Detection of a Soluble Form of BACE-1 in Human Cerebrospinal Fluid by a Sensitive Activity Assay

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Background: Formation of deposits of the insoluble amyloid β-peptide is believed to be causally related with neurodegeneration in Alzheimer disease (AD). The β-peptide originates from a larger amyloid precursor protein (APP) by the action of proteolytic enzymes. The first proteolytic event leading to amyloid formation is the cleavage of APP by the membrane-bound aspartyl protease BACE-1, also known as memapsin-2. Inhibition of BACE-1 is thought to be a therapeutic approach to AD. Measuring BACE-1 activity in biological samples would be useful to elucidate the mechanism of AD and for development of AD drugs.

Methods: We developed a sensitive and specific activity assay for BACE-1. The assay is based on a genetically engineered proenzyme that is specifically activated by BACE-1. The resulting active enzyme is measured with a chromogenic substrate. The use of 2 coupled reactions produces a detection limit as low as 0.4 pmol/L.

Results: The assay detected BACE-1 activity in extracts of human brain tissue as well as, unexpectedly, in human cerebrospinal fluid (CSF). Gel electrophoresis and Western blotting identified the BACE-1 present in CSF as a truncated soluble form of the originally membrane-bound BACE-1.

Conclusion: Detection of the soluble form of BACE-1 in CSF, a relatively easily accessible biological fluid, may be useful for monitoring the effects of drug candidates in vivo and may have diagnostic or prognostic applications.

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Alzheimer disease (AD) is by far the most common form of dementia, accounting for ~90% of all dementia cases. The hallmark pathology of AD is the presence of extracellular deposits of insoluble protein material, known as amyloid plaques, in the brains of affected individuals. These plaques consist predominantly of a small peptide, β-amyloid peptide (I), which is formed from the amyloid precursor protein (APP) by the sequential action of 2 proteases. The initial step in amyloid formation is the cleavage of APP by β-APP cleavage enzyme (BACE-1; also known as memapsin-2 or Asp-2), which produces a large soluble APP fragment and a small membrane-bound fragment (2–5). The latter fragment is further cleaved by γ-secretase, yielding the β-amyloid peptide and a small, rapidly degraded cytosolic metabolite. There is evidence that the accumulation of β-amyloid peptide in the brain and plaque formation have a causal relationship with AD; therefore, inhibition of BACE-1 could be a therapeutic intervention for AD [for reviews, see Refs. (6–8)].

BACE-1 is a transmembrane aspartyl protease that is able to cleave APP at the SEVKMDAEFR sequence. BACE-1 is synthesized as a 501-amino acid-long proprotein [relative molecular mass (Mr) 56 000] and processed to a 456-residue fully enzymatically active membrane-bound form. The extracellular part has 412 residues and contains the 2 active site aspartyl residues at positions 93 and 289. The protein is anchored in the membrane via a 21-residue membrane-spanning region and has a 23-residue palmitoylated cytoplasmic tail (2, 9, 10). BACE-1 occurs both on the cell membrane and...
on intracellular membranes. Recently it has been shown that a soluble form of BACE-1 occurs in cell culture, which is very likely formed from the membrane-bound form in a process known as shedding. There is evidence that the metalloproteinase ADAM10 is involved in this shedding (10, 11). To date, the occurrence of soluble forms of BACE-1 has been observed only in cell culture in vitro.

Current assays for BACE-1 activity are mostly based on hydrolysis of an internally quenched fluorescent peptide substrate. The peptide substrate sequence can be based on natural sequences, such as those in APP, on mutant APP forms, or may be the result of a screening and optimization process. Methods based on such substrates are suitable for measuring activity in relatively pure BACE-1 preparations and have been used successfully in, e.g., inhibitor screening (12–14), but in general, both the sensitivity and selectivity are too low for detection of BACE-1 activity in biological samples. Several years ago, we developed an assay platform for sensitive detection of the activity of proteolytic enzymes (15). The principle of this platform is as follows. The protease to be measured converts a proenzyme into an active enzyme (detection enzyme), and the activity of the latter is detected (Fig. 1). This 2-step approach provides considerable signal amplification, leading to very sensitive assays with either a colorimetric or fluorescent read-out. The proenzyme used in this assay is a modified engineered protein, adapted in such a way that it can be activated by the target protease to be measured. Several protease assays have been developed, based on this principle (16–18).

