score, as covariance in a basic model. Initially, we performed a logistic analysis using these six factors. Subsequently, in order to find additional risk factors, we sequentially inserted and removed variables such as the Barthel Index, QOL, concomitant psychiatric/nervous disorders, and E-PASS score to the logistic model, and evaluated whether these variables were independently associated with the development of postoperative delirium after adjustment for the six basic factors.

RESULTS

Eighty-seven patients (54.7%) were regarded as having delirium based on a NEECHAM score of less than 20 within 10 days after surgery. When examining changes in the median score in all registered patients, there was a decrease in the NEECHAM score between the first and fourth postoperative days, but it gradually increased thereafter (Figure 1). We investigated risk factors per variate, regarding a postoperative NEECHAM score of 20 points as the cut-off value. Postoperative delirium was associated with an advanced age (more than 80 years), reduction of the preoperative ADL (low Barthel Index value), a preoperative NEECHAM score of 27 or lower, an MMSE score of 25 or lower, the presence of concomitant psychiatric disease, urinary incontinence, a history of excitation/hyperactivity, the preoperative risk score (PRS) and comprehensive risk score (CRS)(E-PASS scores), the number of inserted catheters, and PCS on preoperative QOL assessment (SF-8). There were no significant differences related to the other factors such as gender, departments (abdominal surgery, orthopedics), concomitant physical disorders, hematology, or physiological test parameters (Table 4).

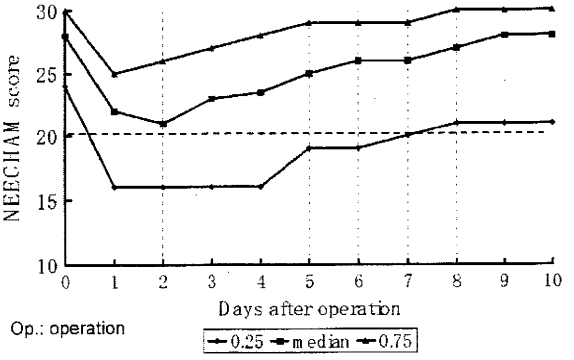

Figure 1. Changes in the NEECHAM score before and after surgery. The score before surgery is expressed as that on Day 0. The upper line indicates the 75 percentile, the middle line the median, and the lower line the 25 percentile. The dotted line represents the cut-off value of the NEECHAM score at which delirium was evaluated as present.

A logistic analysis with six basic model factors showed the association of age (a 1-year increase elevated the risk of postoperative delirium 1.2-fold), gender (males: 3.9-fold), and MMSE (a 1-point decrease elevated the risk 1.2-fold) with the development of postoperative delirium. The above logistic analysis was additionally performed by dividing the subjects with respect to age (younger than 80 years, 80 years or older), MMSE (less than 25 points, 25 points or higher), and the preoperative NEECHAM score (less than 27 points, 27 points or higher). In patients aged more than 80 years, the risk of postoperative delirium was 3.1 times higher than that in those aged younger than 80 years. In those with an MMSE score of less than 25 points, it was 4.0 times higher than that in those with an MMSE score of 25 or higher. In those with a preoperative NEECHAM score

Table 4. Statistically significant factors associated with low NEECHAM score

	Coefficient	Chi-square value	*p*-Value
Age, >80		44.3	<0.01
Barthel Index, <65/65–95/100		12.9	<0.01
NEECHAM, <27		40.1	<0.01
MMSE, <25		40.8	<0.01
Concomitant psychiatric disease, yes/no		5.5	0.02
Urinary incontinence, yes/no		19.5	<0.01
History of excitement, yes/no		9.1	0.01
Numver of catheters (post-operation), continuous	0.23		<0.01
PRS, continuous	2.65		<0.01
CRS, continuous	1.76		<0.01
QOL (PCS), continuous	−0.03		0.04

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Table 5. Multivariate analysis for basic six models

	Odds ratio (95% CI)	*p*-Value
Age, >80	3.14 (1.35–7.26)	<0.01
Male	2.86 (1.09–7.47)	<0.01
Type of surgery, orthopediatrics	0.70 (0.24–2.07)	0.52
Type of anesthesia, general	1.11 (0.26–4.71)	0.89
Preoperative MMSE, <25	3.96 (1.52–10.39)	<0.01
Preoperative NEECHAM, <27	5.33 (1.84–15.31)	<0.01

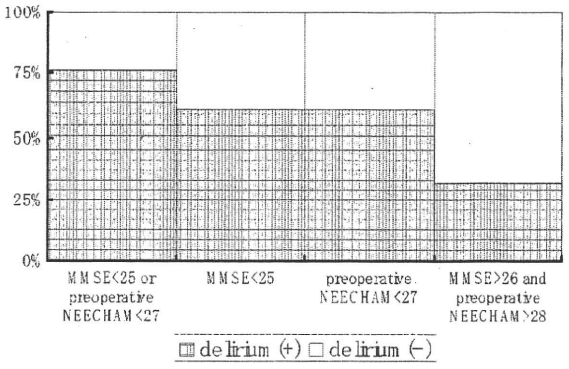

Figure 2. Close association between the MMSE/preoperative NEECHAM scores and the development of delirium.

of less than 27 points, it was 5.3 times higher than that in those with a NEECHAM score of 27 or higher (Table 5). In groups with an MMSE score of less than 25 points or a preoperative NEECHAM score of less than 27 points, the incidence of postoperative delirium was 76% (Figure 2).

When examining factors associated with the development of postoperative delirium, a QOL parameter, PCS of SF-8, was associated with its development after correction with six basic factors ($p = 0.04$). There was no association with other QOL parameters such as MCS of SF-8 or EQ5D. The global E-PASS score (CRS, $p = 0.09$) was weakly associated. Among the factors comprising CRS, surgical invasiveness (surgical stress score: SSS) was associated with the development of postoperative delirium ($p = 0.09$). When classifying the SSS into three categories, the risk of postoperative delirium in the highly invasive surgery group was significantly higher than that in the less invasive surgery group ($p = 0.03$). On the other hand, another factor comprising the CRS, the preoperative physical status, was not associated. The other factors for which an association was noted on univariate analysis were not associated with postoperative delirium after correction with the six basic factors by multivariate analysis.

DISCUSSION

In this study, we employed the NEECHAM Confusion Scale for delirium diagnosis and severity assessment (Neelon *et al.*, 1996). The NEECHAM score is useful for evaluating postoperative delirium in elderly inpatients (Schuurmans *et al.*, 2003), and is available for medical staff (Matsushita *et al.*, 2004); three categories, cognitive information processing, behavior, and physiological control, are assessed. The most unfavorable condition during a 24-h period is regarded as the day's condition. The full score is 30 points, and the severity increases with a decrease in the score. Patients showing a NEECHAM score of 28 points or higher are regarded as having no problems, those showing scores ranging from 21 to 27 points as having mild delirium, and those showing a NEECHAM score of 20 points or lower as having severe delirium. Furthermore, the NEECHAM Confusion Scale has high internal consistency, and is reliable between examiners. It is correlated with the DSM-IV criteria (Immers *et al.*, 2005). Therefore, it is available for delirium diagnosis and monitoring.

E-PASS, which was employed to evaluate surgical-related risks, was developed by Haga *et al.* (1999) as a parameter for predicting the incidence of postoperative complications. Several studies have reported its usefulness in various surgical fields (Tang *et al.*, 2007; Yamashita *et al.*, 2006; Oka *et al.*, 2005). E-PASS consists of three items: PRS, SSS, and CRS. PRS is calculated using a specific formula based on the performance status (PS) (Furue, 1986) and American Society of Anesthesiologists (ASA) physiological status classification (American Society of Anesthesiologists Task Force, 1996), which are used to evaluate anesthesia, the preoperative state, surgical invasiveness, the duration of surgery, volume of intraoperative blood loss, and blood pressure changes, in addition to an index regarding the presence or absence of preoperative concomitant disorders. The SSS is calculated based on the body weight, operation time, and width of the surgical field. The CRS is a parameter for comprehensively evaluating the PRS and SSS (refer to Appendix). For the evaluation of postoperative delirium in elderly patients, preoperative physical (malnutrition, etc.) and mental (cognitive hypofunction) fragility must be considered. In clinical practice, complex evaluation is difficult, and data interpretation is complex. In this study, combination of the NEECHAM Confusion Scale and E-PASS facilitated evaluation; it was simple to interpret results expressed as numerical data.

We serially examined postoperative psychiatric symptoms/behavioral abnormalities using the NEECHAM Confusion Scale. Delirium or a delirium-like state persisted until 3–4 days after surgery, and then subsided. Previous studies indicated that the incidence of postoperative delirium increased with age; the incidences were 12% in patients aged more than 65 years and 35% in those aged more than 80 years (Knill, 1990; Hirsch, 1995). Amemiya *et al.* (2007) reported that, among patients aged more than 75 years who underwent elective surgery for gastric/colorectal cancer, the incidence of postoperative delirium in those aged more than 80 years was 1.3 times higher than that in those aged younger than 80 years. As indicated for an age-related increase in the incidence of dementia, the fragility of the central nervous system may be an important risk factor for the onset of delirium. A study reported that postoperative delirium became severe after 12–24 h (Olympio, 1991). This tendency is also observed in patients with delirium after alcohol cessation (Victor and Wolfe, 1973). Furthermore, the results of uni- and multivariate analyses suggested that the preoperative MMSE and NEECHAM scores influence the postoperative NEECHAM score. The incidence of postoperative delirium was approximately 80% in patients showing a preoperative MMSE score of 25 points or lower or a NEECHAM score of 27 points or lower. Thus, the NEECHAM Confusion Scale may be useful for evaluating the risk of postoperative delirium, the postoperative state, and treatment response.

In this study, significant risk factors for postoperative delirium included an advanced age, the presence of preoperative cognitive hypofunction, and ADL reduction. However, the presence of hematological abnormalities, such as anemia, a decrease in the serum albumin level, and an increase in the serum BUN level, was not a significant risk factor. Furthermore, differences in fields such as abdominal surgery and orthopedics were also not associated with the onset of delirium. On the other hand, univariate analysis showed that CRS involving surgical invasiveness was associated with its onset, suggesting that surgical invasiveness influences the development of postoperative delirium in elderly patients. Previous studies have reported the association of postoperative delirium with the type of anesthetics (Parikh and Chung, 1995) and volume of intraoperative blood loss (Marcantonio *et al.*, 1998; Böhner *et al.*, 2003). However, surgery-related stress may complexly act; it is important to comprehensively evaluate various associated factors. E-PASS, which we employed in this study, involves almost all factors that may contribute to surgery-related risks. This procedure should be introduced to evaluate such risks.

