Amino Acids (2008) 35: 339–343
DOI 10.1007/s00726-007-0618-9
Printed in The Netherlands

Amino Acids

Protein expression of BACE1, BACE2 and APP in Down syndrome brains

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Received August 16, 2007
Accepted October 8, 2007
Published online December 29, 2007;

Summary. Down syndrome (DS) is the most common human chromosomal abnormality caused by an extra copy of chromosome 21. The phenotype of DS is thought to result from overexpression of a gene or genes located on the triplicated chromosome or chromosome region. Several reports have shown that the neuropathology of DS comprises developmental abnormalities and Alzheimer-like lesions such as senile plaques. A key component of senile plaques is amyloid β-peptide which is generated from the amyloid precursor protein (APP) by sequential action of β-secretases (BACE1 and BACE2) and γ-secretase. While BACE1 maps to chromosome 11, APP and BACE2 are located on chromosome 21. To challenge the gene dosage effect and gain insight into the expressional relation between β-secretases and APP in DS brain, we evaluated protein expression levels of BACE1, BACE2 and APP in fetal and adult DS brain compared to controls. In fetal brain, protein expression levels of BACE2 and APP were comparable between DS and controls. BACE1 was increased, but did not reach statistical significance. In adult brain, BACE1 and BACE2 were comparable between DS and controls, but APP was significantly increased. We conclude that APP overexpression seems to be absent during the development of DS brain up to 18–19 weeks of gestational age. However, its overexpression in adult DS brain could lead to disturbance of normal function of APP contributing to neurodegeneration. Comparable expression of BACE1 and BACE2 speaks against the hypothesis that increased β-secretase results in (or even underlies) increased production of amyloidogenic Aβ fragments.

Furthermore, current data indicate that the DS phenotype cannot be fully explained by simple gene dosage effect.

Keywords: Amyloid precursor protein – β-site APP-cleaving enzyme – Down syndrome – Protein expression

Introduction

Down syndrome (DS), which is caused by an extra copy of chromosome 21 (trisomy 21), is the most frequent genetic cause of mental retardation with a prevalence of 1:700 live births. The cerebral cortex of DS brains is of normal thickness after cortical histogenesis has ended at 18 weeks of gestation, but abnormal cortical stratification is observed as early as midgestation or 22 weeks *in utero* (Schmidt-Sidor et al., 1990). This implicates that neuropathology of DS is associated with developmental abnormalities of the central nervous system (Wisniewski and Kida, 1994). By the fourth decade of life, individuals with DS display neuropathological features (i.e. cerebrovascular amyloidosis, senile plaques, neurofibrillary tangles and degeneration of basal forebrain cholinergic neurons) similar to individuals with Alzheimer's disease (AD; Epstein, 1995; Wisniewski et al., 1985).

A key component of amyloid fibrils isolated from cerebrovascular and senile plaques in both, DS and AD, is the 4-kDa amyloid β-peptide (Aβ; Takashima et al., 1981). This amyloid protein is derived from the large membrane-spanning precursor molecule, amyloid precursor protein (APP). APP can be processed into several different biologically active compounds, such as the secreted form, sAPP, which has been shown to have neurotrophic activities, and the aggregating forms, Aβ42, which is the most toxic. The sAPP has been reported to potentiate the effects of nerve growth factor on cell differentiation (Wallace et al., 1997) and has a potent neuroprotective action on cultured neurons (Mattson et al., 1993).

A membrane-associated aspartyl protease (also called β-site APP-cleaving enzyme 1; BACE1 or Asp2) was shown to exhibit all of the properties expected of a β-secretase that produces Aβ by cleaving the APP at the N terminus of Aβ (the β site) (Hussain et al., 1999; Sinha et al., 1999; Vasser et al., 1999; Yan et al., 1999). In searching an additional aspartyl protease, BACE2 (also called Asp1, DRAP or memapsin 2), which is a close

homolog of BACE1, has been identified (Saunders et al., 1999; Yan et al., 1999). BACE2 is also shown to cleave APP at the β site and more efficiently at a different site within Aβ (Farzan et al., 2000; Hussain et al., 2000; Lin et al., 2000). While BACE1 maps to chromosome 11, BACE2 and APP are located within the so-called Down syndrome critical region on chromosome 21, providing a logical link between BACE2 and APP processing (Saunders et al., 1999; Acquati et al., 2000).

