
42 The Amyloid Precursor Protein V717I Mutation Increases Susceptibility to Cell Death in a Cholesterol-dependent Manner

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INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by abnormal accumulation of amyloid β -peptide ($A\beta$) in the form of senile (or amyloid) plaques, neurofibrillary tangles and diffuse loss of neurons and synapses in the neocortex, hippocampus and other subcortical regions of the brain. Genetic linkage studies have identified two genetic polymorphisms that seem to operate as risk factors in late-onset AD (Saunders et al., 1993; Blaker et al., 1998) and three causative genes that are directly involved in the development of early-onset AD (FAD). The latter include the amyloid precursor protein (APP) (Goate et al., 1991), presenilin 1 (PS1) (Sherrington et al., 1995) and presenilin 2 (PS2) (Levy-Lahad et al., 1995). They all seem to accelerate the generation and deposition of $A\beta$, which in turn is thought to lead to neurodegeneration (for general review, see Selkoe, 1999).

The precise sequence of events underlying AD-associated neurodegeneration still remains unknown. Both apoptosis and necrosis have been shown to occur in AD, but apoptosis is currently thought to be the most prominent event, at least in the early phases of the disease (for review, see Nijhawan et al., 2000). The exact mechanism of programmed cell death in AD still remains elusive. *In vitro*-generated $A\beta$ is able to induce apoptosis and cell death in cultured cells, including hippocampal neurons (Loo et al., 1993; Yankner, 1996). In addition, FAD mutant forms of APP, PS1 and PS2 have been shown to activate the programmed cell death and potentiate the effect of apoptotic stimuli (Kovacs et al., 1999; Thinakaran, 1999; Wolozin et al., 1996).

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Recent data have linked cholesterol to A β generation and aggregation. Specifically, studies with cultured cells and transgenic mice expressing FAD-associated APP have shown that cholesterol metabolism is able to modulate APP processing and A β generation/aggregation (Bodovitz and Klein, 1996; Simons et al., 1998; Howland et al., 1998). In addition, both APP and A β are, at least in part, associated with cholesterol-rich domains in cell membranes (Lee et al., 1998; Parkin et al., 1999; Refolo et al., 1991).

In the present study we have used a genetic approach to assess the mechanism by which cholesterol regulates A β generation. Specifically, we have used Chinese hamster ovary (CHO) cells having a defect in the molecular pathway that regulates the expression of the genes involved with the uptake and biosynthesis of cholesterol (for review, see Chang et al., 1997; Brown and Goldstein, 1999). Here we have identified a novel link between intracellular cholesterol compartmentation, FAD-associated forms of APP and cell death.

MATERIALS AND METHODS

CELL LINES AND CONSTRUCTS

Wild-type (WT), 25RA and AC29 CHO cell lines were stably transfected with V717I (APP-V717I, London mutation) APP expression construct, containing G418 as selection marker. Clones were selected based on similar levels of expression of APP-V717I. 25RA and AC29 cells were a generous gift of Dr T.Y. Chang, Dartmouth Medical School, Hanover, NH.

CHOLESTEROL DETERMINATIONS

For total cholesterol determination, 80–90% confluent cells were washed twice in Dulbecco's phosphate buffered saline (PBS; Sigma Chemicals, St. Louis, MO) and extracted in chloroform:methanol (2:1, vol/vol). The chloroform phase was dried, resuspended again in chloroform and assayed using the enzymatic assay from Sigma Chemicals.

For the determinations of intracellular pools of free and esterified cholesterol, cells were incubated in the presence of [1-¹⁴C]acetic acid (Amersham Pharmacia Biotech, Piscataway, NJ) *ad equilibrium* (for 3 days), then washed twice in PBS and extracted as above. The chloroform phase was dried, resuspended again in chloroform and applied, together with standards, to silica gel-G thin layer chromatography (TLC). Plates were developed in hexane:ethyl ether:acetic acid (87:20:1, vol/vol/vol) and visualized in iodine vapor. Spots were scraped and counted in a liquid scintillation counter (Puglielli et al., 1995).

