

Alzheimer's soluble amyloid β is a normal component of human urine

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Abstract Soluble A β (Sa β) is normally present at a low concentration in human plasma and cerebrospinal fluid. Although the factors involved in the regulation of Sa β plasma levels are still unknown, we have explored its excretion in the urine as one of the possible homeostatic mechanisms. The presence of Sa β in the urine was investigated via immunoprecipitation experiments with anti-A β antibodies followed by detection and identification by immunoblot, MALDI mass spectrometry and sequence analysis. Soluble A β (4.3 kDa) immunoreactivity was present in the urine of normal donors, Down's syndrome individuals as well as in patients with renal disorders exhibiting glomerular or mixed proteinuria. Edman degradation of the immunoprecipitated material yielded the intact A β N-terminus and mass spectra analysis indicated the existence of a major component at *m/z* 4327, corresponding to the molecular mass of A β 1-40. Semi-quantitative data obtained from the immunoprecipitation experiments indicate that under normal conditions the daily excretion of intact Sa β in the urine represents less than 1% of the circulating pool.

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Key words: Alzheimer's disease; Down's syndrome; Proteinuria; Sa β

1. Introduction

Amyloid beta (A β) is the major constituent of the fibrils deposited into senile plaques and cerebral blood vessels of patients with Alzheimer's disease (AD), Down's syndrome (DS), Hereditary Cerebral Hemorrhage with Amyloidosis of Dutch origin and normal aging (reviewed in [1]). Although originally thought to be an aberrant degradation product of its precursor molecule β PP, this peptide is now known to be a normal soluble component (Sa β) present at very low concentration in plasma and cerebrospinal fluid (CSF) [2–5]. The major form of Sa β , A β 1-40, is homologous to the amyloid protein extracted from cerebrovascular lesions, although minor Sa β species with heterogeneity and length similar to the A β components deposited in senile plaques were also found in circulation [5,6]. Soluble A β was also described as a normal component in brain parenchyma, from where it can be extracted in aqueous solutions. Interestingly, when soluble fractions of AD and DS brain homogenates were compared with control brains, an increased amount of Sa β was detected [7,8].

This increase appears to precede the appearance of A β deposits [9], suggesting that Sa β species may indeed represent immediate precursors of the deposited fibrils. However, the origin of the A β deposited in the brain is still debatable; it may be locally synthesized, it may come from the circulation, or a combination of both sources.

Soluble A β circulates in plasma associated mainly with lipoprotein particles, specifically high-density lipoproteins (HDL), co-localizing with apolipoproteins J (apoJ), E (apoE) and A-I (apoA-I) [10]. All these apolipoproteins exhibit high binding affinity for A β peptides [11–13]. ApoJ, in particular, displays a very high-affinity interaction for non-aggregated forms of the A β peptides and has been shown to maintain and stabilize the peptide solubility *in vitro* [14]. Several lines of evidence indicate that the blood-brain barrier (BBB) has the capability to regulate the brain uptake and clearance of Sa β . For the brain uptake, at least two different receptor-transport mechanisms have been identified. One involves the peptide when it is complexed to apoJ, a proposed carrier molecule for circulating Sa β species. A 36-fold higher uptake than that of the extracellular space marker sucrose was found for the complex A β -apoJ in the guinea pig vascular-brain perfusion model. The receptor implicated in the BBB transport was identified as gp330 or megalin, the receptor for apoJ [15]. The other receptor transport mechanism pertains to the uptake of free A β peptides and was studied in guinea-pigs [16,17], squirrel monkeys [18], mice [19] and rats [20]. The uptake of the free peptide, although one-fourth of that of the complex A β -apoJ, is saturable and specific in the guinea-pig brain perfusion model, compromising cell surface molecules that are not yet fully identified. One possible candidate is the receptor for advanced glycation-end products (RAGE), a recently described receptor for A β species [21].

The BBB also appears to regulate the clearance of brain Sa β species. Infusion of ¹²⁵I-labeled A β 1-40 into one lateral ventricle in the rat brain has shown that in as little as 3.5 min 30% of the peptide was cleared from ventricular CSF. Ten minutes after infusion ~70% of the radioactivity disappeared from the brain and was recovered in the blood, liver, kidney and urine [22]. In order to clarify to which extent the urinary excretion of Sa β might be an important mechanism for Sa β clearance from the circulation, we have investigated its presence in urine from normal donors, in specimens from DS patients with normal levels of urinary proteins as well as in samples from individuals with different degree of glomerular or mixed glomerular-tubular proteinuria. Down's syndrome urines were studied since it is known that plasma Sa β levels are elevated in these patients [23]. The methodology used herein, immunoprecipitation, immunoblot analysis, matrix-as-

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sisted laser desorption/ionization mass spectrometry (MALDI-MS) and N-terminal sequence clearly identified the presence of A β 1-40 as the major Sa β specie of human urine.

