The Long Term Adenoviral Expression of the Human Amyloid Precursor Protein Shows Different Secretase Activities in Rat Cortical Neurons and Astrocytes*

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Recombinant adenoviruses were used for the expression of human amyloid precursor protein (APP) of Alzheimer’s disease in primary cultures of rat cortical neurons and astrocytes. The catabolic pathways of human APP were studied 3 to 4 days after infection, when the equilibrium of APP production was reached. Although the expression of human wild type APP (WtAPP) by rat neurons induced the production of both extracellular and intraneuronal amyloid peptide (Aβ), Aβ was not detected in the culture medium of rat astrocytes producing human WtAPP. Because a low β-secretase activity was previously reported in rodent astrocytes, we wondered whether modifications of the APP amino acid sequence at the β-secretase clipping site would modify the astrocytic production of Aβ. Interestingly, rat astrocytes produced high amounts of Aβ after expression of human APP carrying a double amino acid substitution responsible for Alzheimer’s disease in a large Swedish family (SwAPP). In both rat cortical neurons and astrocytes, the β-secretase cleavage of the human SwAPP occurred very early in the secretion process in a cellular compartment in which a different sorting of SwAPP and WtAPP seems unlikely. These results suggest that human WtAPP and SwAPP could be processed by different β-secretase activities.

Alzheimer’s disease is the most common form of dementia. The major hallmarks of this neurodegenerative disorder are intraneuronal neurofibrillary tangles as well as senile plaques containing an extracellular amyloid core associated with reactive astrocytes (1). The amyloid peptide (Aβ)1 is the major constituent of the amyloid deposits (2) and is derived from the amyloid precursor protein (APP) (3). The processing of human APP has been widely studied in transfected cells (4, 5). These cellular models have identified different proteolytic pathways for the processing of APP. A portion of APP is cleaved by α-secretase within the Aβ region. This non-amyloidogenic pathway, which precludes the formation of full-length Aβ, occurs during the processing of APP to the plasma membrane as early as in the trans-Golgi network (6, 7). This cleavage releases the N-terminal ectodomain of APP containing the first 17 amino acids of Aβ into the culture medium of transfected cells.

Another fraction of newly synthesized APP appears at the plasma membrane. Following endocytosis of this transmembrane APP, the cleavage by β-secretase at the N terminus of Aβ generates a C-terminal fragment of APP that contains the entire Aβ sequence. A second protease activity called γ-secretase cleaves this C-terminal fragment of APP to release the full-length amyloid peptide, which is detected in the extracellular medium (8).

In transfected cells, the cellular trafficking of APP plays a key role in the choice between the two catabolic pathways of the protein, since the non-amyloidogenic pathway occurs during the processing of APP to the plasma membrane, whereas endocytosis of APP is required for Aβ production (9). Because the intracellular trafficking of APP in transfected cells could be completely different from that in neuronal cells, it was important to study the metabolism of APP in neuronal cells.

So far as neuronal cells are concerned, two cellular models have been recently utilized. Cell lines derived from neuroblastoma (10) or carcinoma (11) allowed the expression of human APP following transfection or infection with a recombinant Semliki Forest virus (SFV) (12, 13). Primary cultures of rat hippocampal neurons have also been used to express human APP following infection by a recombinant SFV. The SFV system allows sufficient amounts of human APP to be produced for biochemical analysis. In this way, it was possible to demonstrate that primary cultures of rat hippocampal neurons produce human extracellular and intracellular Aβ by different mechanisms (14). At least one intraneuronal location for β-cleavage of human APP is within the endoplasmic reticulum (15). It is therefore evident that rat neurons process human APP differently from transfected cells. Although the SFV system allows a high production of human APP into neuronal cells, it induces a pronounced cytopathic effect a few hours after infection (16). The influence of this cytopathic effect on the short term expression and metabolism of APP remains questionable. In addition, glial cells are infected much less efficiently than the neuronal SFV than human APP, making a comparison between glial cells and neuronal cells rather difficult.