To determine cholesterol distribution in the plasma membrane, cells were pre-incubated in the presence of radiolabeled acetate, as described above, and then subjected to cholesterol oxidase (Sigma Chemicals) immediately before extraction (Puglielli et al., 1995). Cholesterol oxidase oxidizes free cholesterol at the 3 β -hydroxyl position to form 4-cholesten-3 β -one (cholestenone), which can then be separated from free cholesterol based on the migration on a silica gel-G TLC. Cholesterol oxidase is not able to cross the plasma membrane in living cells and therefore only has access to the pool of cholesterol in the plasma membrane (Lange, 1992).

LIPOPROTEIN-DEFICIENT SERUM

Lipoprotein-deficient serum (LDS) was produced after elimination of total lipoproteins from fetal bovine serum (FBS) (Brown and Goldstein, 1974). Briefly, FBS was brought to a density of 1.25 g/ml with KBr and centrifuged in a Beckman Vti 65 rotor at 45 000 rpm for more than 8 h at 4–10 °C. Total lipoproteins (VLDL, IDL, LDL, and HDL) were discarded at the top of the tubes and LDS was then dialyzed against 15 l NaCl 0.15 M, EDTA 0.3 mM, pH 7.4, for 36 h at 4 °C with five changes. Total cholesterol levels in LDS dropped by more than 97% after lipoprotein elimination.

A β DETERMINATIONS

For A β determination, cells were grown in six-well tissue culture plates (Fisher Scientific Co, Agawam, MA). The day before the experiments, cells were incubated in 1 ml fresh medium. After 24 h, the medium was collected and A β_{total} and A β_{42} levels were assessed by a sandwich-ELISA assay (A β ELISA Core Facility, Center for Neurological Diseases, Harvard Institutes of Medicine, Boston, MA).

CELL VIABILITY

Cell viability was assessed using ethidium bromide coupled to calcein AM staining (LIVE/DEAD Viability/Cytotoxicity Kit, Molecular Probes, Eugene, OR).

RESULTS AND DISCUSSION

25RA cells have a mutation in the sterol regulatory element binding protein (SREBP) cleavage-activating protein (SCAP), the central regulator of cholesterol metabolism (reviewed in Brown and Goldstein, 1999). Consequently, these cells are resistant to downregulation of cholesterol synthesis by sterols and have an increased content of cell cholesterol (for review, see

Chang et al., 1997; Brown and Goldstein, 1999). We decided to use 25RA cells to assess the role of cholesterol compartmentation in the generation of A β . For this purpose, we first characterized cholesterol distribution and metabolism in both wild-type (WT) and 25RA (25RA) Chinese hamster ovary (CHO) cells and then stably transfected them with APP-V717I, a FAD-associated mutant form of APP (London mutation).

When compared to WT, 25RA cells had a ~six-fold increase in cholesteryl-esters (1310 ± 87 vs. 206 ± 32 mg/g protein) with comparable levels of free cholesterol (162 ± 11 vs. 252 ± 32 mg/g protein). Approximately 60% of total free cholesterol was found in the plasma membrane (vs. 40% of control cells). Intracellular membrane cholesterol was mostly associated to fractions containing Golgi markers in both WT and 25RA cells.

We next stably transfected WT and 25RA cells with APP-V717I and several clones were selected, based on similar levels of expression of APP-V717I (data not shown). Transfection with APP-V717I did not alter intracellular cholesterol distribution (data not shown). 25RA-APP-V717I cells showed increased levels of both A β_{total} (~1.7-fold) and A β_{42} (~2.6-fold) in the medium (Figure 42.1). The A β_{42} /A β_{total} ratio also increased by ~1.5-fold. Stable transfection of the above cell lines with wild-type APP yielded similar results with regard to A β generation (manuscript in preparation). These results confirm data already present in the literature indicating a positive correlation between cellular cholesterol levels and A β generation (Racchi et al., 1997; Howland et al., 1998; Frears *et al.*, 1999; Mizuno *et al.*, 1998). In addition, these results also suggest that the pool of cholesteryl-esters, and not free cholesterol, may be the direct mediator of such correlation.

In order to analyze whether or not we could revert the increased production of A β , we next depleted 25RA cells of cholesterol. Cholesterol depletion was achieved by growing cells in lipoprotein-deficient serum (LDS)-containing medium. The use of LDS, instead of fast-acting cholesterol binding molecules, like cyclo-dextrins, is preferred since it is not associated with cell damage. After six passages in LDS-containing medium, 25RA cells showed an ~67% decrease in total cholesterol. Such an effect was determined by a marked decrease in cholesteryl-esters (Figure 42.2). Even if we observed a redistribution of free cholesterol from the plasma membrane to intracellular membranes, the overall levels of free cholesterol remained unchanged (Figure 42.2).

