ11-28 (IBL 10027)、Aβ 40 (CALBIOCHEM 171608)、Aβ 42 (CALBIOCHEM 171609)、Aβ40 (WAKO 299-56701 kit)、Aβ42 (WAKO 299-56701 kit)、である。Aβ40の抗体とAβ42の抗体は互いに交差性は無く、Aβ11-28の抗体は双方共全て染めると言われている。光顕にて神経細胞などの細胞内の染色を検討した。

②同じ大脳側頭葉皮質を使用して、30μm厚さの切片をmicroslicerで作成し、浮遊法でAβ40(CALBIOCHEM 171608)抗体を用いて免疫染色を行った。この切片を細切トリミングしてepon包埋し、超薄切片でDAB陽性の構造の超微形態像を通常の電顕で検討した。これはpre-embedding法の免疫電顕である。

C. 研究結果

①Aβ40抗体では、従来言われていた老人斑の核の部分の他、萎縮した神経細胞の胞体(図1)、反応性に肥大したと思われるastrocyteの胞体、小さな血管の壁細胞の胞体に、微細顆粒状に陽性であった(図2, 3)。皮質下白質の一部のastrocyteの胞体にも顆粒状に陽性であった。

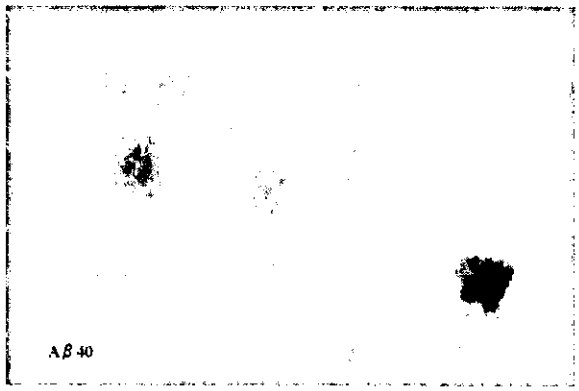

図1:Aβ40抗体を用いた免疫染色。老人斑の核の部分の他、萎縮した神経細胞の胞体に陽性である。

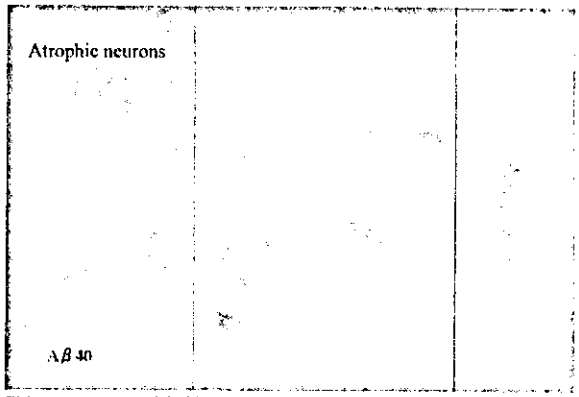

図2:Aβ40抗体を用いた免疫染色。萎縮した神経細胞の胞体内に陽性であり、中央の図では小血管壁が微細顆粒状に陽性である。

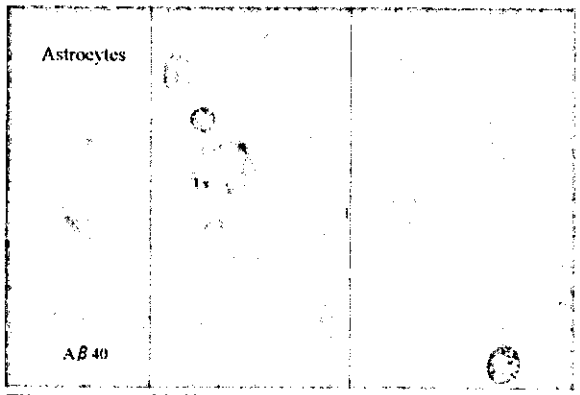

図3:Aβ40抗体を用いた免疫染色。Astrocyteの胞体に微細顆粒状に陽性であり、Aβ42抗体を用いた免疫染色の粗大顆粒状とは明らかに異なる形態を示した。

Aβ42抗体では、老人斑全部の他、microgliaや肥大したと思われるastrocyteの胞体の中に、顆粒状或は粗大顆粒状に陽性になった(図4)。Aβ40抗体を用いた免疫染色の微細顆粒状とは明らかに異なる形態を示した。

全てのβアミロイドを染めるAβ11-28抗体では、上記の2抗体の両方の所見を染色した。

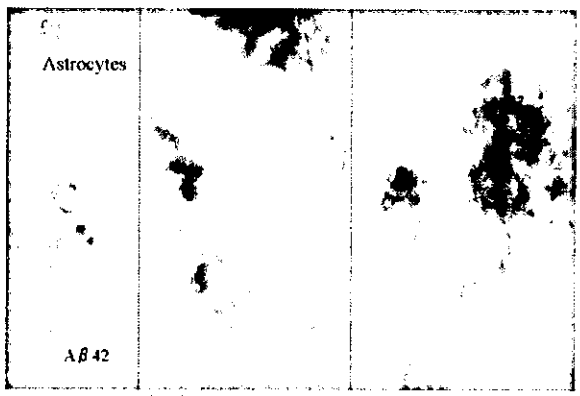

図4:Aβ42抗体を用いた免疫染色。

②Aβ40抗体を用いたpre-embedding法の免疫電顕では、astrocyteや神経細胞の胞体内の微細顆粒状に陽性顆粒は認められたが(図1,2)、これらは細胞内の微細構造に対応していた。またmitochondria内に電子密度の高い顆粒dense bodyが見られた。