To explain the impact of extra chromosome 21 on the pathology of DS, the gene dosage effect hypothesis has been proposed, but several investigators including our group have challenged this hypothesis. Although analysis of the sequence of chromosome 21 has been essentially completed, the molecular and biochemical mechanisms underlying the pathology of DS are still unknown. To gain an insight on the expressional relation between β-secretases and its substrate, APP during the development of DS brain, we investigated protein expression levels of BACE1, BACE2 and APP in cerebral cortex of fetal brain with DS and controls at 18–19 weeks of gestational age. In addition, we evaluated those in frontal cortex from adult individuals with DS showing AD-like pathology to elucidate the link between chromosome 21 and AD-neuropathology.

Materials and methods

Brain samples

Cerebral cortices of fetal brain with DS (4 females) and controls (4 females) at 18–19 weeks of gestational age were obtained from Dr. Mara Dierssen (Genes and Disease Program, Genomic Regulation Center-CRG, Barcelona, Spain) and Dr. Joan Carles Ferreres (Department of Pathology UDIAT-CD, Corporacis Sanit'ria Parc Tauli, Sabadell, Barcelona, Spain). Postmortem frontal cortex samples (superior frontal gyrus) of human adults were obtained from Dr. Nigel J. Cairns (MRC London Brain Bank for Neurodegenerative Diseases, Institute of Psychiatry) (Table 1). All the DS patients were karyotyped and possessed trisomy 21. A formal cognitive assessment of dementia in DS was not performed. In all DS brains there were abundant and extensive beta-amyloid deposits, neurofibrillary tangles and neuritic plaques. Normal brains obtained from individuals with no history of neurological or psychiatric illness were used as controls. The major cause of death was bronchopneumonia in DS and heart disease in controls. Brains were dissected, coronal slices snap frozen and stored at −70 °C until use.

Table 1. Autopsy data for adult brain samples

	DS	Control
N (male/female)	8 (6/2)	9 (6/3)
Age (years)	55.88 ± 7.97	61.89 ± 8.16
Postmortem interval (h)	32.38 ± 20.70	38.78 ± 18.87

Antibodies

Three primary antibodies for APP (N-terminal, Sigma, USA), BACE1 (C-terminal, Exalpha Biologicals Inc., USA) and BACE2 (N-terminal, Exalpha Biologicals, Inc., USA) were purchased. Secondary antibody coupled to horseradish peroxidase was purchased from Southern Biotechnology Associates Inc. (USA).

Western blotting

Brain tissues ground under liquid nitrogen were homogenized in homogenate buffer consisting of 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% (v/v) Tween 20, 1 mM PMSF and 1 tablet of protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) at 4 °C and centrifuged at 12000 × *g* for 10 min. The BCA protein assay kit (Pierce, USA) was applied to determine the concentration of protein in the supernatant. Samples (10 μg) were mixed with the sample buffer (100 mM Tris-HCl, 2% SDS, 1% 2-mercaptoethanol, 2% glycerol, 0.01% bromophenol blue, pH 7.6), incubated at 95 °C for 15 min and loaded onto a 7.5% ExcelGel SDS homogenous gel (Amersham Pharmacia Biotech, Sweden).

Electrophoresis was performed with Multiphor II Electrophoresis System (Amersham Pharmacia Biotech, Sweden). Proteins separated on the gel were transferred onto PVDF membrane (Millipore, Bedford, USA) and the membranes were blocked in blocking buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20 and 2% non-fat dry milk). The membranes were incubated for 2 h at 21 °C with diluted primary antibodies (1:1000 for APP, BACE1 and BACE2). After washing 3 times for 15 min with blocking buffer, membranes were probed with diluted secondary antibody (1:2000 of goat anti-rabbit IgG (H + L)-HRP) for 1 h. Membranes were washed 3 times for 15 min and developed with the Western blot chemiluminescence reagents (NEN™ Life Science Products Inc., USA).

Statistics

The densities of immunoreactive bands were measured by RFLPscan version 2.1 software program (Scanalytics, USA). Between group differences were calculated by nonparametric Mann–Whitney *U*-test using GraphPad Instat2 program and the level of significance was considered at $P < 0.05$.

Results

In fetal brain (Fig. 1), protein expression of BACE2 and APP was comparable between DS and controls. Anti-APP (N-terminal) antibody detected several bands of APP immunoreactivity between 60-kDa and 150-kDa, which could be assigned to full-length APP, modified by post-translation processing and APP cleavage products (or sAPP generated by both α- and β-secretase). BACE1 was increased (178%), but did not reach statistical significance ($P = 0.0571$). In adult brain (Fig. 2), protein expression of BACE1 and BACE2 was comparable between DS and controls. However the sum of APP immunoreactivity between 60-kDa and 150-kDa was significantly increased (183%, $P < 0.001$); APP immunoreactivity between 75- and 150-kDa were remarkably increased (272%, $P < 0.001$) and the 60-kDa band was also significantly increased (144%, $P < 0.05$). There was no correlation between each expression level and either age or postmortem intervals in both fetal and adult brain samples (data not shown).