KEY POINTS

- Postoperative delirium was associated with an advanced age (more than 80 years), low preoperative NEECHAM and MMSE scores, the preoperative QOL, and E-PASS.
- An MMSE score of less than 25 or a preoperative NEECHAM score of less than 27, the incidence of postoperative delirium was 76%.
- E-PASS and the NEECHAM score facilitate assessment of the risk of postoperative delirium in elderly patients, contributing to early prevention/treatment.

Thus, many studies have investigated the risk of postoperative delirium. However, it is important to comprehensively evaluate its risk in individual patients. This is much more important in elderly patients. However, complex procedures are difficult on bedside evaluation. Furthermore, the accuracy of numerical data is required so that the results of evaluation may be reflected by prevention/treatment. The NEECHAM Confusion Scale and E-PASS, which we used in this study, are useful for evaluating the risk of postoperative delirium in elderly patients and initiating prevention/treatment.

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APPENDIX

Equations for E-PASS scores: PRS, surgical stress score (SSS), and CRS (Haga *et al.*, 1999).

(1) $PRS = -0.0686 + 0.00345 \times 1 + 0.323 \times 2 + 0.205 \times 3 + 0.153 \times 4 + 0.148 \times 5 + 0.0666 \times 6$

X1, age; X2, presence (1) or absence (0) of severe heart disease; X3, prsence (1) or absence (0) of severe pulmonary disease; X4, presenc (1) or absence (0) of diabetes mellitus; X5, PS index (0–4); X6, ASA physiological status classification (1–5). Severe heart disease was defined as heart failure of New York Heart Association Class III or IV, or severe arrhythmia requiring mechanical support. Severe pulmonary disease was defined as any condition with a% VC of less than 60% and/or a FEV1.0% of less than 50%. PS index was based on the definition by Japanese Society for Cancer Therapy (VC, vital capacity; FEV, forced expiratory volume).

(2) $SSS = -0.342 + 0.0139 \times 1 + 0.0392 \times 2 + 0.352 \times 3$

X1, blood loss/body weight (g/kg); X2, operation time (h); X3, extent of skin incision (0: minor incisions for laparoscopic or thoracoscopic surgery (including scope-assisted surgery); 1: laparotomy or thoracotomy alone; 2: both laparotomy and thoracotomy.

(3) $CRS = -0.328 + 0.936(PRS) + 0.976(SSS)$

第2回 アルツハイマー病の治療薬－現状と将来展望

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1. アルツハイマー病

アルツハイマー病は、細胞内外への線維性構造物の蓄積を認める、いわゆる“原因蛋白の蓄積病”をその基本病態とする。このアルツハイマー病の病理過程でみられる最も早期の変化は細胞外に認められ、βアミロイド(Aβ)を主要構成成分とする斑状の嗜銀性構造物（老人斑）として知られている。アルツハイマー病患者脳では何らかの原因でこの本来可溶型の生理的Aβの産生・分解・クリアランスの代謝機構が破綻し、不溶性の高まった病的Aβが脳実質に蓄積し、老人斑としての脳アミロイド蓄積・沈着をきたすと考えられている。こうした形成機序から、従来、老人斑自体は免疫原性に乏しく、蛋白分解にも抵抗性で生理的機能のない負の産物ととらえられ、長らくその除去を目的とした治療標的からは除外されていた。しかし、典型的老人斑においては、脳在住のミクログリアが活性化されており、アストロサイトとともに炎症性サイトカインや補体、その他の生体防御因子を産生・分泌していることが知られ、老人斑は低レベルの炎症が持続する慢性炎症病変と捕らえる考え方や、老人斑自体が生体防御反応の結果としてこうした炎症を惹起しているとの考え方が提唱され、アルツハイマー病治療標的の幅が広がってきた。しかしながら、アルツハイマー病の治療戦略の標的はその病態生理に基づいており、その理解は本稿を考える上で不可欠である。

2. アルツハイマー病の病態生理

アルツハイマー病の病態生理においては、Aβの重合ならびに神経細胞毒性発現が、神経原線維変化、神経細胞脱落へと続く一連の病的カスケードの引き金となり、認知症を引き起こすとの考え方が広く受け入れられている。このアミロイドカスケード仮説[1]（図1）を支持する理論的根拠は、

(1)Aβの脳内蓄積は老人斑として形態学的に捉えうる最も早期の変化であり、神経原線維変化、神経細胞脱落に先行する

(2)Aβの前駆体をコードするアミロイド前駆体蛋白amyloid precursor protein (APP)遺伝子の変異や重複をもつ家族性アルツハイマー病が存在する

(3)これまでに家族性アルツハイマー病の原因として発見されたAPP遺伝子変異・重複及びプレセニリン遺伝子変異の全てによって、Aβの産生異常が誘導される

(4)培養神経細胞や動物実験において、重合したAβは細胞毒性やタウ蛋白のリン酸化亢進を引き起こす

(5)重合したAβは記憶に関連するシナプス電位である海馬における長期増強(Long Term Potential, LTP)を阻害し、シナプス毒性を発揮する

(6)Aβの脳内異常沈着を示すAPP遺伝子導入トランスジェニックマウスに抗原としてAβを投与することで（Aβワクチン療法)、Aβの脳内沈着が抑制されるとともに、このモデルマウスが示す様々な機能障害(学習・記憶障害など)も軽減する

(7)このモデルマウスに直接Aβに対する抗体を投与すると（受動免疫療法)、Aβの脳内沈着には影響することなく、記憶障害が可逆的に回復する

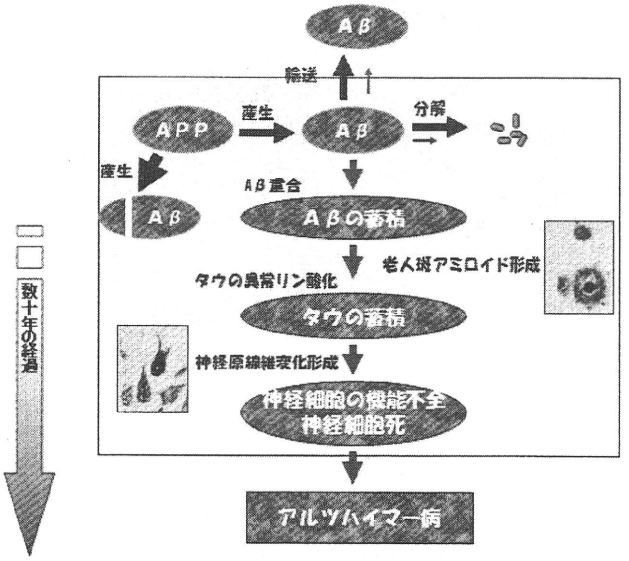

図1 アミロイドカスケード仮説

との知見であるが、厳格な意味でこのアミロイドカスケード仮説に修正が加えられる結果となった。現在、老人斑を構成するアミロイド線維よりもむしろ重合Aβ（Aβオリゴマー）がアルツハイマー病患者脳における神経細胞障害誘導の物質的基盤と考えられ、新たにAβオリゴマーカスケード仮説として市民権を得ている。

3. アルツハイマー病治療薬の現状

前述したアルツハイマー病の病態生理をもとに、アルツハイマー病治療薬・発症抑止薬の研究が行われている。大多数がアミロイドカスケード仮説の上流に位置するAβを標的とした薬剤開発であるが、現状（図2）は対症療法の域をでず、臨床現場ではその力不足が否めない。