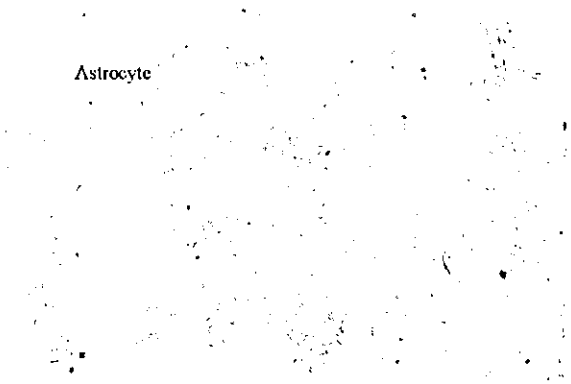

図5:Aβ40抗体を用いたpre-embedding法の免疫電顕。Astrocyteにlipofustinの他、陽性の微細顆粒が散在し、細胞内の微細構造に対応。

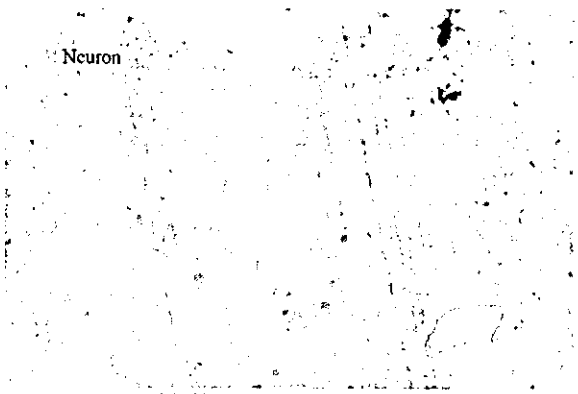

図6:Aβ40抗体を用いたpre-embedding法の免疫電顕。図5のastrocyteと同様である。Mitochondria内に電子密度の高い顆粒dense bodyが見られる。

D. 考察

神経細胞、astrocyte、血管壁の胞体内に微細顆粒状にβアミロイド特にAβ40が認められた。これらの細胞内のβアミロイドは細胞内の微細構造であるendosome-lysosome系、或はER-Golgi系に対応していると考えられる。

Microgliaや一部のastrocyte内に認められた粗大顆粒状βアミロイドは、神経細胞、astrocyte、血管壁の胞体内に認められた微細顆粒状のものとは異なり、貪食或は取り囲まれたものと考えられる。

E. 結論

細胞内Aβはアストロサイト、ニューロン、血管内皮細胞に微細顆粒状に存在し、endosome-lysosome系、或いはER-Golgi系に対応していると考えられる。

F. 健康危惧情報

なし

G. 研究発表

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分担研究報告書

シナプス機能障害を標的としたアルツハイマー病新規治療法の開発

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研究要旨　アルツハイマー病（AD）は高齢化社会の保健・福祉・生活衛生に大きな影を落とす神経変性疾患であり、その高い有病率、長い罹病期間、障害の重大さから大きな社会問題となっており、その克服はきわめて優先度が高い研究課題である。　本研究の目的は、ADの認知障害に関連するシナプス障害に焦点を当ててその病態メカニズムを明らかにし、シナプス障害を阻害する画期的な治療法開発をめざすことにある。　家族性ADの原因遺伝子であるプレセニリンとβAPPの機能はそれぞれAβを形成するγ切断を担う酵素と基質であることが示唆されている。このADの遺伝子機能の産物であるAβは脳内に蓄積するより前に可溶性の多量体を形成していると考えられているが、本研究ではAβの多量体のシナプス障害のメカニズムの解明とそれを標的とした薬剤開発戦略を提案することを目標とする。

Aβオリゴマー産生には新規産生 Aβと Aβフィブリルが関与していると考えられる。検討の結果、Aβオリゴマー産生には線維形成後のAβフィブリルの溶解過程に比較して新規産生 Aβが大きく関与している可能性が示唆された。　また新規産生 Aβのうち Aβ40に比較して Aβ42の作用が大きい。　このことはAβオリゴマー産生を阻害するためには、Aβ42産生あるいはAβ42の凝集を阻害する必要があることを示唆している。トランスレーショナル・リサーチの一環として、Aβ42の産生を特異的に減少させるようなγセクレターゼ修飾薬のスクリーニングを行った。　NSAIDのうち効果のある薬剤と効果のない薬剤の構造から推測した候補薬剤４０種類を選択した。　我々はAβ42産生を特異的に減少させるにもかかわらず Nβ、AICD、NICD を産生する切断には殆ど影響させない新規 compound W をスクリーニングの結果えた。　このコンパウンドは「Aβ42産生を特異的に下げる」理想的なγセクレターゼ修飾作用を持つ。

キーワード；　アルツハイマー病、アミロイドβ蛋白、オリゴマー形成、シナプス毒性

A. 研究目的

アルツハイマー病（AD）の治療法開発は現実的な研究課題となりつつある。世界的に見ても現時点での治療戦略はアミロイド沈着の抑制という観点に絞られてきており、γセクレターゼ阻害剤、βセクレターゼ阻害剤、アミロイドワクチン療法などが試みられている。本研究ではAβ産生・オリゴマー形成・プロトフィブリルなどの凝集体形成を阻害するアルツハイマー病治療法の根拠となるメカニズムを解析しその結果を利用して現在の薬剤開発の問題点を克服し新規薬剤探索の根拠となる方法論とアッセイ系を確立するために実施した。

B. 研究方法

1）アミロイド凝集実験

アミロイドβ蛋白（１－４０）（Bachem

社から購入）をHartley DM, 1999らの方法によりインキュベートしアミロイド・フィブリルを生成させた。 簡単に記すと、1mM NaOHで溶解後、酸性条件下のアミロイドβ蛋白にさらにアルカリ溶液を加えることにより急激に中性条件にし、バッファーを加えた後３７度で決められた時間静置した。インキュベーション終了後のサンプルはテーブルトップの遠心機でフル・スピード１０分間遠心した後上清と沈査分画に分離した。 沈査分画は適度に水で薄めた後、炭素蒸着したグリッド上で乾燥させ、酢酸ウランを添加した後電子顕微鏡で観察した。上清分画はサイズ・イクスクルージョン・カラム（SEC）Superdex 75 PC3.2/30 column (Amersham)で分離した。