In the present work we describe a new, sensitive, and specific activity assay for BACE-1, based on the above-mentioned principle. This assay could detect BACE-1 activity in homogenates of human brain tissue, and surprisingly, we discovered a soluble form of BACE-1 in human cerebrospinal fluid (CSF). The possibility of detecting BACE-1 activity in CSF could open new opportunities for monitoring AD-related processes in vivo.

**Materials and Methods**

**PREPARATION OF MODIFIED PROCASPASE-3**

We obtained a cDNA coding for procaspase-3 in *Escherichia coli* from the American Type Culture Collection. This plasmid was used as a basis for construction of similar plasmids coding for a variety of caspase-3 variants with modifications in their activation sequence. To enable rapid construction of variants, 2 new restriction sites, BamHI and EcoRI, flanking the coding region of the activation cleavage site, were introduced in this plasmid by PCR using the following primers: forward, 5′-GATA TCAAGGAATTCATGGTGTGGATGATGACATGCCG-3′; reverse, 5′-GGATCTCTTCTCAATGCGCACTCCAG-3′. After digestion of this new plasmid with BamHI and EcoRI, novel sequences coding for new protease cleavage sites can be introduced by use of 2 oligonucleotides having BamHI- and EcoRI-compatible overhangs on their 5′ and 3′ ends, respectively. From this plasmid, we used appropriate oligonucleotides to make 3 constructs coding for different procaspase-3 detection enzymes, each having a sequence potentially cleavable by BACE-1 (Table 1 and Fig. 2). All plasmids were verified by sequencing. The 3 resulting constructs were used to transform *E. coli* [strain BL21 (DE3) pLys]. Cultures were grown at 37°C to an absorbance of 0.45–0.7, cooled to 30°C, and induced with 1 mmol/L isopropylthio-β-galactoside. After shaking for 45 min at 30°C, cells were centrifuged (10 min at ~5000 g and 4°C) and either used directly or stored frozen at −20°C. Bacterial pellets were mixed with lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 300 mmol/L NaCl, 10 mmol/L imidazole] and 20 mL/L Bugbuster™ (Novagen), incubated at 4°C while stirring for 30 min, and centrifuged at ~5000 g for 10 min at 20°C. The supernatant was loaded on a nickel-Sepharose column (Amersham Biosciences; 2-mL column volume for 30–50 mL of original culture volume); washed with 20 column volumes of 50 mmol/L Tris-HCl (pH 8.0), 300 mmol/L NaCl, 20 mmol/L imidazole (wash buffer); and eluted with elution buffer [50 mmol/L Tris-HCl (pH 8.0), 300 mmol/L NaCl, 250 mmol/L imidazole]. The peak fractions were desalted by use of a PD-10 column (Amersham Biosciences) equilibrated with 10 mmol/L Tris-HCl (pH 7.0), 1.5 mmol/L NaCl, and 0.1 g/L Brij-35. Gel electrophoresis showed that all 3 caspase-3 variants were almost exclusively in the single-chain M, 32 000 proforms.