We have constructed recombinant adenoviruses that allow the long term expression of human APP into primary cultures of rat cortical neurons and astrocytes without cytopathic effect.
We found that human wild type APP (WtAPP) is processed in a distinct manner in rat cortical neurons and astrocytes. Although rat cortical neurons produce both extracellular and intracellular Aβ from human WtAPP, the amyloidogenic pathway of human WtAPP was not detected in astrocytes. On the contrary, astrocytes produce high amounts of Aβ from human APP carrying a double amino acid substitution responsible for Alzheimer’s disease in a Swedish family (SwAPP). In both neurons and astrocytes, the β-cleavage of SwAPP occurs early in the secretion process. This suggests that WtAPP and SwAPP could be processed by different β-secretase activities.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The anti-MAP2 rabbit serum has been previously described (17). The anti-GFAP rabbit polyclonal antibody was provided by DAKO (Denmark). The L6 is an anti-Aβ rabbit polyclonal antisera. The FCA18 anti-Aβ serum (a generous gift from F. Checler) has an absolute requirement of the first aspartyl residue of the amyloid peptide (18). The 3H5 mouse monoclonal antibody raised against the anionic extracellular domain of APP has been described (19). The W02 mouse monoclonal antibody raised against the Aβ 5–8 sequence was a generous gift of K. Beyreuther (20). The epitopes of the W02 and 3H5 anti-Aβ antibodies are shown in Fig. 5.

**Cell Culture**—Rat cortical neurons were prepared from the brain of 17-day-old Wistar rat fetuses and cultured in neurobasal medium supplemented with 2% B-27 and 0.5 mM L-glutamine (Life Technologies, Inc.) in culture dishes previously coated with poly(lysine) (10 μg/ml in phosphate-buffered saline) (21).

Rat cortical astrocytes were prepared from the brain of 1-day-old newborn Wistar rats (22) and cultured in Dulbecco’s modified Eagle’s medium culture medium supplemented with 20% fetal calf serum, penicillin (50 IU/ml), streptomycin (50 μg/ml), and 0.05% glutaraldehyde for 5 min and incubated with X-gal solution (5b) following the metabolization of o-nitrophenyl β-D-galactopyranoside as described (26).

APP metabolites were analyzed by immunoprecipitation and Western blot as described (27). Neurons and astrocytes cultured on 3-cm dishes were infected with either AdRSVWtAPP or AdRSVsWAPP. The culture medium and the cellular extracts were harvested during the following days. The protein concentration in cell lysates was measured using the BCA method (Pierce-USA) and bovine serum albumin as a standard. Human APP was detected in Western blot analysis of 25 μg of cell lysate proteins or 25 μl of culture medium with the 3H5 (19) or the W02 (20) monoclonal antibodies using the ECL Western blotting detection reagents (Amersham Pharmacia Biotech-Nederland). The immunoprecipitate was analyzed by Western blot on a 4–12% Nupage gel (NOVEX-USA) using the W02 (20) antibody. Quantification of APP and Aβ Production—APP was quantified in immunoblotting experiments using the 3H5 monoclonal antibody (19), a secondary 125I-labeled goat anti-mouse Ig (Amersham Pharmacia Bio-tech-Nederland) and exposure of immunoblots to phosphorimaging plates (Cyclone, Packard Instrument Co.). The same batch of radiolabeled antibody was used to quantify Aβ immunoprecipitated by the L6 antibody and detected in immunoblotting by the W02 monoclonal antibody (20). A standard curve ($r = 0.9987$) was generated by immunoprecipitation and immunoblotting of appropriate synthetic Aβ standards diluted in the same volume of identical culture medium. Quantification of immunoblots was performed using the Optiquant software.