２）細胞培養

HEK293細胞に野生型プレセニリンとスウェーデン変異型ベータアミロイド蛋白前駆体を恒常的に発現させ、定法により培養した。

３）分泌アミロイドベータ蛋白のウェスタンブロッティング及びマススペクトロミー解析

培養上清をそれぞれ回収後、アミロイドベータ蛋白抗体(4G8)で免疫沈降、10-20%トリス-トリシンゲルでSDS-PAGEを行った後、ニトロセルロース膜に転写した。 定法によりアミロイドベータ蛋白抗体(6E10)を用いてフィルムに感光させた。 またサンプルの半量を同様に免疫沈降した後、飽和アルファ-シアノ-4-ヒドロオキシシナミ酸を含むTWA(トリフルオロ酢酸:水:アセトニトリル=1:20:20)に溶解し、マススペクトロミーで解析した。

３）*de novo* アミロイドベータ蛋白定量・定性システム

野生型プレセニリンとスウェーデン変異型ベータアミロイド蛋白前駆体を恒常的に発現した細胞を回収し、定法により粗膜分画を抽出した。 粗膜分画を37℃, 30分間インキュベートした。 100,000ｘｇで超遠心し回収した上清と沈降物を超音波破砕の後、再度超遠心して回収した上清を4G8で免疫沈降し、2)の方法でウェスタンブロッティング及びマススペクトロミー解析を行った。

C. 研究結果

昨年度、Aβオリゴマー生成に通常の40アミノ酸残基のAβよりも長さの長いAβ42が重要な働きを持つこと、およびAβ4、３，２量体が比較的安定であることをSECおよびSDS-PAGE解析で示した。本年度は、同様の解析手段を用いてAβモノマーを試験管内で凝集させる過程とオリゴマー産生メカニズムについて検討した。

オリゴマー形成過程について

Aβモノマーを試験管内で凝集させるとAβオリゴマー形成段階に一時的にAβモノマーが減少することが明らかになった。Aβオリゴマーがさらに凝集しAβ凝集体を形成してしまうと再びAβモノマーがSEC上に認められることを見出したが、この事実はAβの凝集過程でAβオリゴマーとAβモノマーの間には一方向性の平衡関係があることを示唆している。さらにAβ凝集体生成後はAβオリゴマーの産生は殆ど認められず、

Aβ凝集体とAβオリゴマーの間の平衡関係もAβ凝集体形成に偏っていた。したがって試験管内反応では SEC で分離されるようなAβオリゴマーはAβモノマーと異なり Aβの凝集過程に一時的に存在する中間体であることが示唆された。この結果を応用するためにフラクション化したアルツハイマー病脳内のAβ凝集物を解析し、Aβオリゴマーまたは蛋白との複合体などの分離を試みたが、きれいな結果は得られなかった。

オリゴマー産生過程について

Aβの凝集がもたらす神経毒性に関連のあるAβ42産生の阻害メカニズムを明らかにするためにプレセニリン/γセクレターゼによる蛋白分解により生じた Aβ、CTFγ、Nβ、NICD の４フラグメント産生の効率と切断部位を同時に測定できる系を作成した。このアッセイ系を用いてAβ42産生低下作用のある（正確には Aβ42 産生を下げ Aβ38 産生を上げる作用）NSAID である sulindac sulfaide(SS)の cell-free アッセイを行った。興味深いことに、SS は Aβ42 産生を減少させるが CTFγの切断部位には影響を与えなかった。同様に SS は F-Nβの C 末端や NICD の N 末端にも影響を与えなかった。この事実は NSAID のγ切断修飾作用が Aβに限局する、すなわちγ40 切断にのみ影響しγ49、S3、S4 には影響しないことを示している。したがって、NSAID 様の Aβ42 産生を下げ Aβ38 産生を上げる薬剤は開発過程でその効果を強くしても Notch シグナル伝達に影響を与えないと考えられた。そこで、本研究では自ら NSAID 様の効果を持つ薬剤のスクリーニングを行うこととした。NSAID のうちγセクレターゼ修飾作用が報告されている薬剤とそれがないとされている薬剤の特徴から新規３０コンパウンドを選択した。培養上清中の Aβおよび cell-free アッセイのスクリーニングの結果その内一つのコンパウンドが「Aβ42 産生を下げ Aβ38 産生を上げる」作用を持つことが明らかになった。このコンパウンドの IC50 値は 20 μM 程度で SS の半分以下であった。この結果は NSAID の作用そのものに限らず構造類縁体がアルツハイマー病治療薬として開発できる可能性を強く示唆している。

D. 考察

沈着アミロイドの除去をアルツハイマー病治療法として確立するためには、除去されて可溶化された Aβが、新規産生された後集積凝集の過程で発揮した毒性を有さないことが条件となる。　我々は Aβ40 ペプチドを用いて「試験管内でアミロイドオリゴマーは単量体アミロイドβ蛋白の重合過程に検出されるがアミロイドフィブリル形成後の平衡状態では生じない」ことを示した。つまり、シナプス障害の原因物質であると考えられる Aβオリゴマーは新規産生 Aβの線維形成過程で中間体として産生されるが、Aβ線維の分解過程ではほとんど産生されない。

上記のように、Aβオリゴマーは新規産生 Aβから出来ると考えられる。　Aβ40 と Ab42 ペプチドを用いて Aβオリゴマー形成速度について検討したところ、Aβ42 由来のオリゴマー形成が圧倒的に早かった。　脳内から代謝される前に産生され、病理作用を発揮する Aβオリゴマーは新規 Aβ42 由来である可能性が示唆された。