2. Materials and methods

2.1. Samples

Twenty-four-hour urine specimens were collected from two normal donors (proteinuria < 5 mg/dl), three patients with glomerular proteinuria (210–600 mg/dl), five patients with mixed glomerular-tubular proteinuria (7–420 mg/dl) and seven cases of DS (proteinuria < 5 mg/dl). DS urines were kindly provided by Dr. K. Wisniewski (Institute for Basic Research at Staten Island) from well-characterized patients who had been subjected to chromosomal analysis. All samples were dialyzed against distilled water using 1000 Da cut-off membrane and lyophilized prior to use.

2.2. Immunoprecipitation

Fifty microliters of paramagnetic Dynabeads M-450 coated with goat anti-mouse IgG (Dyna) were allowed to interact for 3 h at room temperature with a mixture of 3 μ l of monoclonal antibody 6E10 (anti-A β 1-17; Senetek) and 3 μ l of monoclonal antibody 4G8 (anti-A β 17-24; Senetek). After incubation, unbound antibody was removed by washing the beads with 10 mM phosphate (pH 7.4) containing 150 mM NaCl (PBS) and 0.1% bovine serum albumin. For the immunoprecipitation experiments, 5 mg of urinary proteins were solubilized in 1 ml of PBS, added to the tube containing the paramagnetic beads coated with anti-A β antibodies and incubated overnight at 4°C. After the incubation, the beads were washed 3 times with PBS, resuspended in Laemmli sample buffer and loaded onto the SDS-polyacrylamide gel. Alternatively, the beads were eluted with 10 μ l of isopropyl alcohol/water/formic acid (4:4:1) mixture and analyzed by MALDI-MS.

2.3. Immunoblot analysis

Immunoprecipitated samples were separated on Tris-Tricine 16% SDS-PAGE and electroblotted onto nitrocellulose membrane (Bio-Rad) using 3-cyclohexylamino-1-propanesulphonic acid (pH 11) containing 10% methanol. Membranes were blocked for 1 h with 5% low-fat milk in PBS containing 0.05% Tween-20 and incubated overnight with monoclonal 6E10 (1:500), followed by horseradish peroxidase-labeled goat anti-mouse F(ab')₂ (1:2000; Amersham). Immunoblots were visualized with an enhanced chemiluminescence (ECL) detection kit and exposed to Hyperfilm ECL (Amersham). The intensity of the bands was semi-quantitatively evaluated using a Umax Power-Look scanner and the NIH Image 1.60 software.

2.4. Sequence analysis

Twenty milligrams of urinary proteins from two cases of mixed glomerular-tubular proteinuria were separated on Tris-Tricine 16% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon P, Millipore) using the same conditions described above. After transfer, the membrane was stained with Coomassie Blue and the 4.3 Kda protein bands were excised and sequenced on a 477A protein sequencer (Applied Biosystems).

2.5. MALDI-MS

Soluble A β from the urine samples immunoprecipitated as described above was eluted from the beads in 10 μ l of water/isopropyl alcohol/formic acid (4:4:1) and subjected to MALDI-MS using the

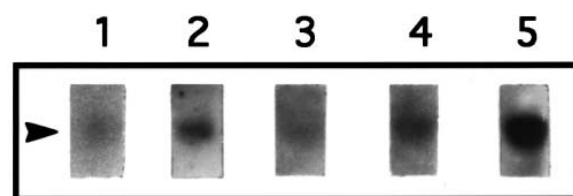


Fig. 1. Western Blot analysis of immunoprecipitated Sa β from urine samples. Fluorograms of the Sa β immunoprecipitated (arrowhead) from 5 mg of urinary proteins from normal donors (lane 1), Down's Syndrome (lane 2) and non-demented individuals with glomerular (lane 3) or mixed proteinuria (lane 4). For comparison, lane 5 displays the immunoreactivity of 10 ng of synthetic peptide A β 1-40 immunoprecipitated under the same experimental conditions.

dried droplet method with α -cyano-4-hydroxycinnamic acid solution as a matrix. Samples were processed at the Mass Spectrometry Facility at the Skirball Institute (NYU Medical Center).

3. Results and discussion

In order to identify the presence of Sa β in the urine, we developed an immunoprecipitation method based on the use of paramagnetic beads coated with goat anti-mouse IgG that were coupled to anti-A β monoclonal antibodies. Amongst the available antibodies immunoreactive with A β we found that the mixture of 6E10 plus 4G8 allowed the highest recoveries and a high sensitivity. Under the conditions tested, immunoprecipitation of 0.2 ng of synthetic A β 1-40 rendered a clearly visible 4 Kda band in the corresponding fluorograms. Parallel experiments using Protein A/G agarose reached the detection limit at 1.0 ng.

For each immunoprecipitation experiment, 5 mg of urinary samples from either normal donors, patients with glomerular proteinuria, individuals with mixed glomerular-tubular proteinuria, or DS patients were used. As indicated in Fig. 1, immunoblot analysis of the immunoprecipitated material after separation in SDS-PAGE revealed the presence of a specific immunoreactive band of about 4 Kda with identical electrophoretic mobility of that of synthetic A β 1-40. Soluble A β immunoreactivity was present in normal donors (proteinuria < 5 mg/dl) (lane 1), although it was consistently more prominent in DS samples exhibiting a similar degree of proteinuria (< 5 mg/dl) (lane 2). It was also detected in samples from patients with mixed glomerular-tubular proteinuria (7–420 mg/dl) (lane 3) as well as predominant glomerular components (proteinuria 210–600 mg/dl) (lane 4).