Surprisingly, 25RA-APP-V717I cells showed a decreased viability in response to cholesterol deprivation. Figure 42.3A shows the growth rate of 25RA-APP-wt and 25RA-APP-V717I grown in FBS- or LDS-containing medium. The growth rate of 25RA-APP-V717I cells reduced progressively from the first to the third week of cholesterol deprivation. This effect was not observed in 25RA-APP-wt or in untransfected 25RA cells, which remained viable for more than 70 days.

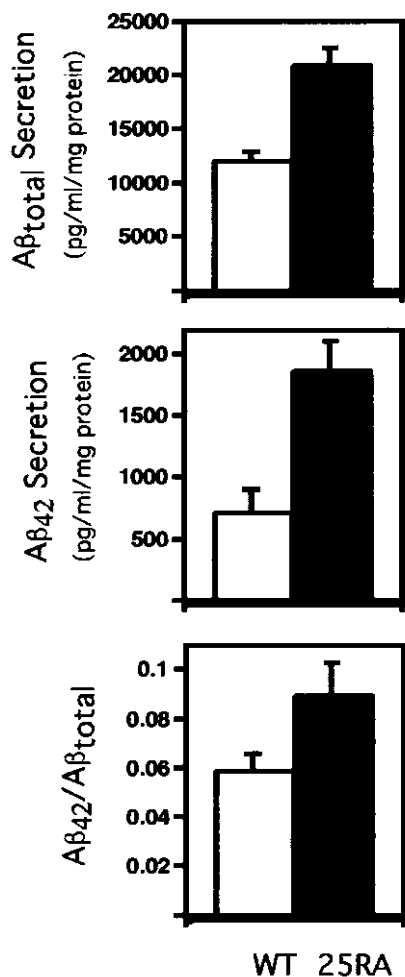


Figure 42.1. A β secretion is increased in 25RA as compared to wild-type CHO cells. WT and 25RA cell lines stably transfected with APP-V717I were grown as described in Materials and methods. A β secretion in the medium was assessed by standard sandwich ELISA. Values are the mean \pm SD of two separate experiments carried out on at least two different clones

Ethidium bromide (for dead cells) plus calcein-AM (for live cells) stainings showed that the reduction in growth rate observed in 25RA-APP-V717I cells was due to marked cellular death (Figure 42.3B). In this set of experiments, we also used AC29 cells stably transfected with APP-V717I. AC29 cells derive from 25RA cells and have the same defect in the SREBP pathway, which leads to increased cholesterol content. In addition, AC29 cells also have a mutated form of acyl CoA:cholesterol acyltransferase (ACAT), the

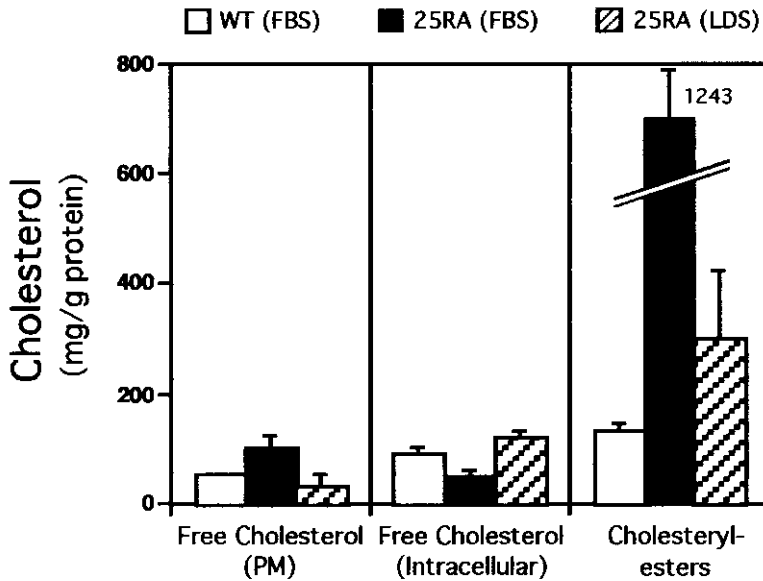


Figure 42.2. Cholesterol depletion reduces cholesteryl-ester levels in 25RA cells. 25RA cells were grown in FBS- or LDS-containing medium for 6 weeks. Cholesterol content and distribution were determined in 80–90% confluent cells. Values are the mean \pm SD of at least three independent determinations. FBS, fetal bovine serum; LDS, lipoprotein-deficient serum; PM, plasma membrane; Intracellular, intracellular membranes