そこで Aβ42 産生を下げることが結果的

に Aβオリゴマー形成を阻害することに繋がると考え、 トランスレーショナル・リサーチの一環としてγセクレターゼ修飾薬のスクリーニングを行った。 具体的には、NSAID のうち効果のある薬剤と効果のない薬剤の構造から推測した候補薬剤４０種類を選択し、セル・フリー・複数基質γ・セクレターゼアッセイ系およびセル・ベースアッセイ系を用いて詳細な性質の検討を行った。

その結果その中の一つの薬剤(compound W)が Aβ42 産生低下能をもつことが明らかになった。 この Compound W の Aβ４２産生を低下させる能力は、sulindac sulfide の約３倍である。 さらに重要なことに Compound W は Aβ４２産生を低下させる。つまりγ40 切断の正確さに影響を及ぼすが、同じβAPP の膜内切断であるγ49 切断に影響を与えない。 また Notch シグナル伝達での細胞内シグナル分子である NICD 産生に直接的にかかわる S3 切断に影響を与えないことが明らかになった。

E. 結論

トランスレーショナル・リサーチの一環として、Aβ42 の産生を特異的に減少させるようなγセクレターゼ修飾薬のスクリーニングを行った。 その結果、新規 compound W をえた。 このコンパウンドは「Aβ42 産生を特異的に下げる」理想的なγセクレターゼ修飾作用を持ち Aβオリゴマー形成を阻害すると考えられる。

F. 健康危険情報

特になし

G. 研究発表

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Intracellular Aβ42 activates p53 promoter: a pathway to neurodegeneration in Alzheimer's disease

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ABSTRACT

The amyloid β-protein (Aβ) ending at 42 plays a pivotal role in Alzheimer's disease (AD). We have reported previously that intracellular Aβ42 is associated with neuronal apoptosis in vitro and in vivo. Here, we show that intracellular Aβ42 directly activated the p53 promoter, resulting in p53-dependent apoptosis, and that intracellular Aβ40 had a similar but lesser effect. Moreover, oxidative DNA damage induced nuclear localization of Aβ42 with p53 mRNA elevation in guinea-pig primary neurons. Also, p53 expression was elevated in brain of sporadic AD and transgenic mice carrying mutant familial AD genes. Remarkably, accumulation of both Aβ42 and p53 was found in some degenerating-shape neurons in both transgenic mice and human AD cases. Thus, the intracellular Aβ42/p53 pathway may be directly relevant to neuronal loss in AD. Although neurotoxicity of extracellular Aβ is well known and synaptic/mitochondrial dysfunction by intracellular Aβ42 has recently been suggested, intracellular Aβ42 may cause p53-dependent neuronal apoptosis through activation of the p53 promoter; thus demonstrating an alternative pathogenesis in AD.

Key words: apoptosis • amyloid β-protein • neurotoxicity

Alzheimer's disease (AD) is the most common dementia in the elderly and is pathologically characterized by remarkable neuronal loss, neurofibrillary tangles, and senile plaques. Amyloid β-protein (Aβ) is the major insoluble protein in senile plaques, and Aβ ending at 42 (Aβ42) is the major depositing species (1, 2). Extracellular Aβ42 is neurotoxic through multiple pathways (3); it is likely that increased extracellular Aβ42 is a major cause of neuronal death in AD. Strong evidence of this is the fact that Aβ42 production is increased by early onset familial AD (FAD)-related mutations in presenilin (PS) 1, PS2, and amyloid β-protein precursor (APP; 1, 4, 5, 6, 7). Therefore, inhibition or clearance of extracellular Aβ42 deposition is a potential treatment for AD (8, 9). Also, because anti-Aβ antibodies slow cognitive decline in AD (10), immunotherapeutic approaches may be a useful treatment (11). However, the validity of the extracellular Aβ cascade theory is still in debate because extracellular Aβ may have neuroprotective effects under physiological conditions (12). An alternative Aβ42 pathogenesis should also be noted. Although Aβ42 produced in endoplasmic reticulum (ER) is physiologically secreted to extracellular space (1, 9, 13), recent pathological studies suggest that Aβ42 accumulates in neurons before plaque formation in AD (14, 15) and Down syndrome (DS; 16, 17). Therefore, an intrinsic Aβ pathway as well as an extrinsic Aβ pathway are widely considered important (18). We also have reported Aβ42 accumulation in neurons, but not in the extracellular space in aged mice carrying a mutant PS1 gene (19), and selective Aβ42 accumulation in neurons undergoing apoptosis in vitro (20). Thus, some perturbation in the intracellular Aβ metabolism might promote neurodegeneration. Recently, Bückig et al. (21) have shown that Aβ42 overproduced in the ER is exported to cytosol, where Aβ42 forms aggresome-like structures, and is partly transferred to the nucleus, an unusual compartment for Aβ42 to reside. Although the incidence of neuronal apoptosis is increased in AD (22, 23), intracellular Aβ42, but not extracellular Aβ, induces neuronal apoptosis in vitro (24). We have found intraneuronal Aβ42 to be linked to apoptosis in AD (25). Although intraneuronal Aβ42 is reported to be associated with dysfunction of mitochondrias (26), lysosomes (27, 28), and synapses (29), intranuclear Aβ42 might have a novel pathogenesis related to apoptosis. P53 protein, which inhibits the cell cycle and also induces apoptosis (30), may play an important role in AD pathology and be associated with cytosolic/nuclear Aβ42. Because, p53-associated neuronal death in cytosolic Aβ42-transgenic (Tg) mice (31), elevated p53 protein levels in AD (32) and DS (33) brains, p53-dependent apoptosis of primary human neurons by Aβ42 injected in cytosol (34), and extracellular Aβ42 neurotoxicity independent on p53 in vitro (35, 36) have been reported. Here, we show that intracellular Aβ42 has a novel effect on the p53 promoter and that this might contribute to neurodegeneration in AD.