Densitometric analysis of the different bands allowed us to estimate the amount of Sa β peptide present in each sample through the comparison with 10 ng of synthetic A β 1-40 peptide immunoprecipitated under the same conditions (lane 5). For the normal donors tested (proteinuria < 5 mg/dl), the excretion of Sa β was calculated at 0.81 ± 0.26 ng/5 mg of

Table 1
Estimation of mean Sa β levels in urine samples

Pathology	<i>n</i>	Proteinuria (mg/dl)	Sa β (ng/5 mg urinary proteins)	Excretion of Sa β (ng/24 h)
Normal donors	2	< 5	0.81 ± 0.26	13 ± 4
Down's syndrome	7	< 5	2.13 ± 0.70	32 ± 11
Glomerular or mixed proteinuria	8	7–600	1.24 ± 0.32	20–2000

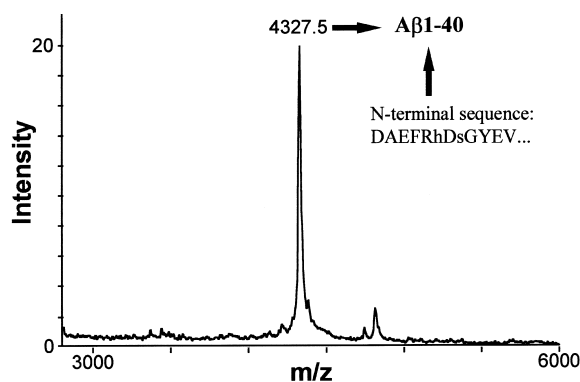


Fig. 2. Mass spectrometry of immunoprecipitated Sa β from urine samples. Aliquots of lyophilized urine samples (5 mg) were immunoprecipitated using monoclonal antibodies 6E10 and 4G8 coupled to paramagnetic beads as described in Section 2. The immunoprecipitated material was eluted using 10 μ l of isopropyl alcohol/water/formic acid (4:4:1) mixture, applied on the spectrometer's probe using the dried droplet method in alpha-cyano-4-hydroxycinnamic acid matrix, and analyzed by MALDI-MS. For calibration purposes, synthetic A β 1-40 was used as standard of mass.

urinary proteins (13 ± 4 ng/24 h). The Down's syndrome patients analyzed (all with proteinuria < 5 mg/dl) excreted a higher amount of Sa β (2.13 ± 0.7 ng/5 mg of urinary proteins, equivalent to 32 ± 11 ng/24 h), which might correlate with their higher plasma concentration [23] as a result of the gene dosage. Taking into consideration that the levels of Sa β in plasma are in the range of 1 ng/ml [4,23] and that under normal conditions the volume of the urine excreted in a 24 h period is around 1–1.5 l, the daily excretion of Sa β in the urine represent less than 1% of the total circulating pool. These values are obviously increased several fold in patients with either glomerular or mixed tubular-glomerular proteinuria; in these cases, where the amount of urinary proteins can reach values of 600 mg/dl or more, the daily excretion of Sa β could reach 2000 ng/24 h. It is possible that when there is loss of permselectivity, Sa β complexes from the circulation could pass through the altered filtration barrier, accounting for the higher excretion of Sa β (Table 1).

The identity of Sa β present in the urine samples was corroborated by N-terminal sequence analysis. The 4.3 Kda bands obtained upon immunoprecipitation of 20 mg of urinary proteins from two cases of mixed glomerular-tubular proteinuria were transferred to a polyvinylidene difluoride membrane and subjected to automatic Edman degradation. The sequences DAEFRxDxGxEV and DAEFRhDsGYEV were obtained, corresponding to the intact N-terminus of A β . No N-terminal heterogeneity was found in the samples analyzed. The mass spectrometry analysis of the immunoprecipitated samples showed a main signal at m/z 4327, corresponding to the molecular mass of native A β 1-40 (Fig. 2). In all the cases studied, the major specie identified in urine was the A β 1-40 in accordance to that specie's predominance in plasma.

The results presented here demonstrate that Sa β is a normal component of human urine. Under normal conditions, where the renal filtration-resorption function is preserved, the amount of intact Sa β excreted in the urine accounts for less than 1% of the total circulating pool, indicating that the majority of the circulating Sa β is catabolized or excreted by a different mechanism. Due to the association of Sa β with HDL

particles, it is possible that Sa β catabolic/excretory pathways may follow those of the lipoprotein particles. If this is the case, it would be expected that the liver would be the key organ involved in the catabolism of Sa β and perhaps in the excretion of the peptide or its degradation products in the bile. Whether urinary Sa β levels are altered in sporadic AD or in familial AD associated with either β PP or presenilin mutations remains to be determined. However, in view of our data (Table 1), potential AD diagnostic methods based on the measurement of urine and/or plasma levels of Sa β should simultaneously evaluate the coexistence of pathological conditions that can alter glomerular filtration and/or tubular resorption.

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