4. 開発途上のアルツハイマー病治療薬

開発途上の治療薬を図3に示す。このうち本稿ではアルツハイマー病の免疫療法に焦点を絞り概説したい。

アルツハイマー病の免疫療法

生体の免疫系を駆使し一旦沈着した脳アミロイド除去を標的とした治療が免疫療法（Aβワクチン療法）

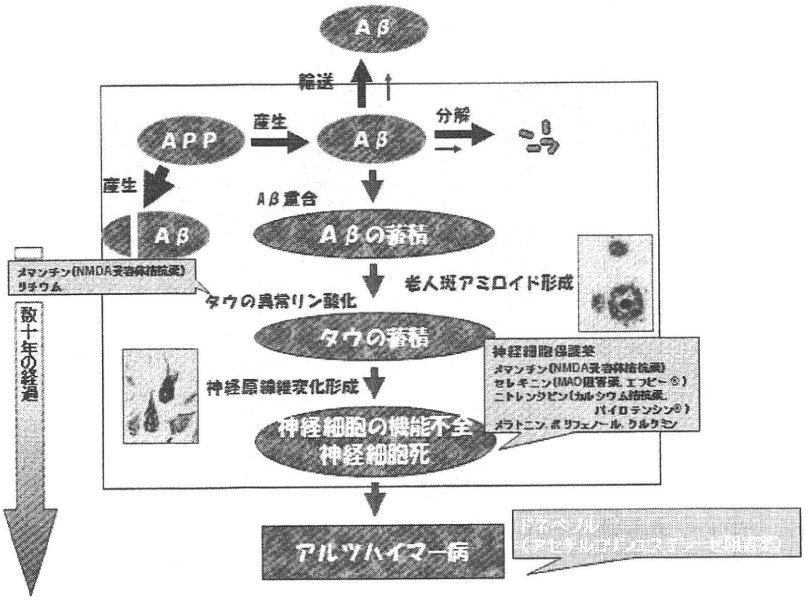

図2 アミロイドカスケード仮説に基づくアルツハイマー病治療薬の現況

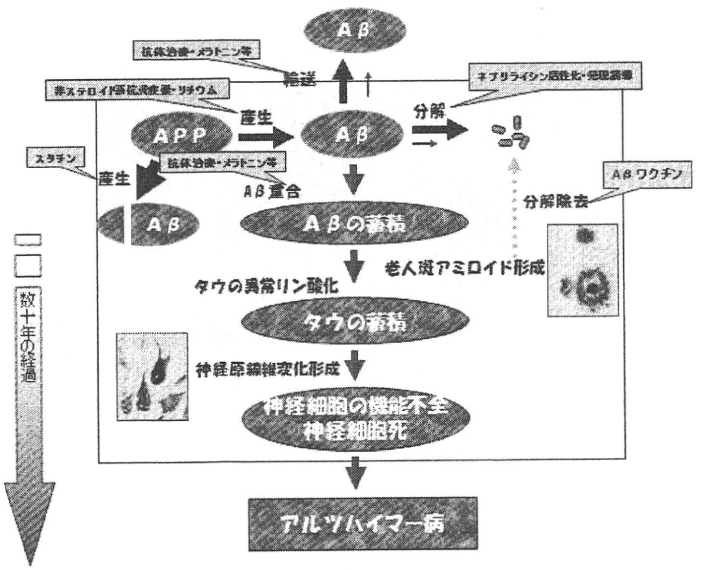

図3 アミロイドカスケード仮説に基づく開発途上のアルツハイマー病治療薬

である。いわゆる老人斑除去を抗体で行う治療法であり、従来の老人斑自体は免疫原性に乏しく、蛋白分解にも抵抗性で生理的機能のない負の産物でありその除去自体できるはずがないとの誤った常識を打破した、いわゆるパラダイム変換に基づきSchenk等[2]により提唱された治療法である。厳格な意味でのワクチン療法とは異なり、アルツハイマー病の免疫療法(Aβワクチン療法)は、Aβペプチドをadjuvantとともに投与し抗体産生を誘導する能動免疫と直接抗体を投与する受動免疫、全身性免疫でなく粘膜免疫を用いた免疫療法の3種を包括した概念として使用されている。

① 能動免疫療法（厳格な定義のAβワクチン療法）

ヒトの変異型APPを強発現させたトランスジェニックマウスを、凝集させた合成Aβで免疫し、脳内に異常沈着したAβが果たして抗体によって排除可能か否かの検討がなされた。その結果、このAβワクチン療法はマウス脳内でのAβ沈着抑制[3]ならびにAβ沈着によると考えられる学習・記憶障害の緩和に有効であることが確認された[4]。Aβワクチンの作用機序としては、①これまで、抗体は血液脳関門を容易に通過しないと考えられてきたが、わずかな抗体がこのバリアーを突破し、脳実質内に異常沈着したAβ凝集塊と結合することがシグナルとなり、ミクログリアによる貪食が促進される。②脳内移行した抗体が直接Aβと結合し、その重合抑制・線維溶解・毒性中和をする。③抗体が血液中からのAβクリアランス誘導を介し、Aβの脳から血液への移行を増加させる、④抗体と結合したAβがその免疫複合体として、Fc受容体を介して血液中に移行する、以上4つの作用機序が想定された。こうした前臨床試験の結果を踏まえ施行されたヒトにおけるAβワクチン(AN-1792)第II相臨床治験は、6%の症例で髄膜脳炎が発症し中止となった。髄膜脳炎が直接の原因となった死亡例は報告されていないが、不幸にして亡くなられた髄膜脳炎合併例[5]・非合併例の2剖検脳による検討から老人斑アミロイドの除去効果が確認された。しかしながら神経原線維変化の除去には至らないことも明らかとなった。残念なことに、その後1年の追跡結果[6]から認知症評価スケール個々の値は、約20%のワクチン反応群（抗体上昇群）とプラセボ投与群との間で有意差を認めなかったが、神経心理学テスト全体の標準化スコア（zスコア）はワクチン反応群で有意によく、また少数ながら同群では脳脊髄液中タウの減少も認められたと報告されている。最近になりAβワクチン(AN-1792)長期経過観察例での剖検所見の報告がなされ、老人斑が消失しても認知症は進行することが明らかとされた[7]。この結果を好意的に解釈すれば、Aβワクチン療法も中等症以上にまで進行してしまうとその認知機能改善治療効果が望めず、やはり予防的な効果を期待して施行すべき治療法である可能性も考えられる。またAβワクチン接種者

に認められた抗体は老人斑認識抗体であった点からは、老人斑を標的としたAβワクチン療法には効果が認められないことへ警鐘をならしたとも考えられる。免疫療法の大事な点はどのような抗体が結果的に産生されるかという点であり、この点は能動免疫療法の克服されるべき重大な弱点である。

② 受動免疫療法（抗体投与療法）

この受動免疫療法が最も注目を集めた所以は[8]、①この抗体投与でアルツハイマー病モデルマウスで発症した記憶障害の回復ができることと、②この発見に端を発し、その病態解析の過程で、実は老人斑Aβアミロイド線維自体のアルツハイマー病発症病態への関与は低く、アミロイド線維形成して沈着・蓄積する前に形成されるAβオリゴマーこそがその本態で、アルツハイマー病の治療標的であることが認識された点にある[9]。これまでさまざまな抗体による受動免疫療法の前臨床試験がアルツハイマー病モデル動物で施行され、その有用性が確認されてきた。老人斑アミロイド除去を標的とする抗体や脳アミロイド除去とは無関係に記憶障害改善効果をも発揮する抗体、さらに老人斑除去のみでなく、Aβのアミロイド線維形成や毒性などの抑制活性を念頭に考案されたAβオリゴマーやシードに対する抗体がこれに含まれるが、これまで沈着した脳アミロイドに結合性を示す抗体を使用した受動免疫療法で微少出血と頻度は低いものの髄膜脳炎の副作用発生がアルツハイマー病モデルマウスで報告されており、使用抗体選択の重要性と副作用を未然に防ぐための教訓として、如何にアミロイドが相当量沈着する前に治療を開始することが出来るか、またそうした対象を如何にして知り得る術をもつかが大事なポイントであることが明らかとなった。

5. アルツハイマー病治療の将来展望

先に開催されたICAD2008ではヒト化モノクローナル抗Aβ抗体2種、nativeな抗Aβ抗体を含有する免疫グロブリン製剤でのヒト第II相臨床治験の途中経過報告がなされ、老人斑を主に認識する抗体では認知機能改善効果が乏しく、抗Aβオリゴマー抗体を含有する免疫グロブリン製剤でのみ認知機能改善効果が得られたことが確認された。本結果からも、いわゆる病態発症を特異的に制御する目的で、生理的な分子には反応しない、病態惹起分子特異的な抗体による治療法の開発が肝要であると考えられ、アルツハイマー病を“治療可能な認知症”とするため、今後より一層研きをかけた抗体治療が世に送り出されることを期待したい。

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PET化学ワークショップ2009

理化学研究所分子イメージング科学研究センター　高橋和弘

2月10日～12日にPET化学ワークショップ2009（通称冬の学校）が行なわれました。今年は、昨年と同じ神戸市六甲山での開催となりました。今回は穏やかな天候に恵まれ人工スキー場を除けば雪はほんのわずかしかありませんでした。とはいえ昨年同様、山の上閉ざされた静かな環境の中で、突っ込んだ議論ができ大いに盛り上がった会でした。

このワークショップは「PET薬剤のルーチンな合成に従事する者が集まり、学術集会では取上げにくい“現場が抱える諸問題”について自由に討議し、PETの現状や日進月歩の“PET化学の新しい展開”に触れる啓蒙的な場を提供する」ことを目的に、平成4年に開始されました。今回は第18回にあたり、108人が参加しました。プログラムは次の通りです。

10日◇　PET化学への招待
◇サイクロメンテ中の被曝事故
◇　各施設のチョッと良いもの
◇　新しい薬剤導入（FLT）
◇　施設運営状況アンケートから
◇　情報交流会
11日◇　フリーディスカッション
◇　耳寄り情報
^{64}Cu、添加物、超純水
◇　「てびき」をナナメに読む脳編
◇　初心者のためのわかる合成装置入門
◇　情報交流会
12日◇　PET施設の放射線管理問題と対応について
◇　核医学認定薬剤師について
◇　製品情報・業界情報

「チョッと良いもの」ではUSBカメラの利用法から始まり分注装置や手軽な遮蔽箱、入手が危ぶまれるアセトニトリルの話題、可溶化剤として多用されるポリソルベート80さらにサイクロトロンの壊し方まで広範な内容に楽しませてもらいました。「薬剤導入（FLT）」では本ワークショップらしい最も現場的な内容に突っ込んだ議論ができました。フリーディスカッションはケーブルカーがメンテナンスのため運休となり、有馬温泉への移動が困難だったため、メイン会場は灘の酒蔵巡りと昨年とはまた違った議論に花が咲いたようです。「耳寄り情報」は気にはなりつつも普段調べることの少ない話題について専門家から教えてもらいました。「てびきをナナメに読む（脳編）」では日頃何となく眺めることの多いPET化学の応用編（臨床利用）を若い発表者に解説していただきました。「わかる合成装置入門」では最近要望が増えつつある多目的合成装置の現状について各社から報告してもらいました。また今年も深夜遅くまで及んだ情報交流会では皆さん有益な議論と親睦を多いに深められた模様です。

FDGブームによるPETセンター数の激増や、分子イメージングの興隆によりPET化学を取り巻く環境も変わりつつあります。その中でこの会への期待が今まで以上に高まっているように思います。このことは参加者の3分の1が初参加だったことからも覗えます。

次回開催案内をご希望の方は簇野（hatanok@nils.go.jp）または高橋（kazu.takahashi@riken.jp）までご連絡下さい。

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Research Report

Microglial activation in brain lesions with tau deposits: Comparison of human tauopathies and tau transgenic mice TgTau^{P301L}

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ABSTRACT

The aim of this study is to clarify the relationship of microglia to phosphorylated tau accumulation and the characteristics of microglial activation in brain lesions of human tauopathies in comparison to mutant tau transgenic (TG) mice. We performed immunocytochemical analyses of brains from six patients with tauopathies, and 24 mice (18 TG mice expressing mutant tau P301L and six non-TG control mice, 11 to 27 months of age) using anti-tau antibodies and various microglial markers. In the tau TG, both semiquantitative severity ratings of microglial activation and an ultrastructural study were performed. In human tauopathies, Iba1- and major histocompatibility complex (MHC) class II-positive activated microglia increased in regions of phosphorylated tau (AT8) accumulation. The immunoreactivity of scavenger receptor class A (SRA) was present in some activated microglia, including phagocytic microglia in Alzheimer's disease (AD). Double-immunofluorescent analysis under a confocal microscope showed activated microglia at the vicinity of AT8-positive cells. Semiquantitative data of the TG and control mice indicated that the immunopositivity of AT8 was closely associated with the number of Iba1-positive microglia in the cortical area. Tau-associated microglia showed rare immunoreactivity for MHC class II antigen and SRA in the TG mice. Ultrastructurally, activated microglia with enlarged cytoplasm were located near neurons containing abnormal cytoskeletons. This comparative study of human tauopathies and tau TG mice

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indicated that microglial activation was closely related to phosphorylated tau accumulation, and that activated microglia of the TG mice may have the low expression of MHC class II and SRA compared with those of human tauopathies.

