THE INTERNATIONAL JOURNAL OF SCIENCE & TECHNOLEDGE

Enhanced Cleavage of Amyloid β Peptides in the Case in Injected Inflammatory Brain of Mice

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Abstract:
The excessive accumulation of Amyloid ß-Peptides (Aßs) in the brain is the causative factor in all genetic as well as sporadic cases of Alzheimer's disease (AD). Two enzymes namely beta-Secretase and gamma-Secretase are involved in the defective cleavage of Amyloid Precursor Protein (APP) and the alpha-Secretase is involved in the normal processing. The core Protein of the Amyloid senile plaques within the brains of afflicted individuals contains Peptide of 39-43 residues, but mostly terminating with residues 40 and 42. The longer $Aß_{42}$ is more abundant in the Amyloid of the neuritic plaques while the shorter Peptides ($Aß_{40}$) found more in the vascular deposits. Factors that lead to the over expression of Aßs are yet to be identified. In the present study, we have shown that the prolonged subcutaneous injection of casein, confirmed the presence of a fibrillary Protein deposits with Amyloid characteristics in chronic Inflammation and associated Systemic Amyloidosis, triggers about 20 times more Abeta accumulation in the mice Brain than that of the control mice and confirmed as well as quantified by RP-HPLC. From the Mass Spectroscopic analysis, we have shown the occurrence of a new type of proteolytic zeta cleavage fragment, a 1-54 residue Abeta in the mice Brain along with the Abeta Peptides.

1. Introduction

In brains of AD subjects, previous investigators (Scheibel et al., 1987) have implicated impairment of the BBB (Blood Brain Barrier) in AD (Hardy et al., 1986). However, recent immunohistochemical studies not only show evidence for BBB impairment in AD, but some aging controls were equally affected. Despite these findings, there appear to be abnormalities in some BBB proteins specific to AD, particularly in cases with marked cerebral amyloid angiopathy (Kalaria and Harik 1989).

Aßs accumulating evidence (McGeer et al., 1989) indicates that a number of proteins associated with the acute-phase response are localized in cerebral Aß –protein deposits of AD. While some appear to be produced *in-situ* in brain, the significance and function of these proteins remain largely obscure. The neocortical amyloid deposits comprise predominantly two type namely neuritic or senile plaques and diffuse or amorphous plaques (Wisniewski et al., 1989). Among these, the amorphous plaques are not revealed by the conventional amyloid stains (Abraham et al. 1988) such as Congo red or thioflavin S. The cerebellar cortex aquires Aß-deposits, but it is intriguing that they are largely restricted to diffuse amyloid plaques.

Multiple alternative sites of cleavage of APP by alpha-secretase generate a variety of Aß peptides, of which Aß40 and Aß42 are the most studied. The FAD mutations in APP, PS1 and PS2 elevate the production of Aß42, normally a minor Aß peptide species, while having variable effects on production of Aß40 (Suzuki et al., 1994). Aß42 is less soluble and has increased aggregative properties, relative to Aß40, which are thought to promote Aß plaque formation (Burdick et al., 1992). The consistency of this observation within cell culture, mouse models and human clinical investigations has led to the amyloid hypothesis and the primary etiological account of Alzheimer's disease (AD) pathology (Hardy and Selkoe 2002).

Aß42 is one of several Aß peptides, all of which are derived by regulated proteolytic Processing of the amyloid precursor protein (APP) (Gandy, 1999). The long, hyperaggregatable Aß42 peptide is apparently essential for initiating the formation of senile plaques (Lemere et al., 1996), an invariant feature of Alzheimer's disease. There is evidence that both the secretor and the endocytic (Perez et al., 1996) pathways contribute to the production of Aß peptides and that intracellular and released Aß peptides might be generated via different mechanisms. However, the relative contributions of each pathway giving raise to overall Aß42 production and the relative pathogenic importance of intracellular and extracellular Aß remain to be resolved (Hartmann et al., 1997).

