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Guinea Pigs as a Nontransgenic Model for APP Processing *in Vitro* and *in Vivo**

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Alzheimer's disease (AD) is characterized, amongst others, by the appearance of vascular and parenchymal β-amyloid deposits in brain. Such aggregates are mainly composed of β-amyloid peptides, which are derived by proteolytic processing of a larger amyloid precursor protein (APP). APP is highly conserved among mammalian species, but experimental studies in rodents are often hampered by the humble APP-processing in the amyloidogenic pathway and by the inability of rodent β-amyloid peptides to form higher molecular aggregates such as soluble oligomers and insoluble β-amyloid plaques. Thus, there is need for in vitro and in vivo model systems that allow identification of factors that increase amyloidogenic APP processing and accelerate β-amyloid plaque formation and testing the potency of pharmacological manipulations to ameliorate β-amyloid load in brain. Transgenic mice that overexpress human APP containing AD-associated mutations that favor the amyloidogenic pathway of APP processing represent such a model. However, mutations of the APP gene are not frequent in AD and, therefore, the mechanisms of β-amyloid plaque formation, the composition of β-amyloid plaques, and the accompanying tissue response in brain of these animals may be different from that in AD. In contrast, guinea pigs express β-amyloid peptides of the human sequence and appear to represent a more physiological model to examine the long-term effects of experimental manipulations on APP processing and β-amyloid plaque formation in vivo. Additionally, APP processing in guinea pig primary neuronal cultures has been shown to be similar to cultures of human origin. In this article we highlight the advantages and limitations of using guinea pigs as experimental models to study APP processing.

KEY WORDS: Alzheimer's disease; amyloid precursor protein; β-amyloid; secretases; aging; animal model.

APP Expression and APP Processing Pathways

There is ample evidence suggesting that increased expression or altered processing of amyloid precursor protein (APP) is one of the early events in the pathogenesis of Alzheimer's disease (AD). APP is a family of glycosylated transmembrane proteins that are ubiquitously expressed but are most abundant in the brain (for review see 1). The APP gene maps to chromosome 21 in humans (2,3) and constitutes a family of different isoforms that are derived by alternative splicing of APP mRNA and named according to their length in amino acids (for review see 4). APP can be processed by both amyloidogenic or nonamyloidogenic pathways. The nonamyloidogenic secretory pathway includes cleavage of APP by an α-secretase within the β-amyloid sequence, which generates a secreted water-soluble 90–100 kD protein while the membrane-anchored C-terminal fragment of APP (P3-CT) remains

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in the cell (5–8). Such α-secretase activity has been attributed to the metalloproteinases ADAM9 (also called MDC9) (9) and ADAM10 (10). The β-secretase, which initiates the generation of β-amyloid peptides by proteolytic cleavage of APP at the N-terminus of β-amyloid (11,12), has been identified recently (β-secretase, also called beta-site APP-cleaving enzyme 1; BACE1) (13–15). The γ-secretase, which finally liberates β-amyloid or P3-peptides, is not yet identified definitely. There is accumulating evidence, however, that presenilins, or a protein complex containing presenilins, possess γ-secretase activity (16–18).

Soluble β-Amyloid Peptides, Oligomers, β-Amyloid Fibrils, and β-Amyloid Plaques—Who Is the Culprit?