**RESULTS**

**Expression of β-Galactosidase in Rat Cortical Neurons or Astrocytes Using a Recombinant Adenovirus**—Recombinant adenoviruses were used as an expression system in primary cultures of both rat cortical neurons and astrocytes as described previously (28). To compare in details the relative efficiency of infection by this vector, rat cortical neurons and astrocytes were infected by a recombinant adenoavirous carrying the LacZ gene (AdRSVβ-gal) at different multiplicities of infection, and the β-galactosidase activity was revealed by histochemistry. Thereafter, neurons were identified by immunocytochemical detection of microtubule-associated protein 2 using an anti-microtubule-associated protein 2 serum, whereas astrocytes were specifically labeled by an anti-α-glial fibrillary acidic protein serum. Those labeling demonstrated that more than 95% of the cells correspond to either neurons or astrocytes. Rat cortical neurons were cultured for 7 days before infection by recombinant adenoviruses. The β-galactosidase activity was observed in the nucleus of infected cells (Fig. 1, A and B) as a result from the fusion of the β-galactosidase with a nuclear localization signal. 63 ± 4% ($n = 25$) of neurons expressed β-galactosidase following infection by AdRSVβ-gal at multiplicity of infection of 10, whereas 59 ± 10% ($n = 25$) of astrocytes showed a blue nucleus at a multiplicity of infection of 1. These results indicate therefore that the relative efficiency of infection by AdRSVβ-gal is about 10 times higher in astrocytes than in neurons. The production of β-galactosidase was measured as a function of time in rat cortical neurons infected by AdRSVβ-gal using o-nitrophenyl β-D-galactopyranoside as a substrate (26). The results presented in Fig. 1C indicate that the produc-
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Fig. 1. Detection of β-galactosidase activity in rat cortical neurons and astrocytes infected by AdRSVβ-gal. Rat cortical neurons (A) and astrocytes (B) were infected by AdRSVβ-gal at a multiplicity of infection of 10 and 1, respectively. The β-galactosidase activity was detected as described previously (28), and cell types were identified by immunocytochemical detection of microtubule-associated protein 2 (A) and anti-glial fibrillary acid protein (B). At indicated times (C), the β-galactosidase activity in the cell lysate of infected neurons and astrocytes was quantified at 420 nm using o-nitrophenyl β-D-galactopyranoside as a substrate (26). Means ± S.D. of three different experiments are shown.

Fig. 2. Production of intracellular APP, extracellular APP and extracellular Aβ by rat cortical neurons infected by AdRSVWtAPP. Rat cortical neurons were infected by AdRSVWtAPP, and the time course of human APP and Aβ generation was measured. At indicated times, extracellular and intracellular human APP was detected by Western blot analysis (WO2) (20) of 25 µl of culture medium and 25 µg of cellular proteins, respectively. Extracellular Aβ was immunoprecipitated by the L6 polyclonal anti-Aβ serum from 1 ml of culture medium; the immune precipitate was then analyzed in Western blot (WO2).

WO2 monoclonal antibody recognizing the Aβ 5–8 amino acid sequence (20). The production of intraneuronal human APP reached a maximum 2 days after infection, whereas the production of extracellular human WtAPP increased up to 3 days after infection (Fig. 2). Although rat cortical neurons produce endogenous APP, this protein was not detected by the WO2 monoclonal antibody, which is specific for the human Aβ sequence (20). It has been previously shown that rodent-cultured neurons are not able to efficiently process rodent APP into Aβ (29). However, when rat cortical neurons were infected by AdRSVWtAPP, they produced extracellular human Aβ (Fig. 2). The kinetic of production of extracellular Aβ (Fig. 2) was very similar to that of APP and was maximal after 3 days of infection. This human APP and Aβ production remained stable as long as the cells survived in culture (up to 7 days after infection). Rat cortical neurons expressing human APP were also able to produce intracellular Aβ (Fig. 3). The production of intraneuronal Aβ has been previously reported in rat-cultured neurons infected by a SFV recombinant for human APP (26). Because of the cytopathic effect of the SFV system, the expression of human APP can be measured only during a very short period of time. Using recombinant adenovirus, we measured the production of intraneuronal Aβ 3 days after infection, when the production of human APP reached a plateau. Interestingly enough, the production of both extracellular and intracellular Aβ by rat cortical neurons during several days did not induce significant neuronal modification.

Fig. 3. Production of extracellular and intracellular human APP and Aβ at indicated times. Extracellular Aβ production reached a maximum 2 days after infection, whereas the production of intraneuronal human APP reached a plateau. Interestingly enough, the production of both extracellular and intracellular Aβ by rat cortical neurons during several days did not induce significant neuronal modification.

Fig. 4. Production of intracellular APP, extracellular APP and extracellular Aβ by rat cortical neurons infected by AdRSVWtAPP. Rat cortical neurons were infected by AdRSVWtAPP, and the time course of human APP and Aβ generation was measured. At indicated times, extracellular and intracellular human APP was detected by Western blot analysis (WO2) (20) of 25 µl of culture medium and 25 µg of cellular proteins, respectively. Extracellular Aβ was immunoprecipitated by the L6 polyclonal anti-Aβ serum from 1 ml of culture medium; the immune precipitate was then analyzed in Western blot (WO2).