Present study was designed to investigate the effect of beta amyloid in *in-vivo* model of systemic amyloidosis. Specifically, the effect of pre-injecting milk casein by subcutaneously weekly five days upto 66 days, which is chronic inflammatory condition, In addition, we have hypothesized that additional APP cleavage fragment Aß54, the first time namely zeta-secretase.

2. Materials and Methods

All experiments were carried out in accordance with the guidelines of the Animal Care and Animal Ethics committee of Central Leather Research Institute.

2.1. Production of Systemic Amyloidosis

Colony-bred adult male Swiss White mice (30-35 g) were used. Animals were selected randomly and caged in groups of five at room temperature (25-35°C) and supplied with commercial pelleted animal feed (marketed by M/s. Hindustan Lever, Bombay, India under the name "Gold Mohur rat feed") and water was provided *ad libitum*. Mice (control n=5, test n=5) were given 0.5 ml of 10% Vitamin free, Casein (1CN Pharmaceuticals, Cleveland, OH, USA) as subcutaneous injection for 66 days (5 days a week) to induce systemic amyloidosis. Control animals were treated with Saline for the same period. Animals were decapitated without anesthesia (group n=5). The systemic amyloidosis is confirmed by ladder formation in the liver. Details of these experiments are given elsewhere (Botto et al., 1997). Immediately after mice were decapitated, brain cerebral cortex was removed on an ice-chilled plate, weighed and stored at -70°C.

2.2. Immunofluoresence Expression of in Mouse Brain Sections

Brain tissue sections were incubated with anti ßAP25-35 for 30 min at 4°C. After blocking the nonspecific binding with 2% BSA solution and washing in PBS, the sections were incubated with FITC conjugated rabbit anti-mouse antibod y (Sigma, USA) for 30 min. at 4°C. Fluorescence pictures were observed using Zeiss fluorescence microscopy (AXIO plan 2, Carl ZEISS NAG f. HBO50, Germany). The FITC conjugated rabbit anti mouse antibo dy was used at a 1:1000 dilution in PBS (pH-7.4) solution.

2.3. RP-HPLC Analysis of Brain Extract

The above SDS insoluble pellet extract containing Aβ peptides were dissolved in 70% formic acid and separated using acidic RP-HPLC buffer systems on a waters system, equipped with an Shimadzu SPD 10A UV-Visible detector (Shimadzu, Kyoto, Japan). Separation were performed using Spherisorb-ODS2 25 cm × 4.6 mm, 5 µm, C18 (Waters, Milford, Massachusetts, USA) silica column. The buffers used were 0.1% trifluoroacetic acid (TFA) in water (buffer A) and 0.1% TFA in acetonitrile (ACN) (buffer B). Solvents were filtered through 0.45 µm nylon filter
(Sartorius, AG, Gottingen, Germany). Samples were eluted with a linear gradient of buffer B at a flow rate at 1 ml/min while monitoring UV absorbance at 220 nm.

2.4. MALDI-Tof Analysis of Aβ Peptides

RP-HPLC fractions were lyophilized and analyzed by using MALDI-TOF (KRATOS, Manchester, UK) mass spectrophotometer. Scanning was performed from mass/charge (m/z) 500-2000. At least 10 scans were summed before transformation of the spectra to give the molecular mass of the peptides.