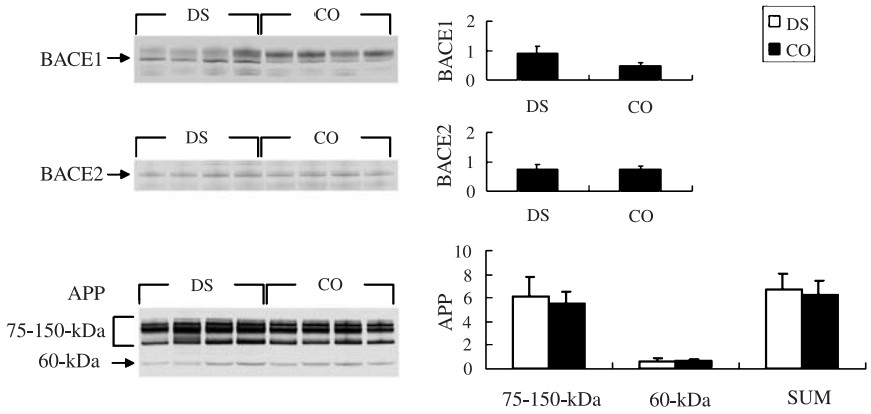

Fig. 1. Expression levels of BACE1, BACE2 and APP in fetal brain. Western blot analysis in cerebral cortex from fetal brain with DS and controls (CO) was performed as described in Materials and method. The immunoreactive bands (BACE1, 60-kDa; BACE2, 60-kDa; APP, between 60 and 150-kDa) were detected in using chemiluminescence reagents. The density of detected bands was measured and calculated by non-parametric Mann–Whitney *U*-test, and the level of significance was considered at $P < 0.05$

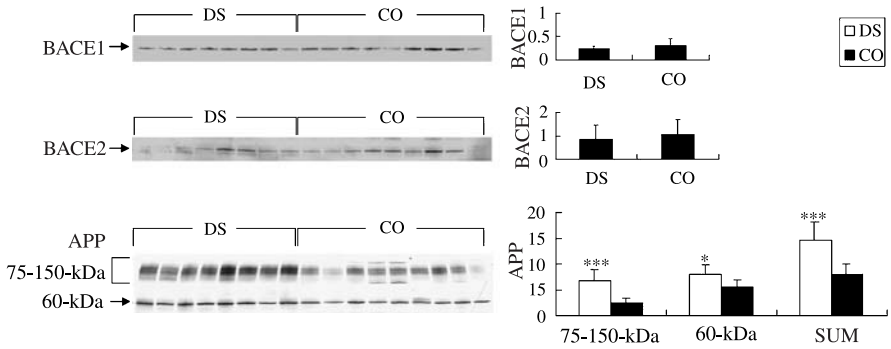

Fig. 2. Expression levels of BACE1, BACE2 and APP in adult brain. Western blot analysis in frontal cortex from adult brain with DS and controls (CO) was performed as described in Materials and method. The immunoreactive bands (BACE1, 60-kDa; BACE2, 60-kDa; APP, between 60 and 150-kDa) were detected in using chemiluminescence reagents. The density of detected bands was measured and calculated by non-parametric Mann–Whitney *U*-test, and the level of significance was considered at $P < 0.05$. $^{*}P < 0.05$, $^{***}P < 0.001$

Discussion

Gene expression during development and more particularly in the developing brain may be of special importance in DS. The phenotype of DS is thought to result from overexpression of a gene or genes located on the triplicated chromosome or chromosome region. To examine the gene dosage effect and gain an insight into the expressional relation between β-secretases and APP in DS brain, we evaluated protein expression levels of "DS candidate genes" – BACE2 and APP- and BACE1 in fetal DS brain. Several findings suggest that APP functions as a growth factor during neuronal development or synaptic plasticity (Takashima et al., 1990; Arai et al., 1997). Cell-surface-expressed cellular APP has a neurite-promoting function, co-localizes with adhesion plaque components and participates in synaptic vesicle recycling (Rossner et al., 1998; Neve et al., 2000). In addition, sAPP from the cell has a potent neuroprotective action on cultured rat hippocampal and septal neurons and in human cortical neurons (Mattson et al., 1993). In fetal DS brain, the numerical density of APP-immunoreactive neurons was found to be higher than in controls, but a high degree of variation precluded statistical significance (Griffin et al., 1998). In the present study, no statistically significant difference was observed in the expression of APP between fetal DS and controls at 18–19 weeks of gestational age. These results suggest that both full-length APP which can play roles as cell surface receptor and sAPP which has been shown to have neurotrophic activities and neuroprotective action could function normally in DS brain of 18–19 weeks gestation.