MATERIALS AND METHODS

Constructs of Aβ40 and Aβ42, transfection, and RT-PCR

Oligonucleotides encoding Aβ40, Aβ42, and reverse-sequence Aβ42 (rAβ42) peptides with initiation (ATG) and stop (TAA) sequences were subcloned into the Hind III site of pTet-Splice (GibcoBRL, Gaithersburg, MD). Transient transfection was performed with lipofectAMINE-PLUS reagent (Gibco BRL). All control cells were transfected with pTet-splice vector only. SKN-SH cells were cultured in serum-free Opti-MEMI (Gibco BRL). Saos2 (p53–/–) and U2OS (p53+/+) were cultured in DMEM (Gibco BRL) containing 5% fetal bovine

serum (FBS) after transfection. For semiquantitative RT-PCR, total RNA was obtained from cells using RNeasy® Mini kit (Qiagen) and treated with RNase-free DNase I (Takara Co., Osaka, Japan) at 25°C for 30 min. cDNA was prepared from 1–5 μg total RNA using a First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Uppsala, Sweden), followed by amplification in a Perkin Elmer GeneAmp PCR system 9700. Each 50–200 ng of cDNA and 10 pmole primer DNAs mixed in the 20 μl HS-Taq reaction solution (Takara Co.) was subjected to 25–30 cycles of 1 min at 94°C, 1 min at 53, 60, or 65°C (annealing) and 1 min at 72°C. Quantitative analyses were done at each appropriate cycle that showed linear amplification (data not shown). Each PCR produced specific bands of 158 (Aβ40), 194 (Aβ42 and rAβ42), 216 (human p53), 232 (guinea-pig p53), 216 (mouse p53), 298 (human β actin), 282 (guinea-pig β actin), 302 (mouse β actin), 77 (MDM2), 97 (p21), 90 (Bax), or 88 (PIG3) nucleotides. Densitometry analysis for quantification was performed using NIH Image 1.62b7.

Immunocytochemical staining and immunoblotting of cultured cells

For immunocytochemical staining, cells on Cell Disks (Sumitomo Chemical Co., Osaka, Japan) were fixed in methanol–acetone (1:1). Fixed cells were treated with 3% H_2O_2 and blocked with non-immune goat serum, followed by incubation with BA-27 at 1:500 and BC-05 at 1:2,000 in PBS. After incubation at 4°C overnight, cells were incubated with anti-mouse IgG antibody and then with peroxidase-anti-peroxidase antibody (DAKO, Carpinteria, CA). The red color was developed with aminoethylcarbazole (AEC). For double immunofluorescent staining, after incubation with the primary antibodies of BC-05 (monoclonal) and anti-p53 antibody (polyclonal, FL393, Santa Cruz Biotech, Santa Cruz, CA) at 1:200, FITC-conjugated anti-mouse IgG (Cappel, Aurora, OH) and Texas Red-conjugated anti-rabbit IgG (Cappel) antibodies were used at 1:100 as the secondary antibodies. Fluorescence was observed with a confocal laser microscope (FLUOVIEW FV300, Olympus Optical Co., Tokyo, Japan). For immunoblotting analyses, the cells were lysed in 2% SDS, and 20 μg of protein was electrophoresed in a 15% SDS-polyacrylamide gel, followed by transfer onto a PVDF membrane (Millipore, Bedford, MA). To markedly enhance the detection of Aβ, the transblotted membrane was incubated in 2.5% glutaraldehyde for 30 min at room temperature and washed with PBS containing 50 mM monoethanolamine for 5 min. The membrane was incubated in 5% skim milk in TBST (25 mM Tris [pH7.6], 150 mM NaCl, 0.1% Tween-20) for 30 min. The membrane was then incubated overnight at 4°C with each primary antibody, i.e., anti-Aβ42 (BC-05), anti-Aβ40 (BA-27), anti-Aβ17-24 (4G8, Signet Pathology Systems, Dedham, MA), anti-β-actin (AC-15, Sigma, St. Louis, MO), anti-p53 (DO-1, Santa Cruz Biotech), anti-MDM2 (Santa Cruz Biotech), anti-p21 (C-19, Santa Cruz Biotech), anti-Bax (Santa Cruz Biotech), anti-PIG3 (Santa Cruz Biotech), anti-human PS1 (N-terminal, Chemicon, Temecula, CA), anti-APP (C-terminal, Sigma), or anti-NSE (H14, DAKO) antibodies in TBST at each recommended concentration. The peroxidase-conjugated secondary antibodies (Pierce, Rockford, IL) were used at 1:20,000, and the membranes were developed using Super Signal West Dura Extended Duration Substrate (Pierce). To obtain Aβ-enriched nuclear extract proteins (NEP), NEP were prepared by a conventional method (37), followed by the removal of high molecular weight proteins and ~10-fold concentration of the proteins performed using Ultrafree tubes with cut-off molecular weights of 10 and 3 kD (Millipore).

Cell viability assay and TUNEL staining

Cell viability was measured with a Cell Counting Kit-F (Wako, Osaka, Japan). Calcein-AM, which specifically produces fluorescence in living cells, was added to the cell cultures, and the fluorescence intensity measured with a multi-well plate reader (CytoFluor II, PerSeptive Biosystems GMI, Clearwater, MN). To test for the specific inhibition of p53-dependent apoptosis, sense and antisense DNAs for p53 mRNA were added to the medium at a concentration of 15 μM (38). Statistical analysis was performed using Kruskal-Wallis and Scheffé tests. For terminal dUTP nick-end labeling (TUNEL) staining, a Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) was used and developed with AEC.