3. Results

In order to identify Aß expression in the brain of the mice affected with systemic amyloidosis, the affected animal brain, spleen and liver tissue section showed increased Congo red stain (Asokan et al, 2014). Whereas in the control mice brain, spleen and liver tissue section showed minimal Congo red stain. The brain sections were treated with antibody raised against $Aß_{25-35}$. The $Aß_{25-35}$ antibody purified by affinity chromatography, then affected animal brain tissue section showed increased staining with Aß antigen. whereas in the control mice such a staining was minimal indicating low expression of Aßs. The brain sections were treated with antibody raised against $Aß_{25-35}$. The $Aß_{25-35}$ antibody purified by affinity chromatography, then affected animal brain tissue section showed increased staining with Aß antigen (Fig. 2b). Whereas in the control mice such a staining was minimal indicating low expression of Aßs (Fig. 2a).
The hexaflouroisopropanol (HFIP) extract of brain has been analyzed by RP-HPLC using the Solvent system 0.1% TFA in water (solvent A) and 0.1% TFA in ACN (solvent B). The RP-HPLC chromatogram of the brain cortex SDS soluble portion is depicted for both control and systemic amyloid affected mice (data not shown), because, the present study focused SDS insoluble fibrillar form of brain affected systemic amyloidotic condition. The RP-HPLC chromatogram of the brain SDS insoluble extract is depicted for both control and systemic amyloid affected mice in Fig. 3a and 3b. The elution pattern of the peptides correlated with the calculated hydrophobicity of eluted peptide sequences (Hopp and Woods., 1981) Aß17-45 being more hydrophobic than $Aß_{43}$, which in turn is more hydrophobic than $Aß_{41}$. The peptides are eluted in the same order of their hydrophobicity, as noted by other workers (Naslund et al., 1994a). After desalting, RP- HPLC fractions collected were subjected to MALDI-TOF analysis. The exact constituents of the mixtures were shown in Fig. 4. The observed mass 4345.6 is assigned to Aß, and the peak due to mass 4530.9 indicate presence of $Aß_{43}$. Another peak with mass 2876.6 is due to $Aß_{17-45}$. In addition to this, a peak due to $Aß_{54}$ appears with molecular mass of 5756.8 indicating a new type of secretase activity. We followed the same nomenclature on APP cleavages that are already included a-, ß -, -, d- and e- cleavage. The new cleavage site proposed in the present work termed "zeta -secretase cleavage site".

4. Discussion

In the present case, the reason for the accumulation of Aßs is not clear. However, it was found that the marked decrease in the cerebral glucose level is observed in the mice with systemic amyloidotic condition, which is due to impaired liver functions (Jessy et al., 1990). The cell stress induced glucose deprivation was known to promote ß-secretase cleavage (Furukawa et al., 1996). Infact, most of the proposed toxicological mechanisms rely on the ability Aß peptide inducing the inflammatory process in AD brain (Paris et al., 2000).

Aß was shown to activate glial cells *in vitro* condition to induce expression of various inflammatory products like IL- 1ß, tumor necrosis factor (TNF-a), nitric oxide (NO) reactive oxygen species, IL-6 and IL-8. However it is not clear whether the cytokine elevation in the brain due to other inflammatory condition will lead to accumulation of Aßs. Cytokine relation is also observed during systemic amyloidosis (Akiyama, et al., 2000).

Inflammation-dependent cerebral deposition of serum amyloid A in a transgenic mouse (mouse that expresses the amyloidogenic mouse saa1 protein in the brain) was recently reported by Guo et al (Guo et al., 2002). The present observation indicates that inflammation and associated systemic amyloidosis will also trigger the Aßs deposition in the brain. The generation of Aß as a proteolysis by-product of an intracellular degradative process is triggered by mutations in the APP sequence, presence of excess amounts of wild type APP, expression of the incorrect isoforms, misfolding or abnormalities in post-translational modification. Other than the over production of APP, accumulation of Aß may also be due to decreased catabolism and/or increased anabolism of the Aßs in the inflammatory conditions (Brugg et al., 1995). In the familial onset of AD, the accumulation is due to 50% over production of Aßs (Golde et al., 1993).

The inoculation of Aß fibrils in primates leads to the onset of Aß like deposits in the animals after six to seven years (Baker et al., 1994). However, it is shown that cerebral Aß can be triggered *in vivo* by a single inoculation of AD brain extract in the transgenic mice (Kane et al., 2000). This result indicates that in the transgenic mice, where the secretase cleavage is prominent, the Aß accumulation is more pronounced suggesting the importance of enhanced secretase cleavage. The exact cause for -secretase cleavage in these conditions is explained by the multiple actions around VIAV sequence (Hardy and Selkoe 2002) (Fig. 1) that results in either $Aß_{41}$ or $Aß_{43}$. Cleavage around this region alone will results in Aßs which can form fibrils.