1. Introduction

The abnormal hyperphosphorylation of tau is a feature common to all diseases with tau filaments. The tau-immunopositive deposits are present in the tau-associated disorders, tauopathies, such as progressive supranuclear palsy (PSP), Pick's disease (PiD), corticobasal degeneration (CBD), frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17). The tauopathies have been characterized by their biochemical features. It has been reported that three-repeat (3R)-tau isoforms predominantly aggregate into filaments in PiD while four-repeat (4R)-tau isoforms aggregate into filaments in PSP and CBD (Buee and Delacourte, 1999). The tau isoform composition of Alzheimer's disease (AD) is a variable mixture of 3R- and 4R-tau (Greenberg and Davies, 1990). The monoclonal antibodies RD3 and RD4 could distinguish the closely related 3R-tau and 4R-tau isoforms with complete specificity (de Silva et al., 2003). The antibody, ET3, is a 4R-tau-specific antibody (Fujino et al., 2005). In non-AD

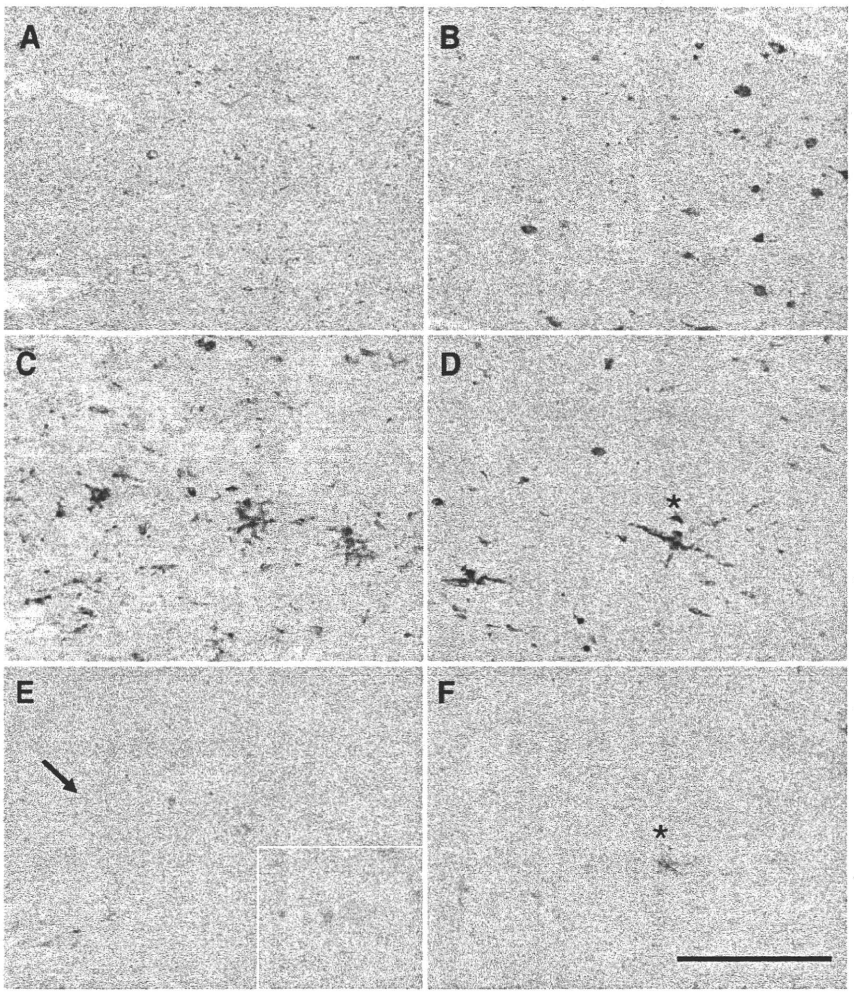

Fig. 1 – Immunohistochemical study of AT8 (A, B), CR3.43 (C, D), and SRA (E, F) on adjacent sections of the frontal cortex of Alzheimer's disease (AD) (A, C, E) and the insular cortex of Pick's disease (PiD) (B, D, F,). The number of MHC class II-positive, activated microglia was much higher than that of SRA-positive microglia in both diseases. When we enlarged the area of AD cortex indicated by the arrow, a small number of SRA-positive, phagocytic microglia were found around senile plaques (E). The asterisks in panel D, F points to highly activated microglia labeled with MHC II and SRA in PiD. All sections were counterstained with hematoxylin. Scale bar = 200 μm.

human tauopathies, abnormal accumulation of tau protein has been shown not only in neurons, but also in glial cells such as astrocytes and oligodendrocytes. The formation of microglial inclusion bodies immunolabeled with phosphorylation-dependent monoclonal antibodies such as AT8 has not been reported in tauopathies, although the expression of tau2 and tau66 in microglia has been reported (Odawara et al., 1995; Ghoshal et al., 2001).

Microglial activation has been reported in a number of AD and transgenic (TG) mouse models of amyloid deposition, including Tg2576 (Frautschy et al., 1998). In AD brains, clusters of activated microglia with MHC class II expression can be found in fibrillary neuritic plaques and to a limited extent in diffuse plaques (Itagaki et al., 1989; Sasaki et al., 1997), and in APP Tg mice, activated microglia are observed around fibrillary Aβ deposits (Sasaki et al., 2002; Morgan et al., 2005). A previous study (Ishizawa and Dickson, 2001) demonstrated the expression of MHC class II molecules in activated microglia in the brains of PSP and CBD. The presence of MHC-positive, activated microglia in the non-AD tauopathies may support the argument that neuroinflammation contributes to pathogenesis of tauopathies. However, it is suggested that neuroinflammation in PSP and CBD is linked to tau pathology in a complicated way, and the pathogenic role of microglia and neuroinflammation may be different in multiple sclerosis, AD or non-AD tauopathies. Increasingly, it is necessary to examine multiple microglial markers from different activation state categories to obtain a more complete picture of the in vivo microglial phenotype. At present, information on microglial activation in non-Alzheimer's tauopathies as well as mutant tau transgenic (TG) mouse models is limited, particularly in the latter condition.

Microglia activation could occur in not only neurodegenerative diseases but also various diseases including local trauma, focal and global ischemia, local and generalized infection, immune-mediated inflammation, and brain tumors. The factors other than tau accumulation that induce

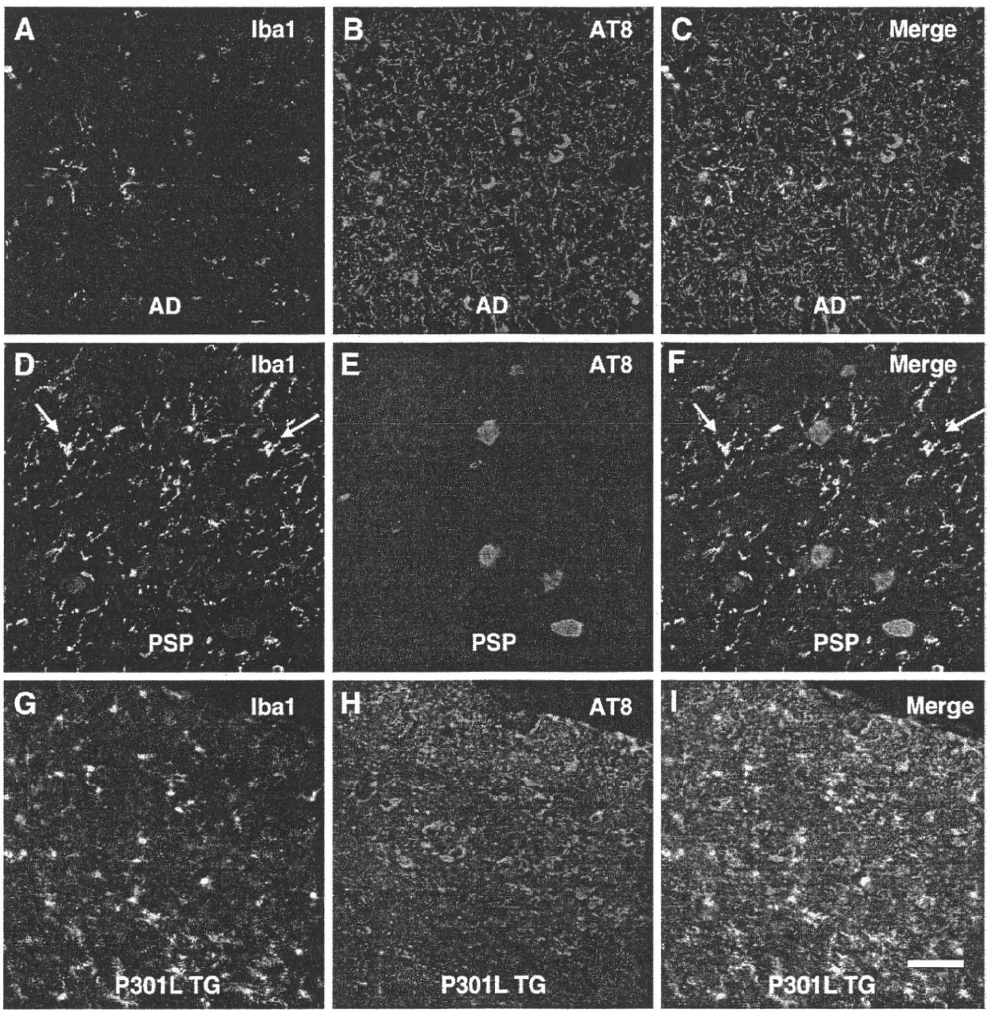

Fig. 2 – Double immunofluorescence of Iba1 and AT8 under a confocal laser microscope in AD (A–C), PSP (D–F), and tau P301L TG mice (G–I). AT8 was absent in microglia, and microglial cluster surrounding AT8-positive cells (a focal accumulation of microglia around AT8-positive structures) was not evident. Arrows (D, F) points to activated microglia in PSP. Scale bar = 50 μm.

microglial activation seem much fewer in the tau TG mice than in human autopsy cases of tauopathies. Therefore, the tau TG mice could be useful to clarify the relationship between phosphorylated tau accumulation and microglial alterations in brain lesions.