It is of particular interest to understand which form of β-amyloid is toxic to neurons or interferes with the learning and memory process even in the absence of neurotoxicity. In early studies, the number of β-amyloid plaques has been shown to correlate with the extent of clinical severity of AD (19–21), a finding that remains controversial (for overview see 22). Later on, studies in AD patients (22,23) and aged animals (24) demonstrated a close correlation between the mean "amyloid load" (i.e., volume of amyloid plaques per volume brain tissue) and the degree of cognitive dysfunction. However, more recently it was demonstrated that passive immunization with an antibody against β-amyloid peptides reduced levels of β-amyloid peptides and reversed memory deficits in APP transgenic mice even while the number of amyloid plaques was not reduced (25,26). This provides some evidence arguing that not the number or the concentration of β-amyloid plaques but the levels of soluble β-amyloid peptides predict the extent of memory deficits. In line with that, soluble β-amyloid levels measured in postmortem brains could distinguish between people who had plaque pathology but no dementia and those who also exhibited clinical dementia (27), and soluble β-amyloid levels have been shown to correlate better with disease severity than do β-amyloid plaque levels (28). In addition, soluble β-amyloid peptides but not amyloid plaques are implicated in spatial learning deficits in APP-transgenic mice (29). The mechanisms by which soluble β-amyloid peptides interfere with learning and memory are not completely understood, but there is evidence of perturbance of hippocampal long-term potentiation in rats (30) and in rat hippocampal slices (31) by β-amyloid oligomers. Additionally, such oligomers have been reported to cause neurological dysfunction independent of neuronal degeneration (32). Another mechanism by which β-amyloid peptides may perturb cognition is the interference with the major steps that constitute cholinergic neurotransmission (for review see 33). For example, solubilized β-amyloid peptides strongly inhibit the potassium-stimulated release of acetylcholine from hippocampal slices (34) and decrease ChAT activity but not acetylcholinesterase activity in the cholinergic SN56 cell line (35). β-Amyloid peptides also decrease the intracellular acetylcholine concentration (36) and impair M_1 receptor–associated signalling (37) in primary septal or cortical cultures. The effects of β-amyloid on reduction of pyruvate dehydrogenase activity (36), the key enzyme for the generation of acetyl-CoA used for neurotransmitter synthesis and the citrate cycle, might explain both cholinergic hypoactivity and metabolic dysfunction of cholinergic neurons after exposure to β-amyloid peptides at low concentrations.

Additionally, the concentration or ratio of distinct β-amyloid species appears to be a critical factor for β-amyloid plaque formation and for the genesis of memory deficits. The C-terminal variants ending at Val40 and Ala42 constitute the majority of β-amyloid proteins present in plaques (38,39). Although β-amyloid 1–42 is more enriched in parenchymal plaques than β-amyloid 1–40, the shorter isoform is more abundantly secreted (40,41). These observations led to the seeding hypothesis, wherein β-amyloid 1–42 serves as seed for plaque formation and β-amyloid 1–40 is incorporated later as β-amyloid peptides deposit in the AD brain (42,43). However, there are remarkable differences in the ability of β-amyloid 1–42 and β-amyloid 1–40 to form β-amyloid fibrils in vivo and in vitro, as discussed in detail by Shin et al. (44).

Transgenic Animal Models of Amyloidogenesis

To understand mechanisms of β-amyloid plaque formation, animal models that mirror that feature of AD are required. Such models are also of great interest for pharmacological studies aimed at slowing down the progression of β-amyloid plaque formation or even removing existing β-amyloid plaques from the brain. However, when establishing rodent models it has to be taken into account that β-amyloid from mice and rats contains three amino-acid substitutions as compared to human β-amyloid (R5G, Y10F, and H13R; see also Fig. 1A). These alterations were shown to influence APP processing (45,46) and the ability of β-amyloid peptides to form secondary structures such as oligomers and fibrils (47,48). This could explain the virtual absence of β-amyloid deposits in normal or aged rodent brain.