5. Conclusion

In conclusion that a new type of secretase involved in the Aß processing which already known gamma -, delta-, and epsillon-secretase site of action. The resulting 54-residue fragment ($Aß_{54}$) is found to be part of amyloid aggregate cleaved by zeta-secretase.

6. Acknowledgments

We thank Council of Scientific and Industrial Research, New Delhi, India for senior research fellowship to Dr. C. Asokan. We also thank Dr. T. Ramasami, Director, Central Leather Research Institute for his kind permission to publish this work. We thank Prof. P. Balaram, Director, Indian Institute of Science for helping us with MALDI-TOF measurements.

7. References

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Annexure

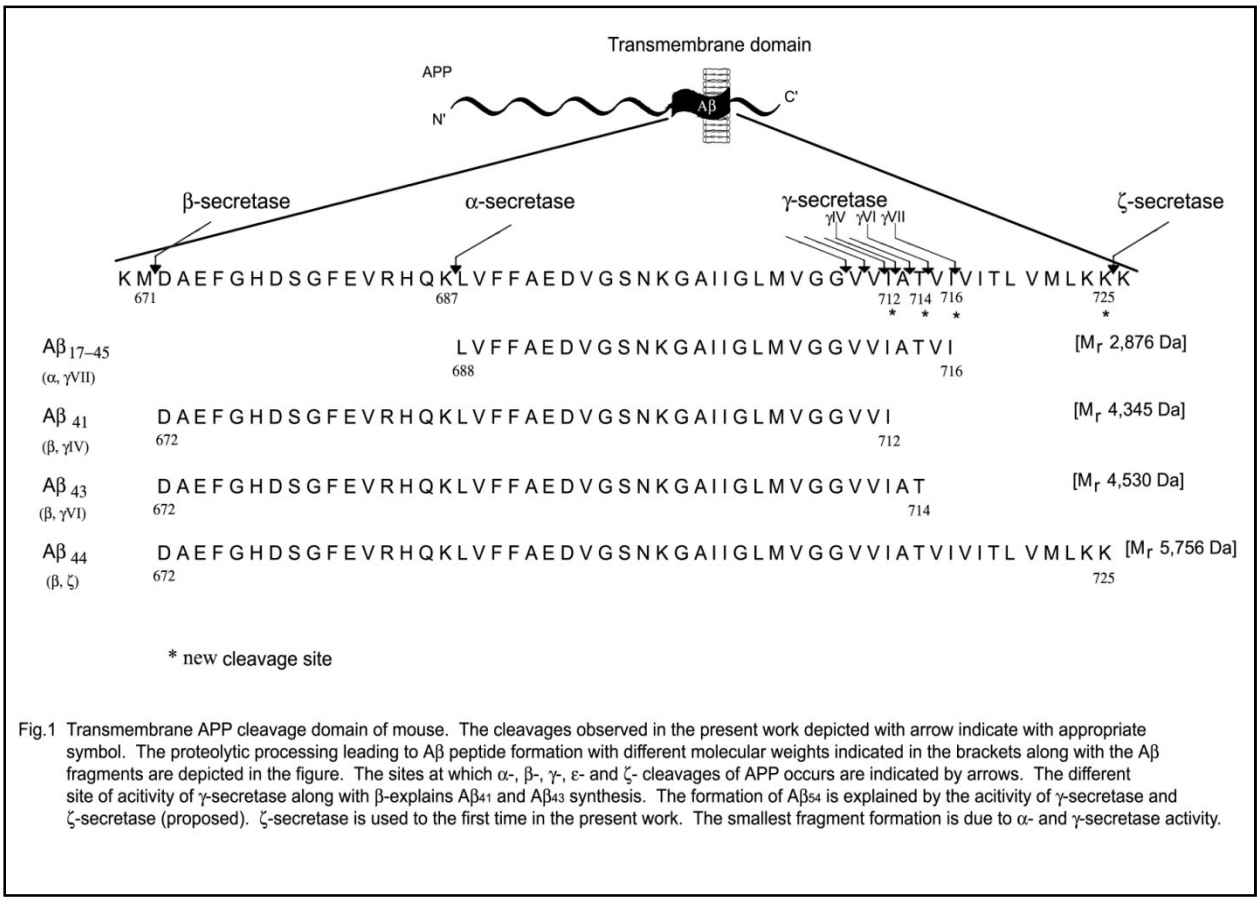