In the present study, we investigated human tauopathies and tau TG mice by immunohistochemistry and electron microscopy using anti-tau antibodies including 3R-/4R-specific antibodies and various microglial markers. The aim of this study to investigate: 1) the spatial correlation between microglial activation and tau accumulation; 2) the comparison of tau TG mice to human tauopathies with respect to the microglial shape and the production of microglial neuroinflammatory mediators.

2. Results

2.1. *Human tauopathies*

Iba1- and MHC class II-positive activated microglia increased in regions of phosphorylated tau accumulation in all the cases of human tauopathies. In the frontal cortex of AD (Figs. 1A,C,E) and the insular cortex of PiD (Figs. 1B,D,F), the number of MHC class II-positive, activated microglia was much larger than that of SRA-positive microglia in the adjacent sections stained with AT8, CR3.43, and SRA antibodies. Higher magnification showed a small number of SRA-positive, phagocytic microglia around senile plaques in AD (Fig. 1D, inset). The number of inducible nitric oxide synthase (iNOS)-positive microglial cells was very limited in each tauopathy case (data not shown).

In the frontal cortex of AD (Figs. 2A–C) and the periaqueductal grey matter of PSP (Figs. 2D–F), double immunofluorescence analysis showed different localizations of the Iba1 and AT8, and double-positive cells were not evident. In addition, there was no evidence of microglial clusters around cells bearing AT8-positive structures such as NFTs, pretangles and neuropil threads, or a direct attachment of activated microglia to tau accumulation.

2.2. *P301L tauTG mice*

At 11 months of age, weak tau staining appeared in the neocortex in one of four TG mice. By 17 months of age, tau-positive neuronal and glial structures were almost consistently observed, and memory disturbance developed, although there was phenotypic variation among the tau TG mice between 11 and 27 months. The hippocampus of the TG mice was labeled with AT8, and 4-repeat tau antibodies, ET3 and RD4, but not with 3-repeat antibody, RD3 (data not shown). In the cerebral cortical section of a 17-month-old P301L TG mouse, microglia were increased in cortical areas where AT8-positive, ET3-positive neuronal structures accumulated, while there was no apparent increase of microglial cells labeled with Iba1 antibody or RCA1 lectin in the areas of mild tau deposition (data not shown). There were no significant differences about the relationship between tau deposition and microglial activation with respect to anatomical regions. The increased microglia labeled with the microglial markers (Iba1 and RCA1) showed morphologically mild activation, revealed by the cytoplasmic enlargement, in the TG mice. The data of semiquantitative evaluation of tau and microglial pathology in cerebral cortical areas of 18 P301L TG mice and six controls are shown in Table 2. There was a positive correlation between the expression score of AT8 and Iba1, $r=0.781$ ($p<0.01$).

Double-immunofluorescent confocal laser microscopy showed an increased number of microglia in the cerebral cortex of TG mice with severe tau deposition, and the absence of tau immunoreactivity in microglia (Figs. 2G–I). Concerning the microglial shape and density, microglia with bushy processes increased in the areas, where AT8-positive cells were sparse, and microglial cluster was not evident around AT8-positive cells.

In the cryostat sections of the hippocampal area of 21-month-old tau TG mice, AT8-positive neurons/glia increased, and mildly activated microglia showed immunopositivity for Mac1 and F4/80, but only rarely for scavenger receptor class A (SRA) (Figs. 3A–D). A small number of Ia-positive microglia were observed in the area with severe AT8 deposition (Figs. 3E, F). We

Table 1 – Antibodies and lectin used in this study

Specificity, epitope	Clone name, type	Source	Dilution	AR
Tau, phosphorylated S202/T205	AT8, mouse MA	Innogenetics, Ghent, Belgium	1/1000–1/200	–
Tau, 3-repeat RD3	8E6/C11, mouse MA	Upstate, Lake Placid, NY, USA	1/3000	+
Tau, 4-repeat RD4	1E1/A6, mouse MA	Upstate, Lake Placid, NY, USA	1/200	+
Tau, 4-repeat	ET3, mouse MA	Dr. P. Davies	1/1000	+
Iba1	Rabbit PA	Wako, Osaka, Japan	1/500	+
Human MHC class II, beta-chains of DP, DQ and DR	CR3.43, mouse MA	DAKO, Glostrup, Denmark	1/50	+
Mouse MHC class II (Ia), I-A and I-E	M5/114, rat MA	Behringer-Mannheim, Mannheim, Germany	1/20	–
Human SRA, CD204	SRA-E5	Trans Gene Inc., Kumamoto, Japan	1/50	+
Mouse SRA	2F8, rat MA	Dr. S. Gordon	1/250	–
Human iNOS	Rabbit PA	CHEMICON, Temecula, CA, USA	1/2000	+
Rat and mouse iNOS	Rabbit PA	CHEMICON, Temecula, CA, USA	1/5000	–
CR3, CD11b and CD18	Mac-1, rat MA	Behringer-Mannheim, Mannheim, Germany	1/20	–
Biotinylated RCA1	β-d-Galactose	Vector, Burlingame, CA, USA	1/200	+

Iba1, ionized calcium binding adaptor molecule 1; MHC, major histocompatibility complex; SRA, scavenger receptor class A; iNOS: inducible nitric oxide synthase; CR, complement receptor; RCA1, *Ricinus communis agglutinin* 1; AR: antigen retrieval; (+): autoclaving (121 °C, 10 min, citrate buffer).

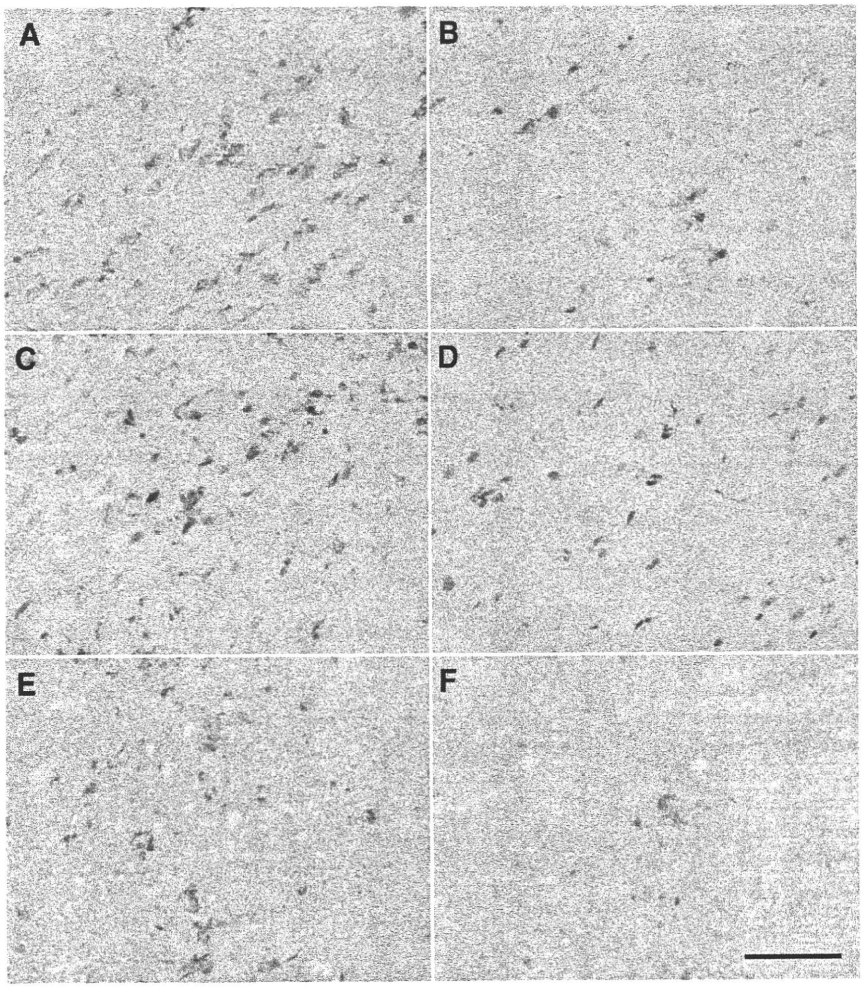

Fig. 3 – Immunohistochemistry in the hippocampus of 21-month-old tau TG mice. Microglia with mild enlargement of their cytoplasm, labeled with Mac1 (B) and F4/80 (C) but rarely labeled with SRA (D), were observed in the subiculum with severe AT8 deposition (A). The pyramidal cell layer with numerous AT8-positive cells (E) had a small number of Ia-positive microglia (F). All the sections were counterstained with hematoxylin. Scale bar = 50 μm.

could not find iNOS reactivity in the transgenic mice (data not shown).

In the ultrastructural analysis of the tau TG mice, we could see microglial cells, which had a small nucleus with dense peripheral chromatin and significantly enlarged cytoplasm containing increased rough endoplasmic reticulum and mitochondria, near neuron containing NFT-like structures (Fig. 4A). Immunoelectron microscopy using Iba1 antibody in P301L TG mice revealed that the cytoplasm of Iba1-immunopositive microglia was filled with lysosomal dense bodies (Fig. 4B). Abnormal filamentous or tubular structures corresponding to NFTs were not found in microglia.

3. Discussion

The present study showed that P301L tau TG mice displayed microglial activation in the grey matter associated with phosphorylated tau deposition similar to those observed in human tauopathies. These include the increase of Iba1-positive microglia and the appearance of microglia expressing MHC class II antigen. Our semiquantitative analysis in the TG mice also demonstrated that the number of Iba1-positive microglia was closely associated with the accumulation of phosphorylated tau. Thus, our findings indicate that microglial activation may be involved in the progression of tauopathies.