Gel mobility shift assay

Synthetic Aβ1-40 and Aβ1-42 were both obtained from BACHEM (Philadelphia, PA), and a BandShift Kit (Amersham Pharmacia Biotech) was used. Both sense and antisense 48-mer oligonucleotides of the p53 promoter region were synthesized (pp53, see Fig. 3*B*). The sense pp53 end-labeled with ^{32}P was annealed with antisense oligonucleotides to form double-stranded pp53 (ds-pp53). About 10,000 cpm of labeled ds-pp53 (~0.1–5 ng DNA) was incubated with synthetic Aβ40 or Aβ42 in 20 μl of 10 mM Tris (pH7.5), 50 mM NaCl, 0.5 mM DTT, 10% glycerol, 0.05% NP-40, and 1 μg poly(dI-dC) for 20 min at room temperature. The incubated samples were then electrophoresed in a 5% polyacrylamide gel, followed by autoradiography. To study the differential binding affinity of Aβ42 in pp53, each region-specific 40-mer sense and antisense oligonucleotides was synthesized.

Magnetic bead collection of Aβ42 and chromatin-immunoprecipitation (ChIP) assay of p53 promoter DNA

To collect Aβ42 using the oligonucleotide and magnetic beads, biotinylated oligonucleotides of HSE-B, HSE-MT, nonHSE-3′, and their antisense oligonucleotides were synthesized. The double-stranded oligonucleotides were coupled with streptavidin-conjugated magnetic beads (Dynabeads® M-280, DYNAL, Oslo, Norway). Approximate 50 μg of coupled beads was mixed with synthetic Aβ40 or Aβ42 peptide under the same conditions as for the gel mobility shift assay. To collect intranuclear Aβ42 from Aβ42-transfected cells, 100 μg of NEP (34) was dialyzed to remove the salts and then was incubated with ~40 μg beads in a total volume of 100 μl. A ChIP assay was performed according to previous reports (39, 40) with minor modifications. For each immunoprecipitation, 1 μg of anti-Aβ antibody was used. PCR for the p53 promoter DNA containing the HSE region was performed for 35 cycles of 1 min at 94°C, 1 min at 53°C and 1 min at 72°C, and produced a specific band of 123 nucleotides.

Luciferase assay

A Dual-Luciferase® Reporter Assay System (Promega, Madison, WI) was used. Wild-type (WT) or mutant (MT) pp53 was subcloned into the Xho-I site of the pGL3-Enhancer vector expressing *Firefly* luciferase (pGL3-E-pp53WT, MT). The pGL3-E-pp53WT/MT with pTet-Aβ40/42, pTet-tTAk and *Renilla* luciferase-expressing pRL-CMV were co-transfected into SKN-SH. Luciferase assay was performed according to the manufacturer's instructions, and

luciferase activities were measured with a luminometer (LUMATLB9501/16, PerkinElmer Inc., Wellesley, MA) 48 h after transfection.

Primary brain cell cultures from fetal guinea pig

Mixed primary brain cell cultures were prepared from fetal guinea pig as previously reported (20). Briefly, embryonic day-30 brains were minced and treated with 0.03% trypsin/0.02% EDTA at 37°C for 3 min, followed by sieving through 67 μm mesh. Cells were then plated on Cell Disks (Sumitomo Chemical Co., Osaka, Japan) and maintained in Opti-MEMI containing 5% fetal bovine serum for 14 days prior to the experiments.

Immunocytochemical staining of Tg mice and human brain tissues

For immunocytochemical staining of human brain, tissues were fixed in 10% buffered formalin. Frontal and temporal cortical tissue blocks were then transferred into 30% sucrose, followed by quick-freezing with dry ice. Each section (10 μm thick) was prepared at the time of use. Tg2576 mice were purchased from Taconic (Germantown, NY). Tg mice sections were fixed in either 10% formalin (Tg2576) or 4% paraformaldehyde (mutant PS1-Tg), followed by paraffin embedding. After blocking endogeneous peroxidase by 0.3% H_2O_2 in methanol for 30 min, Mouse to Mouse Block (SCYTEK Laboratories, Logan, UT) was used to reduce the background in immunostained mice brain tissues. Sections were autoclaved at 120°C for 20 min in 0.01M citrate buffer (pH 6.0), and incubated with primary antibodies in TBS containing 5% skim milk and 0.1% Tween-20 overnight at 4°C. The primary antibodies were diluted at 1:2,000 (BC-05), 1:1,000 (4G8), and 1:200 (anti-p53, FL393, Santa Cruz). Incubation with HRP-conjugated ENVISION+ secondary antibodies (DAKO) at room temperature for 1 h and developing with DAB were used to detect antigens. Counterstaining with hematoxylin was performed for 10–30 s. For immunofluorescent staining, as well as in primary cultures, FITC-conjugated anti-mouse IgG (Cappel) and Texas Red-conjugated anti-rabbit IgG (Cappel) antibodies were used. Lipofuscin autofluorescence was blocked by 0.1% Sudan Black B in 70% ethanol (25). For counting numbers of Aβ42 plaques and neurons, hippocampal tissues from six AD and four age-matched control brains were immunostained with BC-05 after autoclave treatment. Numbers of Aβ42 plaques, cytosolic Aβ42 positive neurons, and both nuclear and cytosolic Aβ42 positive neurons were counted in six separated fields (×200 magnification) at the CA1 sector.

Human brain tissues for immunoblotting

Human brain tissues (frontal cortices) were obtained from five AD cases and five age-matched normal controls. The ages (years) and sex (M, male; F, female) of AD cases A1-A5 were 79F, 82F, 83M, 79M and 79F, respectively; while those of control cases C1-C5 were 72M, 78M, 90M, 74F, and 76F, respectively. Clinical features and pathological findings were used for the diagnosis of AD. Tissues were homogenized in 2% SDS, and 30 μg of total protein was immunoblotted.