Fig.1 Transmembrane APP cleavage domain of mouse. The cleavages observed in the present work depicted with arrow indicate with appropriate symbol. The proteolytic processing leading to Aβ peptide formation with different molecular weights indicated in the brackets along with the Aβ fragments are depicted in the figure. The sites at which α-, β-, γ-, ε- and ζ- cleavages of APP occurs are indicated by arrows. The different site of acitivity of γ-secretase along with β-explains $Aβ_{41}$ and $Aβ_{43}$ synthesis. The formation of $Aβ_{54}$ is explained by the acitivity of γ-secretase and ζ-secretase (proposed). ζ-secretase is used to the first time in the present work. The smallest fragment formation is due to α- and γ-secretase activity.

Figure 1

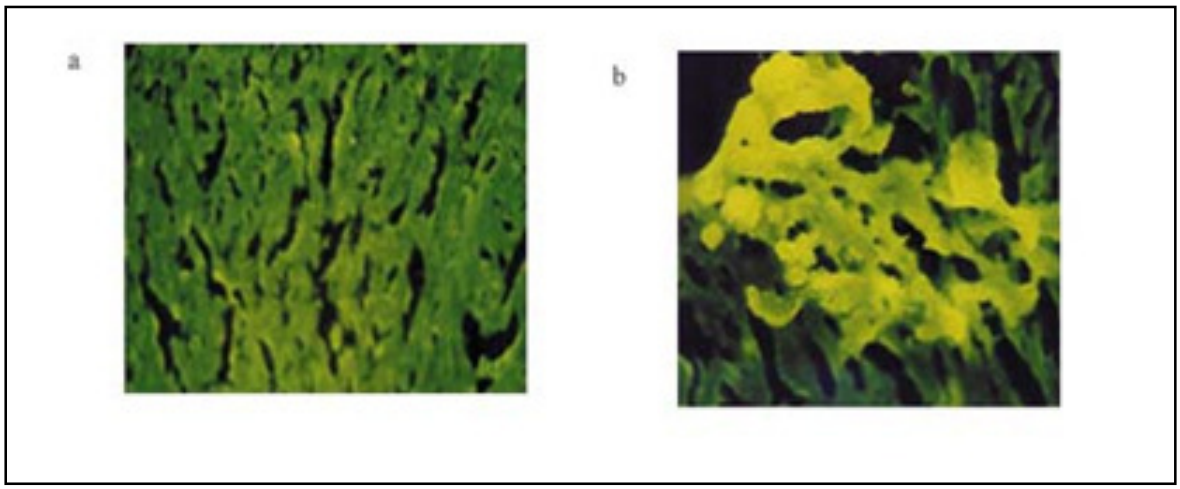

Figure 2: Immunostaining of Abeta's in the brain (a) control (b) brain with chronic inflammation of mice. Original magnification x40.