**ASSAY OF BACE-1 ACTIVITY IN SOLUTION**

BACE-1 (0–125 μg/L) was incubated overnight at 25°C with various concentrations of procaspase-3 variants 1.1, 1.2, and 1.3 in wells of a 96-well microtiter plate in 50 μL...
of BACE buffer [10 mmol/L sodium acetate (pH 5.0), 1.5 mmol/L NaCl, 0.1 g/L Brij-35]. At the end of incubation, we added 50 μL of detection buffer [100 mmol/L Tris-HCl (pH 8.0), 1.5 mmol/L NaCl, 0.1 g/L Brij-35] containing the caspase substrate AspGluValAsp-p-nitroanilide (DEVD-pNA; 1.6 mmol/L; BioSource) to each well, followed by 20 mmol/L dithiothreitol (DTT). The subsequent color formation was monitored for 1–4 h in a plate reader set to record the absorbance at 405 nm (ΔA405 nm/h). Activities were generally expressed as (ΔA405 nm/h) × 1000. In our previous assays based on the same principle, both activation of the proenzyme and detection of the activity of the activated proenzyme with a chromogenic peptide substrate were performed simultaneously, and the relationship between color formation and incubation time was parabolic. In the present assay, conversion of procaspase by BACE-1 is much slower than caspase-mediated conversion of the chromogenic peptide substrate and requires overnight incubation, which makes performing the rapid color formation reaction simultaneously impractical. In addition, the optimum assay conditions for BACE-1 activity (pH 5.0) and caspase activity (pH 8.0) require separation of the 2 enzymatic steps. This led us to develop an assay that is no longer parabolic, but has a linear relationship with incubation time.

**IMMUNOCAPTURE ASSAY FOR BACE-1 ACTIVITY**

An IgG fraction was purified from goat anti-rabbit serum (Cedar Lane Laboratories) by affinity chromatography on protein G-Sepharose (Amersham BiSciences). Rabbit anti-BACE-1 antibody (TNO-BACE-1-71) was purified by affinity chromatography from antiserum prepared by immunizing a rabbit with a synthetic peptide corresponding to residues 46–62 from the NH2 terminus of human BACE-1. Wells of flat-bottomed 96-well microtiter plates (Costar; high binding type 1) were coated overnight at 4 °C with 100 μL of the purified goat anti-rabbit immunoglobulin at 10 mg/L in carbonate buffer, pH 9.6 (Sigma), and washed 4 times with wash buffer [10 mmol/L sodium acetate (pH 5.0), 1.5 mmol/L NaCl, 0.1 g/L Brij-35]. Rabbit anti-BACE-1 IgG (0.4 mg/L) in wash buffer was added and incubated for 2 h at 37 °C in a humidified chamber, followed by 4 wash steps with wash buffer.

Soluble truncated recombinant BACE-1 was diluted in binding buffer (wash buffer containing 1 g/L bovine serum albumin) at 0–6 μg/L, and 100 μL was added per well as a control line. Human CSF samples or human brain extracts were diluted at least 10-fold in binding buffer, and 100 μL of each of these dilutions was added to the plate. After incubation for 2 h at 4 °C, the wells were washed 4 times with wash buffer, and 50 μL of detecting enzyme (procaspase-3 variant 1.1, 1.2, or 1.3) at 30 mg/L in wash buffer was added and incubated overnight at 25 °C. At the end of this incubation, 50 μL of 100 mmol/L Tris-HCl (pH 8.0), 1.5 mmol/L NaCl, 0.1 g/L Brij-35, 20 mmol/L DTT, 1.6 mmol/L DEVD-pNA was added and the plate was incubated at 25 °C. The color formation was monitored at 405 nm in a plate reader.

**PREPARATION OF BIOLOGICAL SAMPLES**

Human CSF samples from 10 individuals, obtained for diagnostic purposes, were used for this study. CSF was obtained by lumbar puncture between the L3/L4 or L4/L5 intervertebral space, using a 25-gauge needle, and collected in 12-mL polypropylene tubes. Within 2 h, CSF samples were centrifuged at 2100 g for 10 min at 4 °C. A small amount of CSF was used for routine analysis, including total cells (leukocytes and erythrocytes), total protein, and glucose. CSF was aliquoted in 0.5- or 1-mL polypropylene tubes and stored at −80 °C until further analysis.