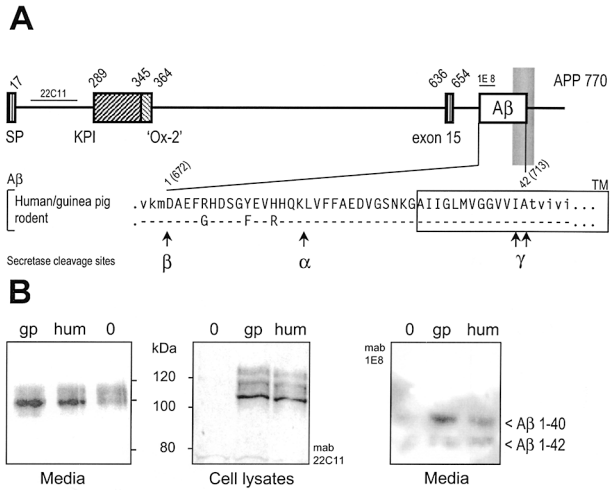

Fig. 1. A, Schematic representation of the APP molecule, depicting the signal peptide (SP), alternative spliced exons, the Aβ and transmembrane regions, and epitopes of antibodies used (22C11, 1E8). The sequence of human/guinea pig and rodent (rat, mouse) Aβ (in capitals) is shown in the lower part, indicating amino acid substitutions and secretase cleavage sites as well as the partial membrane insertion of Aβ. The similarity of APP orthologues in different species is greater than 90% troughout the molecule with exception of the signal peptide and the 'Ox-2' homology domain, which do show higher divergence. The R676G exchange is a key factor for the reduced amyloidogenic processing of rodent APP (ref. 45). **B,** Heterologous expression of gpAPP in human SY5Y neuroblastoma cells. Western blot analysis of secreted (left panel) and cellular APP (middle), as well as Aβ detection in conditioned cell culture media of SY5Y cells transfected with gpAPP695 (gp), human APP695 (hum), or vector (pCEP4) only control (0) utilizing monoclonal antibodies 22C11 (APP blots) and 1E8 (Aβ). Analysis was performed as described elsewhere (ref. 58). There are virtually no differences between human and gpAPP detectable.

Therefore, valuable models of β-amyloid plaque formation have been developed by overexpression of human APP constructs containing APP-associated mutations that favor the amyloidogenic β-secretase pathway of APP processing (see e.g. 49–54). These mice have been used to test therapeutic strategies aimed at reducing β-amyloid plaque formation, for example, by treatment with antiinflammatory compounds and by immunization approaches. However, mutations of the APP gene are not frequent in AD and, therefore the mechanisms of β-amyloid plaque formation, the molecular composition of β-amyloid plaques, and accompanying tissue response in brain of these animals may be different from that in the AD brain.

The Guinea Pig as a Nontransgenic Animal Model of Amyloidogenesis

As discussed above, in rodents the β-amyloid sequence differs from that of human β-amyloid by three amino acid substitutions. There are, however, other animal species expressing β-amyloid peptides identical to the human β-amyloid sequence (55) and some of them (hamster, guinea pig, and rabbit) appear to be well suited for experimental studies in vitro and in vivo. Before such animals could be used for biochemical analysis of APP processing in vivo, general properties of APP, its expression profiles, and APP processing pathways in vitro need to be documented. Therefore, in the first step, we cloned and sequenced the entire coding region of guinea pig APP (gpAPP) and used this construct for recombinant expression of gpAPP in human neuroblastoma cells.

Sequence analysis of gpAPP cDNA revealed similarities of approximately 90% and 97% to human APP at gene and protein level, respectively, a complete conservation of protein domain structure in human and gpAPP (for details see 56), and alternative splicing that occurs exclusively at exons 7, 8, and 15, generating an isoform pattern equivalent to human APP (for schematic presentation see Fig. 1A). The APP695 isoform is most abundant in guinea pig brain, whereas the longer isoforms are predominantly expressed in peripheral organs such as muscle and liver (56).

In Vitro Studies

When recombinant gpAPP was expressed in human SY5Y neuroblastoma cells, we observed comparable amounts of amyloidogenic processing products as compared to transfected human APP, indicating that there are no intrinsic sequence-specific factors that influence gpAPP processing (Fig. 1B). Likewise, in guinea pig cerebrospinal fluid (CSF) approximately 80%–90% of β-amyloid peptides were of the 1–40 species, which mirrors the situation in human CSF (57).