This study illustrated the presence of activated microglia expressing Iba1, Mac1, F4/80, RCA-1 lectin and MHC class II protein (Ia antigen) in brain lesions rich in tau phosphorylation in P301L TG mice, which were generated by us as tau mutant (P301L) transgenic mice expressing an FTDP-17 mutation within the longest form of tau (2N, 4R) (Murakami et al., 2006). This is the first study to demonstrate multiple microglial markers in tau TG mice. By 17 months of age, tau-positive neuronal and glial structures were almost consistently observed, and memory disturbance developed. However, there is a general feature of phenotypic variation among our transgenic animal lines, as observed in this study. In other P301L TG mice, progressive white matter pathology (Lin et al.,

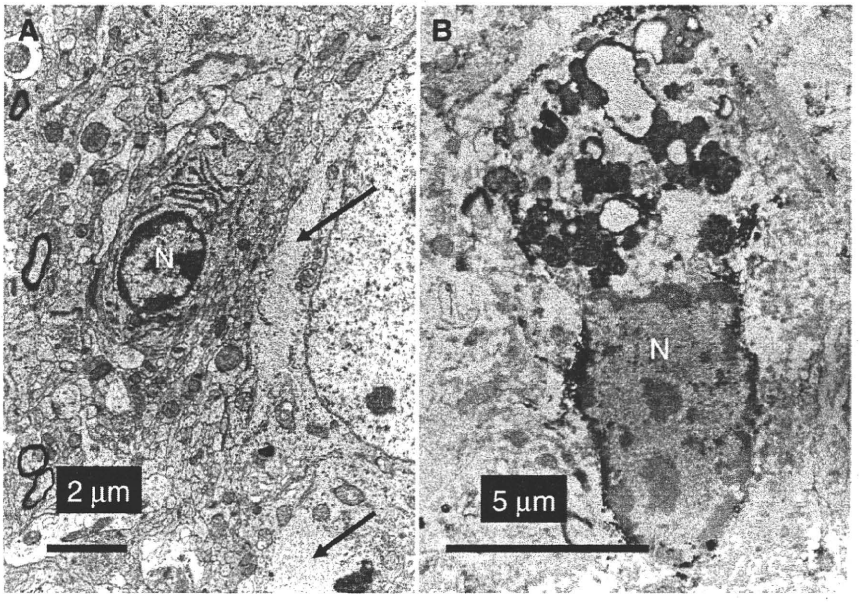

Fig. 4 – Ultrastructurally, microglial cells showed activation with slightly enlarged cytoplasm and lysosomal, dense bodies in the hippocampus of P301L mice (21-month-old). (A) Microglia with significantly enlarged cytoplasm containing increased rough endoplasmic reticulum and mitochondria were found near neurons containing abnormal, tubular structures (arrows). (B) Iba1 immunostaining revealed labeling of microglial cells filled with lysosomal dense bodies. No abnormal filamentous or tubular structures were observed in microglia. N: Nuclei of microglia.

2005), gene expression profiles (Ho et al., 2001) and apoptosis (Zhao et al., 2003) have been examined. Microgliosis has been reported in some lines of TG mice expressing mutant tau, such as P301S (Bellucci et al., 2004) and R406W (Ikeda et al., 2005); however, the microglial pathology in TgTau^{P301L} mice had not been investigated in detail in previous studies.

Our results of human tauopathies confirmed the findings of the previous reports that MHC class II-positive, activated microglia were present in some areas with tau burden in AD and non-AD tauopathies (Paulus et al., 1993; Kobayashi et al., 1999; Arnold et al., 2000; Ishizawa and Dickson, 2001). Regarding MHC class II protein expression, the expression in our tau TG mice seemed less marked than that in non-AD tauopathies. This might be related to the difference of the species and TG mouse model, since MHC class II expression is much less easily induced on microglia in the mouse (Perry, 1998) and microglia in TG mice overexpressing beta amyloid protein are less highly activated than those in human AD (Schwab et al., 2004). Another possibility is that microglial MHC class II expression is in part attributable to factors such as hypoxia or infection prior to patient death.

The present study demonstrated the different expression of SRA on microglia in AD, non-AD tauopathies, and P301L TG mice. A small cluster of phagocytic microglia expressing SRA could be observed around the senile plaques in AD, while cluster formation of phagocytic microglia was not evident in PiD or PSP. Bornemann et al. (2001) demonstrated increased immunoreactivity for SRA on the borders of beta amyloid deposits in the TG mice overexpressing the amyloid precursor protein. Thus, increased expression of SRA on microglia might be due to extracellular deposits of beta amyloid in AD. So far, SRA expression of microglia in non-AD tauopathies and tau TG mice is unclear. The expression of SRA is not found in resting microglia, but in activated microglia, preferentially in phagocytic microglia. This phenomenon may be attributable to our observation that SRA was expressed in highly activated microglia in PiD. Alternatively, the expression of SRA on microglia was very rare in P301L TG mice in this study. There may be a difference between non-AD tauopathies and tau TG mice with respect to microglial SRA expression. In addition, morphological study made in this study indicated that the presence of phagocytic microglia (brain macrophages) was not evident in P301L TG mice. However, those findings do not rule out phagocytic activity of microglia in the TG mice model, since SRA is not the only marker for a microglial phagocytic phenotype (e.g., expression of Fc receptors is also generally considered to suggest phagocytic capability) and our electron microscopic evaluation of P301L TG mice identified numerous secondary lysosomes in microglia.

In the present study, there was no evidence of microglial clusters or attachment around neurons containing pretangles, NFTs, Pick bodies or neuropil threads in both tauopathies and the TG mice by immunoperoxidase and double immunofluorescence analyses using antibodies, such as AT8, Iba1, MHC class II, and SRA. The trigger for microglial activation in tauopathies still remains unclear. *In vitro* studies have investigated the effect of microglial stimulators, including lipopolysaccharide, interferon-gamma (IFN-gamma) and colony stimulating factors, and IFN-gamma has been shown to be a particularly potent microglial stimulant (Sasaki et al., 1989). However, no evidence of IFN-gamma stimulation on microglial activation has been reported in vivo, except in a study of multiple sclerosis

(Takeuchi et al., 2006). *In vitro* studies have shown that microglia are activated by beta amyloid (Lue et al., 2001), prion protein (Brown et al., 1996), and alpha-synuclein (Zhang et al., 2005); however, the effect of tau in microglial activation in culture systems is not yet known. In vivo the deposition of phosphorylated tau is intracellular except ghost tangles. Thus, the activation of microglia in tauopathies might result from a mediator such as ATP or cytokines/chemokines produced locally, not from direct interaction with phosphorylated tau.

The present study indicated that phosphorylated tau epitope (AT8) was absent in microglia, as shown by double-immunofluorescent confocal microscopy using Iba1 antibody, which is the most sensitive marker for microglia. Microglia labeled with monoclonal antibodies against conformational epitope of tau, tau2 and tau66, has been noted in AD, PSP, CBD, and in Lewy body disease (Odawara et al., 1995; Ghoshal et al., 2001). The expression of tau2 and tau66 in microglia does not seem to be disease-specific, since tau2-reactive microglia has been observed around ischemic foci of cerebral infarction and in inflammatory lesions of different pathologies (Uchihara et al., 2000, 2005). In addition, microglial cells are not labeled with Tau1, Alz50, or AD2, all of which reveal astrocytic and oligodendroglial pathology. No AT8-positive microglia were observed in PiD in sections subsequently stained with HLA-DR antibody (Schofield et al., 2003). The lack of AT8 in microglia might be due to the difference of cell origin and/or a paucity of molecules associated with microtubules. However, it is conceivable that the absence of phosphorylated tau or NFTs in microglia may not be indicative of functional normal microglia, since microglia become dysfunctional with aging and the deterioration of microglia is aggravated by genetic and environmental risk factors.

Importantly, what role activated microglia play in the pathogenesis and disease process of tauopathies should be addressed. The bystander damage hypothesis claiming microglial autotoxicity leaves unanswered issues in chronic CNS neurodegenerative diseases. However, microglia may play an important role in amplification of neuronal injury in tauopathies, and microglial dysfunction, "diseased microglial cells", may contribute to the slow degeneration of neurons and astrocytes. It is of particular significance that in the CNS, iNOS expression has been shown to provoke neuronal cell death. This study indicated that the expression of iNOS in activated microglia is not significant in tauopathies and P301L TG mice, in agreement with the results of PSP (Komori et al., 1998). Our immunocytochemical and electron microscopical results that activated microglia were present at the vicinity of tau-positive neurons suggest that activated microglia might be involved in microglial–neuronal interactions at the proximal neurites and synapses of the affected cells. An understanding of the role of microglia in synaptic signaling is still elusive, but microglia may play an important role like synaptic stripping (Trapp et al., 2007), N-methyl-D-aspartate (NMDA) receptor-mediated synaptic responses (Moriguchi et al., 2003) and releasing extracellular signaling molecules. Microglia may produce not only a direct toxic or trophic effect to neuron, but also an indirect one through the effect of astrocytic potential function in synapse. Recently, Yoshiyama et al. (2007) have shown that synapse loss, impaired synaptic function and microglial activation precede tangle formation in P301S TG mice, where tau pathology accelerated compared to P301L TG. In fact, it is the loss of synaptic contact that leads directly to the personal devastation, i.e. dementia (Terry, 2000). Thus, microglial activation might be early manifestation of neurodegeneration or neuroinflammation, and might be involved in dysfunction of synaptic signaling in tauopathies. Our P301L TG mice were reported to demonstrate memory disturbance at 11–12 months of age (Murakami et al., 2006). This study using immunohistochemistry and Iba1 antibody could not demonstrate that microglial response precedes the appearance of AT8 immunoreactivity or memory disturbance. To address the question of when microglial activation occurs, a further study of microglial pathology using morphologic and morphometric techniques in many P301L TG mice of young age groups should be performed.

In neurodegenerative diseases, different "activation states" of microglia exist in which microglia may selectively upregulate neuroprotective and/or neurotoxic molecules and depend on the activation environment, including the cell type and signal molecules. Yoshiyama et al. (2007) reported that activated microglia may be neurotoxic because immunosuppression with FK506 attenuated tau pathology and increased lifespan. Microglial production of trophic molecules, such as glia-derived neurotrophic factor and brain-derived neurotrophic factor, and neurotoxic molecules, such as NO and proinflammatory cytokines, as well as neuronal survival should be investigated quantitatively in tau TG mice, including FK-506-treated tau TG mice. Combined with our previous study (Murakami et al., 2006), this study suggests that microglial production of "trophic effectors" may be not enough to rescue injured neurons at the advanced stage of tauopathies.