Human brain tissue was obtained from The Netherlands Brain Bank. Autopsies were performed on donors with written informed consent from the donor or from direct next of kin. All procedures were approved by the local ethics committee of the Free University of Amsterdam. Human brain tissue samples (white matter) were

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**Table 1. Oligonucleotides used as inserts in BamHI- and EcoRI-cleaved procaspase-3 expression plasmids.**

<table>
<thead>
<tr>
<th>Prodomain-large subunit</th>
<th>cleavage sequence</th>
<th>small subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt H2N___LGGIE</td>
<td>D</td>
<td>SVGDDD....</td>
</tr>
<tr>
<td>1.1 H2N___LGGIE</td>
<td>GSKTEEISVNLDAFFE</td>
<td>HDSEF</td>
</tr>
<tr>
<td>1.2 H2N___LGGIE</td>
<td>GSEIDLVM</td>
<td>DEF</td>
</tr>
<tr>
<td>1.3 H2N___LGGIE</td>
<td>GSSEISYEVERF</td>
<td>E</td>
</tr>
</tbody>
</table>

Fig. 2. Schematic representation of the constructed procaspase-3 variants.

The sequences in solid rectangles represent in all constructs the wild-type procaspase-3 N-terminal large and C-terminal small subunit sequences, respectively. Shown in the dotted rectangles are the activation cleavage sequences of wild-type (wt) procaspase-3 (a single aspartic residue) and the 3 engineered procaspase-3-derived BACE-3 substrates. The activation cleavage positions in the constructs are indicated with filled arrowheads.
The soluble truncated recombinant BACE-1 was either obtained from Enzyme Systems Products or produced at Discovery Technologies, Novartis AG Basel. Recombinant cathepsin E was from R&D Systems (cat. no. 1294-AS), and porcine renin was obtained from Sigma (cat. no. R-2761). Recombinant cathepsin D and BACE-2 were produced at Novartis Pharma AG Basel. The BACE-1 inhibitor P10-P4' Stat (Val) was purchased from Bachem (cat. no. H-4848), and the BACE-1 inhibitor ANJ886 was prepared at Novartis. Anti-BACE-1 antiserum (cat. no. B0681) was purchased from Sigma.

**Materials and Methods**

**Development of a Sensitive Assay for BACE-1 Activity**

For the BACE-1 assay we used a modified procaspase-3 as detection enzyme. Wild-type single-chain procaspase-3 can be activated by cleavage after residue Asp175, giving a 2-chain active molecule [for a review see Ref. (19)]. We found that insertion/replacement of the original activation sequence between the 2 chains of procaspase-3 by an alternative activation sequence that is recognized and can be cleaved by the protease of interest gives a procaspase that can be activated by the target protease. We designed several modified procaspase-3 substrates (Fig. 2), based on reported BACE-1 cleavage-recognition sequences (12–14, 20, 21). Using standard molecular biology techniques, we constructed bacterial expression vectors coding for the various modified procaspase-3 molecules. The corresponding proteins were expressed in *E. coli* and purified.

**Comparison of BACE-1-Specific Procaspase-3 Substrates**

Three modified procaspase-3 variants were tested for their suitability as BACE-1 substrates. We found that each of the 3 variants was recognized and cleaved by BACE-1, giving caspase activity. We did, however, observe considerable differences in activation and background activities among the 3 different procaspase detection enzymes (Fig. 3). The signal obtained with substrate 1.2 was highest, followed by the signals obtained with substrates 1.3 and 1.1; however, the background activity, as measured without addition of BACE-1, was much lower for substrate 1.3 than for substrates 1.1 and 1.2. Thus, substrate 1.3 had a much higher signal-to-background ratio (116) compared with substrates 1.2 and 1.1 (57 and 5.8, respectively). We therefore used substrate 1.3 for further development of a BACE-1 activity assay.

**Results**

**Development of Assay for BACE-1 Activity**

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**Cross-reactivity with Other Proteases**

We also evaluated the activity of 4 other aspartyl proteases—BACE-2, cathepsin D, cathepsin E, and renin—with substrate 1.3. We found that all of these proteases had considerable activity with this substrate (Table 2). We thus concluded that in this format the assay can be used for only well-defined purified BACE-1 preparations, such as those used, for example, in inhibitor screening, but not for measuring BACE-1 activity in complex biological samples.