RESULTS

Elevation of p53 mRNA levels by intracellular Aβ42

To study the effects of cytosolic Aβ accumulation, we made constructs that express Aβ40, Aβ42, or reverse-sequence Aβ42 (rAβ42) in cytosol under a tetracycline (TC)-sensitive promoter and transiently expressed them in the SKN-SH human neuroblastoma cell line (Fig. 1*A*, upper). Each Aβ mRNA was expressed in the respective culture and was clearly inhibited by TC (Fig. 1*A*, lower). ELISAs showed no increases in extracellular Aβ (data not shown). Cytosolic accumulation of Aβ40 or Aβ42 was clearly seen in the cells (Fig. 1*B*). The specificity of BA-27 and BC-05 for Aβ40 and Aβ42, respectively, was shown by immunoblotting (Fig. 1*B*, right). Interestingly, Aβ42 immunoreactivity was also seen in the nucleus of the cells 48 h after transfection (Fig. 1*B*, right, lower panel). These cells may have been degenerating, because lots of Aβ42-transfected cells were undergoing apoptosis at 48 h (see Fig. 2*B*, *C*). The immunoreactivities were absorbed by the respective Aβ peptides (data not shown). To check whether some amounts of Aβ were present in the nucleus of each transfected cell, we performed immunoblotting of NEP after enrichment of 3–10 kD proteins (see Materials and Methods). Both 4 kD soluble Aβ40 and Aβ42 were similarly detected in NEP of the cells 24 h after transfection (Fig. 1*C*). However, oligomeric or fibrillar Aβ42 was not detected in NEP (data not shown). In addition, we found approximately six- and twofold elevations of the p53 mRNA level in Aβ42- and Aβ40-transfected cells, respectively, whereas rAβ42 did not have this effect (Fig. 1*D*). Aβ42 mRNA levels were elevated from 5 h after transfection, followed by a parallel elevation of p53 mRNA levels (Fig. 1*E*). Taken together, an increase in p53 mRNA expression seems to be tightly linked to cytosolic Aβ expression, especially Aβ42. In other human neuroblastoma cell lines (LAN-5, SH-SY5Y), smaller increases in p53 mRNA levels were observed (data not shown).

P53-dependent apoptosis caused by intracellular Aβ42

MDM2, p21 (WAF-1), Bax, and PIG3 are well-known target genes for p53 (39). We found 2- to 4-fold increases in their mRNA levels, and 1.6-fold (MDM2, Bax, PIG3) or 3-fold (p21) increases in their protein levels in Aβ42-transfected cells (Fig. 2*A*). Since p53 overexpression causes apoptosis in SKN-SH (38), we measured the cell viability, which was significantly ($P<0.01$) reduced to ~20 and 40% in Aβ42- and Aβ40-transfected cells, respectively, but not in rAβ42-transfected cells (Fig. 2*B*). Cell death was inhibited by TC. Further, consistent with Zhang et al. (34), the addition of actinomycin D (ActD) or a caspase inhibitor (CI), Z-VAD-fmk, inhibited cell death ($P<0.01$), indicating that de novo protein synthesis and caspase activation were required and that it was apoptosis. Moreover, antisense p53 (AS-p53), but not sense p53 (S-p53), DNA inhibited cell death, suggesting p53 dependence. TUNEL staining showed many positive cells in Aβ42-transfectants, a lesser number of positive cells in Aβ40-transfectants and no positive cells in control cultures (Fig. 2*C*). To further confirm the p53 dependence, we studied Saos2 (p53–/–) and U2OS (p53+/+) osteosarcoma cells. In U2OS, p53 mRNA was increased ~2-fold by Aβ40, and ~3-fold by Aβ42 and p53 protein was increased ~1.2-fold by Aβ40 and ~1.5-fold by Aβ42; however, p53 mRNA and protein did not appear in Saos2 (Fig. 2*D*). The relatively lower alteration in p53 protein levels compared with mRNA levels may be due to the

effect of p53 protein degradation. Viability of Aβ42-transfected U2OS cells decreased significantly to ~60% (P<0.01), but Aβ42-transfected Saos2 cells were unaffected (Fig. 2*E*). Antisense p53 DNA inhibited cell death in U2OS. As Saos2 and U2OS readily died in the serum-free medium that were used for SKN-SH, we cultured them in 5% FBS-containing medium after transfection; the relatively lower effect on p53 mRNA expression and vulnerability of U2OS compared with SKN-SH would be due to the differences in medium conditions.