Barbiero et al. (2003) reported western blot findings on two DS fetal brain samples and one control sample and BACE2 was overexpressed in DS. No statistical evaluation could be carried out, however, and the finding remains anecdotal therefore and does not contradict our results.

Both BACE1 and BACE2 cleave APP at the β-secretase site and lead to the generation of Aβ. However, while BACE2 has been suggested to have a non-amyloidogenic function in the secretory pathway (Fluhrer et al., 2002), BACE1 is thought to be the major β-secretase for generation of Aβ peptides by neurons (Cai et al., 2001). Additionally, the coexpression of BACE1 and APP in human cortical neurons could provide evidence for a role of BACE1 as authentic β-secretase (Marcinkiewicz and Seidah, 2000). In our study, the expression level of BACE1 was increased in fetal DS brain, without reaching statistical significance, whereas BACE2 was comparable between fetal DS and controls. Interestingly, in a study by Teller et al. (1996) the soluble Aβ42 protein was present in some fetal DS brains from 21 weeks of gestation age while undetectable in others. APP mRNA expression, however, did not differ between DS fetuses with and without presence of soluble Aβ42 (Teller et al., 1996). Considering our current results and the report of Teller et al. (1996) protein expression level of BACE1 and BACE2 may not be necessarily related to regulation of APP metabolism. In addition, our finding does not support the gene dosage effect hypothesis for DS phenotypes (Engidawork and Lubec, 2001; Cheon et al., 2003a–d; Ferrando-Miguel et al., 2004; Shin et al., 2006; Gulesserian et al., 2007).

In our study, protein expression of APP in adult DS brain was significantly increased. However, protein levels of BACE1 and BACE2 were comparable between DS and controls. These results are consistent with other studies suggesting that the neuronal overexpression of APP dose not induce the overexpression of its metabolising enzymes in neurons (Rossner et al., 2001; Yasojima et al., 2001), although Holsinger et al. (2002) reported increased BACE1 immunoreactivity in AD brain (2002). BACE1 levels were not significantly different in AD compared with control brain (Yasojima et al., 2001). Additionally, BACE1 protein levels did not differ significantly between control and transgenic Tg2576 mice showing amyloid pathology (Rossner et al., 2001). Taken together, as regional expression pattern of BACE1 mRNA did not correlate with the distribution of Aβ deposits (Bigl et al., 2000), our results suggest that increased expression of β-secretases is not necessary for enhanced generation of Aβ peptide. Methodologically, markers for neuronal (neuronal specific enolase) and cell (actin) density were comparable between DS and controls in both fetal and adult brain ruling out expression differences by neuronal and/or cell loss in DS (data not shown).

The full-length APP or sAPP normally functions as a cell surface signalling molecule and growth factor, or neuroprotective factor, respectively. In the normal healthy cell, it appears that both amyloidogenic and non-amyloidogenic APP processing pathways operate and that a precise balance between these pathways is maintained (Wasco and Tanzi, 1995). The disruption of this normal signalling function of APP and balance of two pathways cause cell cycle abnormalities in neurons, and these abnormalities are at least one cause of the neurodegeneration and consequent dementia in AD and DS (Neve et al., 2000). However, APP gene dosage effect in DS per se cannot be ruled out as a specific risk factor for neurodegeneration. Additional factors like reactive oxygen species generation and/or altered cellular redox status may compromise the ability of regional neuronal subpopulations for neural network formation and/or cause enhanced vulnerability to a host of various stress factors.

Based on our results, we conclude that APP overexpression seems to be absent during the development of DS brain up to 18–19 weeks of gestational age, but its overexpression in adult DS brain could lead to the disruption of normal function of APP resulting in neurodegeneration. The balance between amyloidogenic and non-amyloidogenic processing pathways of APP could be regulated by more complicated mechanism and many other factors, which have not been identified so far. The present study speaks against the hypothesis that increased Aβ generation is solely due to increased protein levels of β-secretases. Furthermore, the current data indicate that the DS phenotype can not be simply explained by gene dosage effect.

Acknowledgments

We thank Dr. Nigel J. Cairns (MRC London Brain Bank for Neurodegenerative Diseases, Institute of Psychiatry) for providing brain tissues. We also thank Dr. Rainer Seidl (Department of Pediatrics, Medical University of Vienna, Vienna, Austria) for critical discussion.

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