**Development of Assay for BACE-1 in Biological Samples**

To obtain the necessary specificity to detect BACE-1 activity in biological samples, we developed an immunocapture assay. In this assay, BACE-1 is captured from solution to a microtiter plate by an immobilized antibody (TNO-BACE-1-71), and after washing, the activity is determined as described above. In this immunocapture format, cross-reactivity with BACE-2, cathepsin D, cathepsin E, and renin was negligible (Table 2). The detection step of the assay can be performed either kinetically or with a fixed incubation time. The lower detection limit is dependent on the duration of the activation step, in which BACE-1 converts the procaspase-derived detection enzyme into active caspase (usually overnight), and the Table 2. Cross-reactivity of aspartyl proteases in BACE-1 solution and immunocapture assay.

<table>
<thead>
<tr>
<th>Protease</th>
<th>In solution</th>
<th>After capture</th>
</tr>
</thead>
<tbody>
<tr>
<td>BACE-1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>BACE-2</td>
<td>115</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>546</td>
<td>1.9</td>
</tr>
<tr>
<td>Cathepsin E</td>
<td>713</td>
<td>0.2</td>
</tr>
<tr>
<td>Renin</td>
<td>36</td>
<td>2</td>
</tr>
</tbody>
</table>
duration of the detection step, in which the resulting caspase-3 activity is determined. The assay is linear with BACE-1 concentration up to 15 \(\mu\)g/L for the 0.5-h detection period and up to 2 \(\mu\)g/L for the 4-h detection period (Fig. 4).

The detection limit, determined as activity exceeding the mean blank activity (\(n = 10\)) by at least 2 SD, varied from 0.2 \(\mu\)g/L (~4 pmol/L) for an overnight activation step and a 0.5-h detection step to 0.02 \(\mu\)g/L (~0.4 pmol/L) for an overnight activation step and a 4-h detection step. We determined intraassay variation by measuring a set of samples 5 times on the same plate with the same reagents. The intraassay CV was 4% at 1 \(\mu\)g/L and 8% at 0.1 \(\mu\)g/L. The interassay CV was 15% at 1 \(\mu\)g/L for 7 assays performed with the same batch of reagents (stored as frozen aliquots at \(-70^\circ\)C) over a period of 1 year. The relationship between measured BACE-1 activity (recombinant BACE-1 added to CSF) with dilution of CSF in buffer was linear for dilutions of 10-fold or higher; a 20-fold dilution of CSF was used routinely. Sample stability was not studied in depth, but we observed a significant loss of activity after repeated freezing and thawing of CSF samples. Therefore, storage in aliquots and avoidance of freezing-thawing appears advisable.

Detection of BACE-1 activity in brain extracts

Human brain tissue was frozen in liquid nitrogen, powdered in a mortar, and subsequently extracted with a buffer containing detergent. The BACE-1 activity in the extract was determined with the immunocapture assay. In all extracts activity was detected that could be inhibited to a large extent by the BACE-1 inhibitors ANJ886 and P10-P4’S (Val), indicating that the observed activity can be attributed to BACE-1. The BACE-1 activities were in the range of 0–10 ng/g brain tissue (Fig. 5).

Detection of BACE-1 activity in CSF

Human CSF obtained from a total of 10 patients and controls was used in the immunocapture assay. Unexpectedly, low but consistent activities could be measured in several of these CSF samples. The activities could be inhibited almost completely by the inhibitors ANJ886 and P10-P4’S (Val) (Fig. 6). The presence of BACE-1 activity in CSF is remarkable because BACE-1 is a membrane-bound protease. The authors of in vitro studies using cell cultures of BACE-1–transfected cells have reported the presence of soluble forms of BACE-1 (10–12), but no such data have been reported for biological fluids. In Fig. 6, samples 1–5 were from a variety of patients without dementia, samples 6–9 were from confirmed AD cases, and sample 10 was from a hydrocephalic patient.

Detection of BACE-1 protein in CSF

When we analyzed human brain extracts and CSF by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Western blotting and detection with an anti-BACE-1 antibody, the presence of BACE-1 in CSF could be confirmed. In CSF, a clear band could be seen with a size similar to that of the truncated recombinant BACE-1 used as the control (Fig. 7) and is in agreement with a truncated soluble form missing the transmembrane and intracellular domains.