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Rat-cultured Astrocytes Infected by AdRSVWtAPP Produce Intracellular and Extracellular APP—When rat-cultured astrocytes were infected by AdRSVWtAPP, human WtAPP was found in both cellular extract and culture medium (Fig. 4). The production of intracellular and extracellular human APP by infected rat astrocytes showed a kinetics very similar to that observed in infected neurons. Although human WtAPP was produced by rat astrocytes to levels comparable with neurons, Aβ was undetectable in both cellular extracts and culture medium (Fig. 4). This absence of Aβ in rat astrocytes expressing human WtAPP could result from the rapid degradation of the amyloid peptide. To test this hypothesis, rat astrocytes were incubated for 24 h at 37 °C in the presence of the culture...
Rat cortical neurons were infected by AdRSVWtAPP. Three days after infection, the infected cells were lysed, and 1 mg of cellular proteins were immunoprecipitated with the L6 polyclonal anti-Aβ serum. The immunoprecipitate was analyzed by Western blot using the WO2 monoclonal antibody.

After infection of rat astrocytes by AdRSVWtAPP, the kinetics of production of APP was analyzed by Western blot (WO2) of 25 μl of culture medium and 25 μg of cellular proteins. Immunoprecipitation of 1 ml of culture medium with the L6 polyclonal anti-Aβ serum and analysis of the immunoprecipitate by Western blot (WO2) does not allow detection of Aβ.

Expression of the Human APP Carrying the Double Swedish Mutation in Rat Neurons and Astrocytes—The astrocytic β-secretase activity has been previously reported to be very inefficient (30). We wondered whether modifications of the APP amino acid sequence at the β-secretase cleavage site could influence the production of Aβ by rat astrocytes. In a large Swedish family with Alzheimer’s disease, the APP gene carries the first aspartyl residue of the amyloid peptide (18). Analysis of this immunoprecipitate in Western blot with the WO2 antibody clearly indicates that rat astrocytes expressing human WtAPP favor the β-secretase cleavage of the human WtAPP. The WO2 antibody (Fig. 5A) was used in Western blot analysis of extracellular medium from neurons expressing either WtAPP or SwAPP. The results presented in Fig. 5B show that the 3H5 antibody detected extracellular human SwAPP and WtAPP, part of the extracellular SwAPP being found at a lower molecular weight, as compared with the WtAPP. This shift in the molecular weight could result from a predominant β-secretase cleavage of the SwAPP. This was confirmed by the analysis of the same culture medium with the WO2 antibody, which only recognizes soluble APP resulting from the α-secretase cleavage. Levels of secreted SwAPP were indeed much lower than WtAPP when detected by the WO2 antibody. This indicates that in rat neurons, SwAPP favors the β-secretase cleavage at the expense of the α-secretase cleavage, as it has been previously reported in other cell types (10). As compared with WtAPP, the expression of human SwAPP by rat cortical neurons significantly increased the production of Aβ in both the culture medium (Fig. 5B) and the cellular extracts (not shown). Quantification of Aβ in the culture medium of rat neurons infected by AdRSVWtAPP or AdRSVSwAPP indicates that rat neurons expressing SwAPP secrete 6.4 times more Aβ as compared with neurons expressing WtAPP (Fig. 6A).

Human SwAPP was expressed in rat astrocytes. The resulting soluble SwAPP was also characterized by a faster migration in SDS-polyacrylamide gel electrophoresis, as compared with the WtAPP (Fig. 5C). The culture medium of rat astrocytes expressing human SwAPP was immunoprecipitated by the FCA18 anti-Aβ serum, which has an absolute requirement of the first aspartyl residue of the amyloid peptide (18). Analysis of this immunoprecipitate in Western blot with the WO2 antibody clearly indicates that rat astrocytes expressing human SwAPP produce Aβ with an N-terminal sequence starting at Asp1. This β-cleavage of human SwAPP by rat astrocytes was also concomitant with a reduction of the α-cleavage, as demonstrated by the analysis of soluble WtAPP and SwAPP with the WO2 antibody (Fig. 5C). Quantification of Aβ in the culture medium of rat astrocytes infected by AdRSVSwAPP indicates that rat astrocytes expressing SwAPP secrete Aβ at levels that are 3.7-fold higher than those measured in the culture medium of neurons expressing WtAPP (Fig. 6B).