In conclusion, microglial activation may be closely related to phosphorylated tau accumulation and a direct contribution of intracellular tau accumulation to the activation of neighboring microglia seems unlikely in human tauopathies and tau TG mice. The presence of activated microglia in the vicinity of tau-bearing neurons suggests that microglia might be involved in microglia–neuronal interaction at the proximal neurites or synapse. The present study serves to validate P301L TG mouse models of tauopathies with respect to microglial pathology. Based on the immunohistochemistry for Iba1, MHC class II and SRA, however, it appears that microglia of the TG mice have weak inflammatory response and low phagocytic activity compared with those of human tauopathies.

4. Experimental procedures

4.1. *Human tauopathies*

Autopsy brains from three AD patients (66, 71, 83 years old at death; two males and one female), two PSP patients (63 and 77 years old, two males) and one PiD patient (a 73-year-old male) were examined according to the ethical rules of Gunma University and the Japanese Society of Pathology. All brains were fixed in 10% buffered formalin, and embedded in

paraffin. For light microscopy and immunohistochemistry, consecutive 5-μm-thick sections, each containing one of the following regions, were studied: cerebral cortex and its subcortical white matter; hippocampus; basal ganglia and insular cortex; mid brain.

4.2. *TG mice expressing P301L human tau*

The TG mice for human mutant tau, P301L tau (proline to leucine mutation at the amino acid number 301 in exon 10), used in this study have been described previously (Murakami et al., 2006). We studied a total of 24 P301L TG mice (eight males and 16 females) between 11 months and 27 months of age. Six non-transgenic littermates (11–27 months old; two male and four females) served as controls. The animals were sacrificed and the brains were immediately excised and processed according to the following procedure. For histological and immunohistochemical analyses, all mouse brains were fixed in 0.1 M phosphate buffer (PB, pH 7.6) containing 4% paraformaldehyde (PFA), and embedded in paraffin. Then, consecutive sections were cut at 5-μm-thick. Some mouse brains were fixed with periodate–lysine–PFA (PLP) fixative at 4 °C for 4–6 h, washed with PBS and embedded in OCT compound (Miles, Elkhart, USA). The samples were cut into 6–8-μm-thick sections using a cryostat (Leica).

4.3. *Immunocytochemical methods*

Immunohistochemistry was performed using the biotin–streptavidin (B–SA) immunoperoxidase method (Nichirei, Tokyo, Japan). For staining of paraffin sections with some antibodies, antigen retrieval was performed by autoclaving (121 °C, 10 min, citrate buffer). The sections were incubated with each of the primary antibodies or biotinylated RCA-1 listed in Table 1 overnight at 4 °C. All sections, except those stained with RCA-1, were incubated with a secondary antibody at room temperature (RT) for 30 min, and then reacted with peroxidase-labeled streptavidin at RT for 30 min. The immunoreaction was visualized with diaminobenzidine (DAB) and briefly counterstained with hematoxylin.

Immunohistochemical analyses for rat monoclonal antibodies against Mac-1, F4/80, Ia antigen, and SRA were performed only on cryostat-cut tissue sections. After incubation with the rat monoclonal antibodies, biotinylated goat anti-rat Ig (diluted 1:20, Tago, Inc., Camerillo, Calif) was used as a secondary antibody, followed by streptavidin–peroxidase complex (Nichirei).

For double immunofluorescence analyses, sections were incubated overnight at 4 °C in a cocktail of the Iba1 polyclonal antibody and the mouse AT8 antibody. After the incubation, a combination of secondary antibodies, Alexa 488-tagged goat anti-rabbit IgG (1:200; Molecular Probe, USA) and Alexa 594-tagged goat anti-mouse IgG (1:200; Molecular Probe) were applied for 30 min at room temperature. Prior to immunolabeling, auto-fluorescence of the lipofuscin granules was blocked with Sudan black B treatment. The sections were examined with a BioRad MRC-1024 confocal microscopic system. As a control, parallel sections treated under identical conditions except for omission of each primary antibody were used.

Table 2 – Semiquantitative evaluation of AT8 and Iba1 immunoreactivity in cerebral cortical areas of P301L TG and non-TG mice

No	Mice	Age (months)	Sex	AT8	Iba1
1	Non-TG	11	M	0	1
2	Non-TG	23	M	0	1
3	Non-TG	24	F	0	1
4	Non-TG	25	F	0	1
5	Non-TG	25	F	0	1
6	Non-TG	27	F	0	1
7	P301L TG	11	F	0	1
8	P301L TG	11	F	0	1
9	P301L TG	11	M	0	1
10	P301L TG	11	M	1	1
11	P301L TG	18	F	1	1
12	P301L TG	18	M	1	1
13	P301L TG	19	F	1	1
14	P301L TG	24	M	1	1
15	P301L TG	24	M	1	1
16	P301L TG	26	F	1	1
17	P301L TG	27	F	1	1
18	P301L TG	21	F	2	1
19	P301L TG	19	F	2	2
20	P301L TG	18	F	3	2
21	P301L TG	17	M	3	3
22	P301L TG	18	F	3	3
23	P301L TG	21	F	3	3
24	P301L TG	25	F	3	3

AT8: 0, absent; 1, mild; 2, moderate; 3, severe.
Iba1: 1, <10 positive nuclei; 2, 10–20 positive nuclei; 3, >20 positive nuclei at ×400 magnification.

4.4. *Ultrastructural analysis*

For the transmission electron microscopy (EM) and the pre-embedding method of immunoelectron microscopy, cerebral cortical tissues of a 21-month-old P301L mouse were processed as described previously (Sasaki et al., 2002). In the transmission EM analysis, ultrathin sections were cut, and then stained with uranyl acetate and lead acetate prior to EM observation. After immunostaining of the immunoelectron microscopy, the ultrathin sections were cut, and were examined using EM with/without staining with uranyl acetate.

4.5. *Semiquantitative analysis of immunolabeling*

Cerebral cortical areas of 14 P301L TG and five control mice were selected for semiquantitative evaluation of tau and microglial pathology. The score of AT8 deposits was assigned as follows: 0 = absent, 1 = mild, 2 = moderate and 3 = severe. For microglial pathology, the microscopic field with the highest density of Iba1-positive microglia was identified and the number of microglia was counted at ×400 magnification. The score was determined as follows: 1=<10 cells, 2=10–20 cells, 3=>20 cells. In this assessment, each microglia had to have an immunopositive nucleus to be counted. Spearman correlation coefficients using a software (SPSS11.5 soft package for Windows) were used to evaluate whether the AT8 immunoreactivity was correlated with the Iba1 immunoreactivity.

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Research Report

Plasma antibodies to Aβ40 and Aβ42 in patients with Alzheimer's disease and normal controls

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Plasma

ABSTRACT

Antibodies to amyloid β protein (Aβ) are present naturally or after Aβ vaccine therapy in human plasma. To clarify their clinical role, we examined plasma samples from 113 patients with Alzheimer's disease (AD) and 205 normal controls using the tissue amyloid plaque immunoreactivity (TAPIR) assay. A high positive rate of TAPIR was revealed in AD (45.1%) and age-matched controls (41.2%), however, no significance was observed. No significant difference was observed in the MMS score or disease duration between TAPIR-positive and negative samples. TAPIR-positive plasma reacted with the Aβ40 monomer and dimer, and the Aβ42 monomer weakly, but not with the Aβ42 dimer. TAPIR was even detected in samples from young normal subjects and young Tg2576 transgenic mice. Although the Aβ40 level and Aβ40/42 ratio increased, and Aβ42 was significantly decreased in plasma from AD groups when compared to controls, no significant correlations were revealed between plasma Aβ levels and TAPIR grading. Thus an immune response to Aβ40 and immune tolerance to Aβ42 occurred naturally in humans without a close relationship to the Aβ burden in the brain. Clarification of the mechanism of the immune response to Aβ42 is necessary for realization of an immunotherapy for AD.

1. Introduction

AD brains are invariably characterized by two pathological features: initial Aβ amyloidosis characterized by extracellular deposition of Aβ42 (43) and Aβ40, and subsequent tauopathy characterized by intracellular accumulation of neurofibrillary tangles consisting of abnormally phosphorylated tau. Since familial AD-linked mutations of amyloid β protein precursor

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(βAPP), presenilin-1 (PS-1), and presenilin-2 (PS-2) increase the extracellular concentration of Aβ42 (43) and protofibril Aβ, these peptides are likely to be initiating factors in the pathogenesis of all types of AD (Hardy and Selkoe, 2002, Selkoe, 2002). Transgenic mouse models reproducing substantial brain Aβ amyloid support these hypotheses, and the appearance of neurofibrillary tangles (NFT) enhanced by Aβ amyloid in Tg2576 x tau P301L double transgenic mice further indicates that Aβ amyloidosis is the most important target for curing AD (Lewis et al., 2001).

Recent studies suggested that Aβ immunotherapy is the most promising among the many candidate therapies for AD. Schenk and others showed that an Aβ42 peptide vaccine clearly reduced the Aβ amyloid burden in transgenic model mice (Schenk et al., 1999; Weiner et al., 2000; Janus et al., 2000; Morgan et al., 2000). Passive immunization using anti-Aβ antibodies was also shown to be effective for reduction of the Aβ amyloid burden (Bard et al., 2000). These findings suggest peripheral antibodies to Aβ may serve a protective role against AD. A detectable increase in antibodies to Aβ42 was observed in about 25% of patients who received AN1792 in a Phase I study (Orgogozo et al., 2003; Nicoll et al., 2003). Analysis of serum samples by ELISA indicated that 15 of 18 patients experiencing meningoencephalitis in a Phase II study had antibodies against Aβ42. CSF antibodies to Aβ42 were present in 6 of 8 patients tested after the onset of encephalitis. However, titers of antibodies to Aβ42 were not correlated with the occurrence or severity of symptoms or relapses (Orgogozo et al., 2003). An autoantibody to Aβ40 was first detected in human B cell lines from AD patients (Gaskin et al., 1993). Naturally occurring antibodies to synthetic Aβ40 were confirmed by ELISA in the CSF and plasma of non-immunized humans and titers were significantly higher in healthy controls than in patients with AD (Du et al., 2001). Titers of anti-Aβ42 peptide antibodies were lower in AD patients compared with healthy individuals (Weksler et al., 2002), or elevated in AD patients and elder transgenic mice (Nath et al., 2003). Naturally occurring anti-Aβ42 antibodies were detected at very low levels by ELISA in over 50% of elderly individuals and at modest levels in 5% of them. Neither the presence nor the amount of naturally occurring anti-Aβ42 antibodies correlated with the presence, or age of AD onset, or the plasma levels of Aβ40 and Aβ42 (Hyman et al., 2001). Normal levels of antibodies to Aβ42 and Aβ40 were present in both AD and control groups, even in a young population (Baril et al., 2004). Thus, the previous reports suggested complex relationships for naturally occurring antibodies to Aβ.