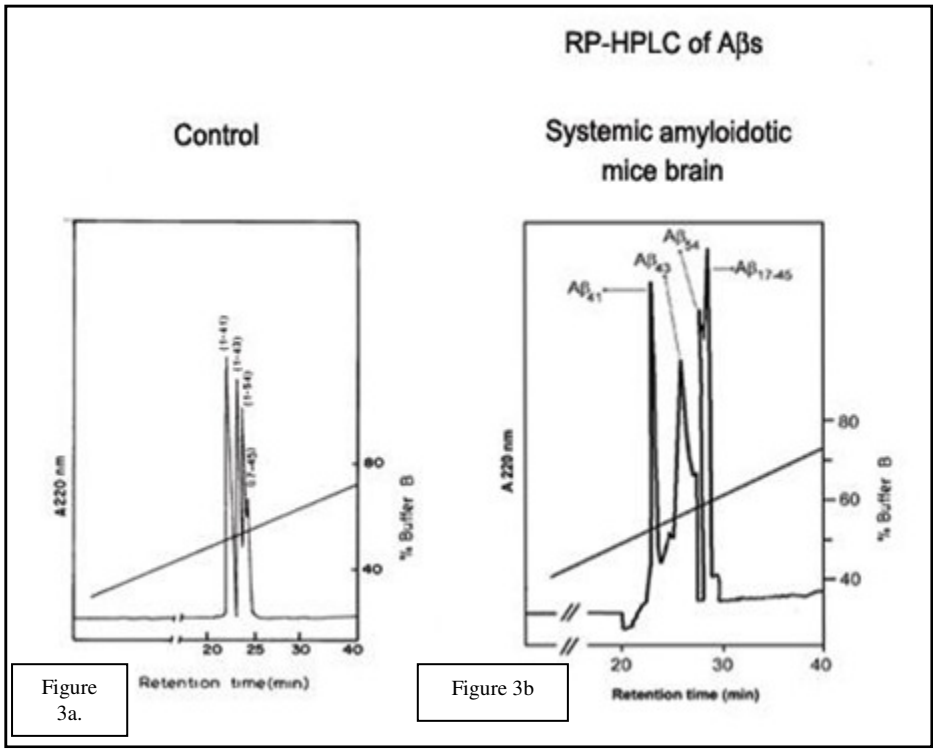

Figure 3: Extracted Aβ_{41}, Aβ_{43}, Aβ_{54}, and Aβ_{17-45} separated by RP-HPLC. The curve (a) represents the elution profile of extract of the control mice brain. The curve (b) represents the elution profile of extract of the mice brain affected by the systemic amyloidosis. Synthetic standards were used to label the Aβ_{41} and Aβ_{43} in the RP-HPLC chromatogram. The left and right-axis indicates the absorption and the gradient profiles respectively.

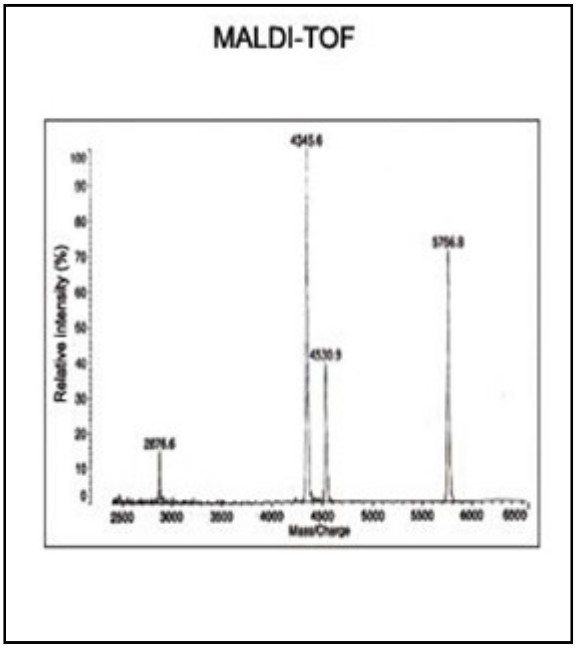

Figure 4: MALDI-TOF analysis of Aβs containing fractions obtained from acidic pH RP-HPLC separation.