GpAPP expression and processing was further characterized in primary cell cultures derived from guinea pig embryonic brain at gestation day 24 (58). We observed that APP expression and β-amyloid formation increased during cultivation in parallel with cellular maturation. At approximately 10–14 days in vitro a stable phase was reached, thus providing a suitable time for analysis. Western blot analysis of conditioned culture medium revealed accumulation of endogenous β-amyloid peptides shown to consist of approximately 80%–90% β-amyloid 1–40 and able to form oligomeric aggregates. Furthermore, it was shown that single neurons retain their typical pattern of APP isoform expression throughout cultivation (58).

Further analysis of guinea pig primary cultures include the effects on APP processing of the protein phosphatase inhibitor okadaic acid (59). In this study we observed a dose-dependent upregulation of intracellular

and secreted APP, while the amount of β-amyloid peptides was decreased (59). These data resemble and extend previous observations of increased nonamyloidogenic APP processing under conditions of increased protein phosphorylation (for review see 4) in a more physiological experimental system.

Fetal guinea pig brain cultures were also used to reveal the role of glycophosphatidylinositol-anchored proteins in secretory APP processing and β-amyloid generation (60). By treatment of guinea pig brain primary cultures with phosphatidylinositol-specific phospholipase C and a series of other experiments it was shown that one or more glycophosphatidylinositol-anchored proteins play an important role in β-secretase activity and in β-amyloid biogenesis.

The human β-amyloid sequence of gpAPP and the significant amounts of β-amyloid peptides present in conditioned medium of primary neuronal cultures derived from guinea pig brain and in guinea pig CSF made it possible to establish novel techniques for the quantification of human β-amyloid peptides using guinea pig biological samples. Clarke et al. (61) developed an immunoprecipitation-HPLC-MS procedure to detect β-amyloid peptides with a comparable sensitivity performance as compared to ELISA assays. The advantages of the MS detection system are a higher specificity and greater flexibility, which yield much more information from a single sample than an ELISA assay. Likewise, a liquid phase electrochemiluminescent detection system introduced by Khorkova et al. (62) for the quantification of β-amyloid peptides and secretory APP fragments offers advantages with regard to assay time and the linearity of the signal across a wide range of concentrations.

In Vivo Studies

The α-secretory, nonamyloidogenic processing of the APP is regulated in part by mechanisms that involve protein kinase C (PKC) (63–65; for review see 4). Disturbances in PKC activation or activation of PKC coupled receptors have been implicated in the increased amyloidogenic APP processing in the brains of AD patients (66,67). To test the hypothesis of a reciprocal relation between secretory APP processing and generation of β-amyloid peptides in vivo, neocortical PKC activity was constitutively overactivated in guinea pig brain by in utero treatment with methylazoxymethanol (MAM). This treatment resulted in increased basal and phorbol ester-stimulated PKC-activity (by up to 70%) and in elevated secretory APP processing (by 35%) in cortex of MAM-treated guinea pigs (68). Subsequently, PKCα and PKCβ1 isoforms were identified as the key regulators of α-secretory APP processing in this experimental paradigm (69). The levels of β-amyloid peptides, however, remained unchanged as compared to control animals, suggesting an independent regulation of α- and β-secretase pathways (68). These results are inconsistent with those demonstrating reduced β-amyloid formation under conditions of increased APP α-secretion in vitro, but corroborate other reports, demonstrating no association between APP α-secretion and β-amyloid generation in vitro (70–73). These data suggest that competition for the substrate APP in the α-secretase and β-secretase pathway only occurs when one pathway is upregulated dramatically. This implies that pharmacological stimulation of PKC-coupled neurotransmitter receptors might increase secretory APP processing but not reduce β-amyloid generation or slow β-amyloid plaque formation.

This hypothesis is supported by observations made by Stephenson and Clemens (74) after the direct stimulation of the PKC-coupled metabotropic glutamate receptor by agonist infusion in the guinea pig hippocampus. The activation of this glutamate receptor failed to reduce β-amyloid levels but rather increased intraneuronal labelling for β-amyloid.