Four days after infection, the production of Aβ by astrocytes...
expressing SwAPP induced an important cellular toxicity, leading to a dramatic loss of cellular proteins. Although variations of the cellular protein content were measured between different cultures of astrocytes, a 95% loss of cellular proteins was measured 4 days after infection of rat astrocytes by AdRSVSwAPP, as compared with astrocytes expressing WtAPP at a similar level (Fig. 7). Altogether, these results demonstrate that the β-secretase cleavage of human WtAPP is very inefficient in rat astrocytes. On the contrary, rat astrocytes contain a β-secretase that cleaves SwAPP with high efficiency.

**DISCUSSION**

We have shown here that recombinant adenoviruses can be used to overexpress proteins in primary cultures of rat cortical neurons and astrocytes. The expression of the transgene can be maintained as long as the cells survive in culture. Although astrocytes are infected more efficiently with recombinant adenoviruses than are neurons, a very similar expression can be achieved in both cell types by using different multiplicities of infection.

The use of a recombinant adenovirus carrying the human WtAPP 695 cDNA sequence allowed us to study the processing of human APP in rat-cultured neurons and astrocytes. The present paper is, to our knowledge, the first to study the long term expression and metabolism of human APP in both rat cortical neurons and astrocytes. Rat cortical neurons expressing human WtAPP produce extracellular and intracellular amyloid peptide during several days. This sustained production of amyloid peptide does not induce any significant neurotoxicity.

Although rat-cultured astrocytes are able to produce high amounts of human WtAPP, large amounts of Aβ were found in the culture medium of rat astrocytes expressing human SwAPP. The production of the amyloid peptide by rat cortical astrocytes induces a very important cellular toxicity. These results demonstrate therefore that, contrary to neurons, a sustained production of amyloid peptide by astrocytes induces a dramatic cellular toxicity.

The production of Aβ from human SwAPP by rat astrocytes could have two explanations. Either the astrocytic β-secretase activity could be located in a cellular compartment that is accessible only to human SwAPP and not to WtAPP or the astrocytic β-secretase activity could cleave human SwAPP and not WtAPP. In both rat cortical neurons and astrocytes, the β-secretase cleavage of the human SwAPP occurs at the expense of the α-secretase cleavage. In mouse neuroblastoma N2a cells, the same observation allowed to conclude that the β-secretase cleavage of the SwAPP occurs in the medial compartment of the Golgi apparatus and disables APP molecules that would normally be cleaved by α-secretase (10). Therefore, the β-secretase cleavage of human SwAPP by rat-cultured astrocytes has to occur very early in the secretion process in a cellular compartment in which a different sorting of WtAPP and SwAPP seems unlikely. Therefore, our results suggest that the amyloidogenic cleavage of human SwAPP in rat astrocytes is performed by a β-secretase that does not cleave the human WtAPP.

In conclusion, we report here the long term expression of human Wt and SwAPP in rat cortical neurons and astrocytes, and demonstrate that the Swedish mutation alters APP processing in both cell types. In neurons, SwAPP is processed in a pathway that increases the production of extracellular and intracellular Aβ. In astrocytes, the production of Aβ, resulting from the expression of SwAPP, induces an important cellular toxicity. The processing of human SwAPP by rat-cultured astrocytes uses a β-secretase activity, which is very inefficient on WtAPP. The dramatic effect of the Swedish mutation on the metabolic processing of APP in astrocytes, in contrast to neurons, suggests that astrocytes could be an efficient model for studying amyloidogenic processing of APP in vivo.
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astrocitic processing of APP suggests that cell types other than neurons could contribute to elevate Aβ production in some FAD cases. It remains, however, that the recent identification of Aβ in the extracellular medium of primary cultures of human astrocytes (32) highlights an important difference between rodent and human astrocytes in the processing of human APP. Nevertheless, coexpression of human WtAPP and protease activities in rat astrocytes will be important for the further characterization and identification of β-secretase activities.

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