Aβ42 bound to heat-shock elements in the p53 promoter

Aβ theoretically forms a β-hairpin shape followed by a helix-turn-helix (HTH) motif (41), an essential motif in the DNA binding domain of heat-shock transcription factors (HSF) (42). Since the p53 promoter contains heat-shock elements (HSE) (43), Aβ42 might directly bind the p53 promoter. Thus, we performed a gel mobility shift assay using a 48-mer p53 promoter oligonucleotide (pp53, see Fig. 3*B*). Aβ42, but not Aβ40, bound pp53 dose-dependently (Fig. 3*A*). Also, the reverse sequence Aβ42 peptide (Aβ42-1) did not bind (data not shown). However, when incubated overnight, Aβ40 did bind pp53 (data not shown). Such Aβ42 binding was not found in two other promoters, namely Oct-1 and EBNA (Fig. 3*A*, left). These bands of the Aβ42-pp53 complex disappeared when electrophoresed on SDS-PAGE, indicating that this complex was not SDS-insoluble (data not shown). An excess of cold pp53, but not non-specific calf thymus DNA, clearly diminished the binding of Aβ42 to labeled pp53, providing evidence of the sequence specificity (Fig. 3*B*, upper). As shown in Fig. 3*B* lower, studies using region-specific 40-mer oligonucleotides revealed that oligonucleotides containing the middle 10 nucleotides in HSE (see HSE-A, B, C), but not nonHSE-5′ or nonHSE-3′, bound Aβ42 showing the same size bands (arrow on the lower right panel), and that HSE-B showed the most remarkable binding. The data indicated that Aβ42 prominently bound the middle part of HSE. Further evidence for the Aβ42 binding site came from the observation that the replacement of the 10-nucleotide sequence from -58 to -49 markedly diminished the Aβ42 binding affinity (see Fig. 4*A*). We next made biotinylated HSE-B to co-precipitate Aβ42 using streptavidin-conjugated magnetic beads. The collecting efficiency of synthetic Aβ42 by HSE-B magnetic beads was much improved when NEP, but not bovine serum albumin (BSA), was co-incubated (Fig. 3*C*, upper). Aβ40 was not recovered because of its lower affinity (data not shown). Consistent with the gel mobility shift assay, nonHSE-3′ collected no Aβ42, and mutant (MT) HSE collected minimal Aβ42 (Fig. 3*C*, middle). Approximately 10 pg Aβ42 was recovered from 100 μg NEP of Aβ42-transfected cells (Fig. 3*C*, lower). Thus, Aβ42 may bind the p53 promoter in cooperation with other unknown nuclear proteins in vivo and might not form SDS-insoluble fibrils in the nucleus. We also performed a chromatin-immunoprecipitation (ChIP) assay. P53 promoter DNA was detected by PCR in eluates recovered from the nuclei of transfected cells by immunoprecipitation with each specific anti-Aβ antibody (Fig. 3*D*). Anti-Aβ40 antibody co-precipitated the p53 promoter, though HSE-B did not co-precipitate Aβ40, which may be due to the cross-linking step and PCR detection in the ChIP assay. Other two different heat-shock promoters of *HSP32* (heme oxygenase-1, HO-1) (44) and *HSP70* (45) were not detected by PCR, suggesting Aβ binding specificity for the p53 promoter (data not shown).

Activation of p53 promoter by Aβ42 in cells

We then made two constructs in which wild-type (WT) or mutant (MT) pp53 were subcloned upstream of *Firefly* luciferase (Fig. 4*A*). The binding affinity of MT pp53, in which 10 nucleotides of the putative Aβ42 binding site were replaced with the sequence-reversed DNA, was 50–60% of that of WT pp53. The luciferase activity in WT pp53 + Aβ42-coexpressed cells increased about sevenfold compared with controls (Fig. 4*B*, left), corresponding well to the approximately sixfold elevation in p53 mRNA levels induced by Aβ42 transfection (see Fig. 1*D*), while Aβ40 showed a smaller effect. This effect was inhibited by TC. In MT pp53-transfected cells, the baseline luciferase activity was dramatically reduced to ~10% of that of WT pp53, and the luciferase activity of MT pp53 + Aβ42-coexpressed cells increased only approximately threefold, which did not reach statistical significance (Fig. 4*B*, right). Thus, the reduction in pp53 activation from sevenfold to threefold corresponded well to the ~50% decrease in the binding affinity of MT pp53 in vitro (Fig. 4*A*).

Hydrogen peroxide-induced intranuclear Aβ42 accumulation and elevated p53 expression in guinea-pig primary neurons

We previously reported that H_2O_2 treatment induces selective Aβ42 accumulation in guinea-pig primary neurons (20). Such cultures provide a suitable system for Aβ study because guinea-pig Aβ has the same sequence as human Aβ, and the authenticity of the intracellular Aβ species has been confirmed by HPLC-mass spectrometry (46). H_2O_2 is known to cause p53-related apoptosis in neuronal cells (47). To check the biological significance of intracellular Aβ42 on the regulation of p53 mRNA expression in native neurons, we examined the alteration of Aβ42 localization after H_2O_2 treatment. As shown in Fig. 5*A*, although intracellular Aβ42 was hard to see under normal conditions (0 h), cytosolic and partly nuclear accumulation of Aβ42 was significantly observed at 6 h after treatment with 1 mM H_2O_2. Subsequently, marked localization of Aβ42 in nucleus was observed at 12 h. In association with intranuclear Aβ42 accumulation (green), p53 protein (red) accumulated in these cells. Neurons intensely positive for both intranuclear Aβ42 and p53 looked to be degenerating because dendrites and axons disappeared and the cells became round-shape (Fig. 5*A*, 12 and 24 h). In fact, in an earlier report we demonstrated many TUNEL-positive cells in these H_2O_2-treated cultures (20). Also, Aβ42 became detectable in NEP by immunoblotting at 12 h (Fig. 5*B*), corresponding to the marked immunoreactivity in the nucleus at 12 h (Fig. 5*A*). Protein levels of both p53 and Bax, a target of p53, started to be elevated at 6 h after treatment; however, levels of β-actin, the internal standard, were not changed (Fig. 5*B*). Similarly, p53 mRNA levels started to be elevated at 6 h after treatment (Fig. 5*C*). Thus, Aβ42 may effect p53 mRNA expression at 6 h, when intranuclear Aβ42 was detectable by immunostaining but not yet by immunoblotting. The data indicate that oxidative DNA damage may induce Aβ42 accumulation in cytosol and then sequentially in the nucleus activating p53 cascade.

Intracellular Aβ42 accumulation associated with p53 expression in Swedish mutant APP-Tg and L286V mutant PS1-Tg mice brain

To study whether similar neurodegeneration occurs in brain, we next checked intracellular Aβ42 and p53 in FAD mutant gene-Tg mice brain. It is known that intracellular Aβ accumulates with