In the Zurich cohort of a Phase II study, patients who generated antibodies to β-amyloid plaques in the plasma as determined by tissue amyloid plaque immunoreactivity (TAPIR) assay showed significantly slower rates of decline for cognitive functions and daily living activities suggesting that antibodies against β-amyloid plaques may be protective against AD (Hock et al., 2002, 2003; Gilman et al., 2005; Bombois

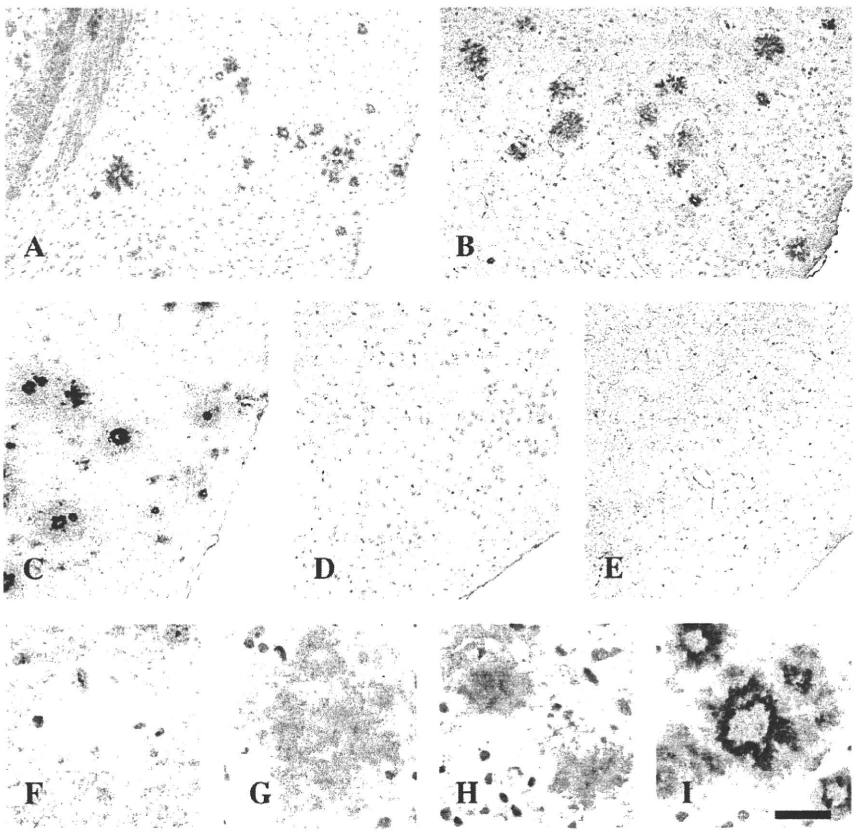

Fig. 1 – TAPIR using plasma and Tg2576 mouse brains. Many senile plaques in Tg2576 brains were labeled prominently in A (AD group, TAPIR grading ++) and B (aNC group, TAPIR grading ++). C: control Aβ immunostaining with Ab9204; no senile plaques were labeled in D (AD group, TAPIR grading –) and E (aNC group TAPIR grading –). F to I are examples of TAPIR grade. F: TAPIR –; G:±; H: +; I: ++. Scale bar=6.25 μm in A–E and 25 μm in F–I.

et al., 2007). Here, we examined 113 AD cases and 155 age-matched normal controls by TAPIR assay in order to clarify the positive rates, antibody characters, correlations with clinical symptoms, and clinical roles of naturally occurring antibodies against β-amyloid plaques. Modification of plasma Aβ40 and Aβ42 concentrations by antibodies to Aβ was also studied based on age- or AD-dependent alterations of plasma Aβ levels.

2. Results

2.1. *High positive TAPIR rate but no difference between AD and aNC groups*

Some plasma samples from AD and aNC groups strongly labeled nearly all amyloid plaque cores (Fig. 1A, B and I; grading ++) as did Ab9201 (Fig. 1C). Other plasma samples from both groups showed negative (Fig. 1D, E, F, grading –), weak (Fig. 1G, grading ±), or positive (Fig. 1H, grading +) labeling. The TAPIR staining was detected by anti-IgG second antibody, but not with anti-IgM or IgA antibodies (not shown), thus TAPIR-positive antibody was shown to be IgG antibody. The specificity of TAPIR-positive antibody was examined by immunoprecipitation of Aβ as described in 2.3. In the AD group, 42 cases (37.2%) were TAPIR –, 20 (17.8%) were ±, 44 (38.9%) were grading +, and 7 (6.2%) were ++. Fifty one of 113 AD patients were ++ and +, suggesting frequent appearance (45.1%) of naturally occurring antibodies to amyloid plaque cores. In the aNC group, 54 cases (34.8%) were TAPIR –, 37 (23.9%) were ±, 44 (28.4%) were +, and 20 (12.9%) were ++. Sixty-four cases of 155 aNC group (41.3%) were TAPIR ++ or +. No significant differences were detected by Mann–Whitney's *U* tests in the positive rates of naturally occurring antibodies to amyloid plaque cores among groups ($p=0.77$), or comparisons between the positive AD group (++ and +), negative AD group (± and –), positive aNC group (++ and +) and negative aNC (± and –) group ($p=0.54$) (Table 1).

2.2. *TAPIR was not correlated with clinical symptoms*

There were no significant differences in gender or mean age in both AD and aNC groups (Table 1). No significant differences were observed in MMS scores and disease duration among the TAPIR –, ±, +, ++ subgroups of AD samples (Table 1 and Fig. 2A, B). There were also no significant differences in the progressive decline of MMS scores among these AD subgroups (Fig. 2C). The presence of naturally occurring antibodies to Aβ as detected by TAPIR may therefore not improve prognosis of AD.

2.3. *TAPIR-positive plasma recognized Aβ40 and FAβ, but Aβ42 very weakly*

As indicated in Fig. 3, freshly prepared Aβ40 and Aβ42 were composed of monomers and dimers. However, formic acid extractable Aβ (FAβ) exhibited polymerization as shown by the higher molecular mass of its oligomers (Fig. 3, left panel). Immunoprecipitation with TAPIR ++/+ plasma obtained from the AD and aNC groups retrieved Aβ40 monomers and dimers as well as higher molecular mass polymers. Immunodetection of monomeric Aβ42 using 6E10 was very weak, whereas no dimeric form of Aβ42 was detected (Fig. 3 right panels). These findings suggest that TAPIR-positive plasma reacts with Aβ, but its reactivity to Aβ42 is very weak.

2.4. *Antibodies to Aβ appeared before Aβ amyloid deposits in the brain*

In order to clarify when these antibodies against Aβ appear, we additionally examined the remaining 50 plasma samples from subjects younger than 43 yeas old in the tNC group. Surprisingly, TAPIR revealed that antibodies to Aβ appeared in a 2 year-old child and also in some young subjects (TAPIR +; Fig. 4A, B and C). TAPIR-positive rates were 57% by 10 years old ($n=7$; 4 TAPIR +), 64% by 20 years old ($n=11$; 6 TAPIR +), 20% by 30 years old ($n=10$; 2 TAPIR +) and 10% by 40 years old ($n=10$; 1 TAPIR +). To confirm further this early appearance of antibodies to Aβ, immunoprecipitation was performed. Essentially identical finding to those seen in the AD and aNC groups were revealed (Fig. 4 D–F). Aβ40 and FAβ monomers and dimers were strongly immunoprecipitated (arrows). However, immunoprecipitation of the Aβ42 monomer was also weak and the Aβ42 dimer was absent in TAPIR-positive plasma from younger controls.

This was also the case in plasma obtained from Tg2576 model mice. Plasma from younger and older Tg2576 mice labeled

Table 1 – Summary of tissue amyloid plaque immunoreactivity (TAPIR) in AD and control groups

	Grade	Cases	rate (%)	Gender (M/F)	Mean age (SD), yr	Mean MMSE (SD)	Mean duration (SD), mo
AD ($n=113$)	–	42	37.2	11/31	75.4 (7.2)	14.7 (7.2)	50.9 (34.4)
	±	20	17.8	5/15	75.0 (8.0)	15.4 (7.3)	39.5 (27.4)
	+	44	38.9	14/30	74.5 (8.2)	14.9 (6.3)	37.5 (24.7)
	++	7	6.2	3/4	77.3 (5.3)	14.7 (6.2)	47.7 (19.7)
aNC ($n=155$)	–	54	34.8	21/33	74.7 (9.5)	29.9 (0.3)	–
	±	37	23.9	16/21	76.0 (8.7)	29.6 (0.5)	–
	+	44	28.4	19/25	77.9 (8.0)	29.7 (0.4)	–
	++	20	12.9	3/17	74.2 (11.8)	29.9 (0.3)	–

In the AD group, 42 cases (37.2%) were TAPIR –, 20 (17.8%) were ±, 44 (38.9%) were +, and 7 (6.2%) were ++; 51 of 113 AD patients were ++ and +, suggesting high rate of TAPIR (45.1%). In the aNC group, 54 cases (34.8%) were TAPIR –, 37 (23.9%) were ±, 44 (28.4%) were +, and 20 (12.9%) were ++; 64 of 155 aNC controls (41.3%) were TAPIR-positive. No significant differences were found in the positive TAPIR rate within each group ($p=0.77$), or in comparisons between the positive AD group (++ and +), negative AD group (± and –), positive aNC group (++ and +), and negative aNC (± and –) group ($p=0.54$). There were no significant differences in gender, mean age, mean MMS score or mean disease duration according to TAPIR grade in both AD and aNC groups. yr: years old; mo: months.