Discussion

Current assay methods for BACE-1 activity are focused mainly on detection of inhibition of the activity of purified BACE-1. The present assay allows detection of BACE-1 activity in extracts of brain, heart, and CSF from human patients.
BACE-1 preparations and are frequently used in screening assays for identification of BACE-1 inhibitors in early-phase drug discovery. These enzymatic activity assays use peptide substrates with sequences that are cleaved by BACE-1 and are based on the sequences in the natural BACE-1 substrate APP; on mutant forms of APP, particularly the “Swedish mutant”, which is cleaved considerably faster than the wild-type APP sequence; or on forms selected by screening peptide libraries. Detection is generally based on fluorescence (12–14).

Apart from these assays, several other cellular assays intended for screening compounds for BACE-1 inhibition have been developed (14, 22, 23), but methods for convenient detection of BACE-1 activity in biological samples with sufficient specificity and sensitivity have not yet been established. In the present work we describe a convenient, rapid, and sensitive method for detection of BACE-1 activity in biological samples, based on immunocapture with a specific BACE-1–binding antibody and a sensitive 2-step detection method based on activation of an engineered proenzyme. The detection limit of this method corresponds to the activity of 0.02 µg/L active recombinant BACE-1. With this new method, BACE-1 activity could be detected in extracts of human brain tissue, and surprisingly, BACE-1 activity could also be detected in human CSF. The presence of a truncated, soluble form of BACE-1 in CSF could be confirmed by gel electrophoresis and Western blotting. The occurrence of soluble forms of BACE-1 has been described previously in cell cultures. In HEK 293 cells transfected with BACE-1 cDNA, a small fraction of BACE-1 was found to be present in the culture medium in a soluble form with a slightly smaller molecular mass (10, 11). There is convincing evidence that metalloproteinases are involved in the formation of the truncated soluble form of BACE-1 in cell culture because, in the presence of metalloproteinase inhibitors, the amount of the soluble form is decreased considerably. Results obtained in experiments with an ADAM10-specific inhibitor indicated that the metalloproteinase ADAM10 converts the native membrane-bound BACE-1 into a truncated soluble form, in a process known as shedding (11). In the present report we describe the occurrence of BACE-1 activity and protein in a soluble form in human CSF. In this limited study, we detected BACE-1 activity in CSF from all 4 AD patients and the 1 hydrocephalic patient tested, whereas such activity was detected in the CSF from only 1 of 5 patients without dementia. This observation might lead to the speculation that BACE-1 activity in CSF differs in AD patients and non-AD patients and merits a more thorough study with a larger number of well-characterized patients and appropriate controls. The measurement of soluble forms of BACE-1 in a relatively accessible biological fluid such as CSF might provide new possibilities for in vivo research on the AD disease mechanism, disease development, and monitoring of disease activity or therapy.

We thank Dr. S. Veenstra, NIBR-Basel, for the synthesis of ANJ886. Human brain materials were obtained through the kind collaboration of The Netherlands Brain Bank.

Fig. 6. Detection of BACE-1 activity in human CSF.

The BACE-1 activity in 10 human CSF samples was determined with the immunocapture assay and expressed as µg of active BACE-1/L of CSF. Samples 1–5 were obtained from a variety of non-AD patients; samples 6–9 represent 4 confirmed AD cases; and sample 10 is from a hydrocephalic patient. Results are the means of duplicate determinations. In the presence of a BACE-1 inhibitor, no activity could be detected in any of the samples (not shown).

Fig. 7. Detection of BACE-1 protein.

Human CSF was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting. Detection was performed with a specific BACE-1 antibody (cat. no. B0681; Sigma), followed by goat anti-rabbit peroxidase-labeled antibody, with detection by luminescence. Lanes 1 and 2: human CSF samples; lanes 3 and 4, truncated recombinant BACE-1 at 50 and 100 µg/L, respectively. The arrow indicates soluble truncated BACE-1 in human CSF.

References

protein by the transmembrane aspartic protease BACE. Science 1999;286:735–41.