On the other hand, these observations are partly contradictory to those made by Beach et al. (75) after increased cholinergic stimulation by systemic administration of the acetylcholinesterase inhibitor physostigmine to guinea pigs for 10 days. In this experimental setup, reduced concentrations of insoluble but not of soluble β-amyloid 1–40 and 1–42 peptides were detected in guinea pig cortex (75), indicating a protection from amyloidogenesis after stimulation of PKC-coupled acetylcholine receptors.

Epidemiological studies demonstrated a strong reduction in the incidence of AD by estrogen replacement therapy in postmenopausal women and in patients treated with cholesterol-lowering statins. The question whether these are direct effects of estrogens and statins was addressed in experimental studies in vivo using guinea pigs. In the first study, female guinea pigs were subjected to ovariectomy followed by estrogen treatment (76). The authors observed an increase in total β-amyloid levels and in the ratio of β-amyloid 1–42 to 1–40 peptides after ovariectomy, which was attenuated by treatment of ovariectomized guinea pigs with 17β-estradiol (76). Another study demonstrated the reduction of cerebral β-amyloid 1–40 and 1–42 levels in guinea pig brain and CSF after systemic treatment with the cholesterol-lowering drug simvastatin (77).

Furthermore, guinea pigs have been used to reveal whether breakdown of the blood-brain barrier after experimental thiamine deficiency leads to the formation of β-amyloid aggregates in brain. However, despite prolonged thiamine deprivation and advanced neurological symptoms, no altered APP or β-amyloid immunostaining was detected (78). Given that the formation of β-amyloid plaques is a process that may require years, this observation is not surprising, but it is not unlikely that manipulations that alter the concentration of β-amyloid peptides could possibly contribute to β-amyloid plaque formation.

CONCLUSIONS

In summary, data from our own work and that of others demonstrate that guinea pigs represent a valuable nontransgenic animal model to follow APP metabolism in vitro and after experimental modulations or pharmacological treatments in vivo. The advantages of guinea pigs over rodents are the human β-amyloid sequence of gpAPP and the higher activity of the β-secretase pathway in guinea pig brain, as shown here for the first time, employing a BACE enzymatic assay (Fig. 2). Additionally, the higher amount of brain tissue and CSF samples that can be collected alleviate the analysis of APP and fragments thereof. Guinea pig β-amyloid peptides form higher molecular structures such as oligomers, which are reported to be involved in the suppression of long-term potentiation and that have the potential of β-amyloid plaque formation in a more chronic process than in APP-transgenic mice.

However, such experiments on amyloid plaque formation in guinea pigs after experimental manipulations are time consuming, the reproduction kinetics of guinea pigs is low (about 70 days of pregnancy, 2–5 offspring per litter), and guinea pigs are not a proper animal species to perform learning or memory tasks. Additionally, in many cases the cross-reactivity of antibodies raised against human or rodent antigens is not known and analysis of gene expression is hindered by the low number of genes sequenced in guinea pigs. Nevertheless, for biochemical studies aimed at understanding APP processing pathways in a more physiological environment than in APP transgenic mice, guinea pigs are an appropriate experimental animal model.

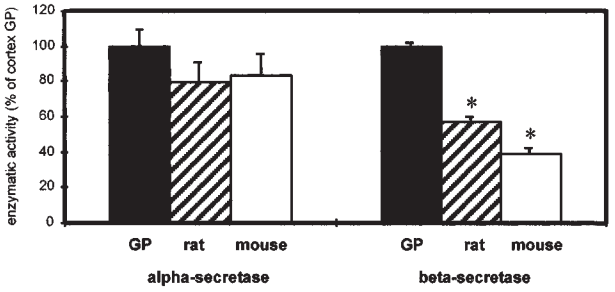

Fig. 2. The enzymatic activity of α-secretase and β-secretase in guinea pig (GP), rat, and mouse parietal coretx as measured by a fluorimetric assay according to the manufacturer's protocol (R&D Systems, Wiesbaden, Germany). Note that the α-secretase activity is similar in all three species, but the β-secretase activity in guinea pig parietal cortex is twice as high as in mouse and rat cortex. *Significantly lower than guinea pig cortex ($P < 0.05$).

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