enzyme that controls cholesterol esterification (reviewed in Chang et al., 1997). Consequently, they have a ~six-fold increase in membrane cholesterol with undetectable levels of cholesteryl-esters. Only <5% of AC29-APP-V717I and untransfected 25RA cells died after 3 weeks in LDS-containing medium (Figure 42.3B). In contrast, >90% of 25RA-APP-V717I cells were not viable under the same conditions (Figure 42.2B). Taken together, these results suggest that depletion of the pool of cholesteryl-esters and not of membrane cholesterol potentiates the susceptibility to cell death in cells expressing FAD-associated mutant forms of APP.

One possible reason for cell toxicity associated with cholesterol mobilization from intracellular pools of cholesteryl-esters is the generation of cholesterol oxides. Cholesterol oxides have been shown to downregulate the universal caspase inhibitor FLIP, activate CPP-32 (caspase 3) and induce DNA fragmentation and chromatin condensation (Sata and Walsh, 1998; Yin et al., 1999). In macrophages, they originate after hydrolysis of cholesteryl-esters, through the action of as-yet identified cholesterol oxidase(s) (Kellner-Weibel et al., 1999). Apoptosis is one of the possible mechanisms of cell death occurring in neurons of AD patients (for review, see Nijhawan et al., 2000). FAD-associated mutant forms of APP, PS1 and PS2 have been shown to

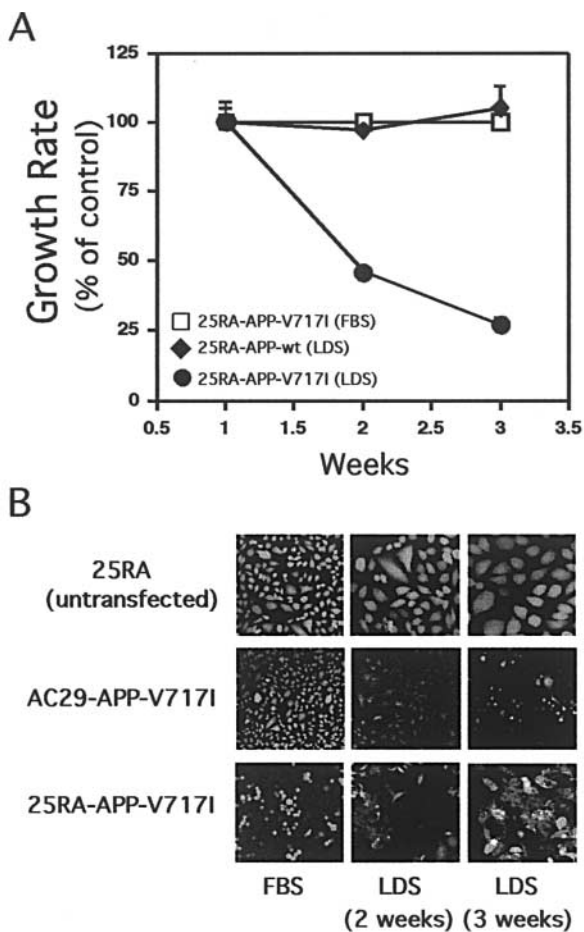


Figure 42.3. Cholesterol deprivation induces cell death in 25RA-APP-V717I cell lines. AC29 and 25RA cell lines stably transfected with APP-V717I were grown in the presence of FBS- or LDS-containing medium for the indicated periods of time. (A) Growth rate was expressed as percentage of 25RA-APP-V717I cells grown in FBS medium. Values are the mean \pm SD of two different determinations from at least three different clones. (B) Cell death was assessed using calcein-AM (alive; green) plus ethidium bromide (dead; red) stainings

activate the programmed cell death pathway and to potentiate apoptotic stimuli (Kovacs et al., 1999; Thinakaran, 1999; Wolozin et al., 1996; reviewed in Nijhawan et al., 2000). However, the exact molecular event that activates apoptosis in AD still remains unknown. The above results suggest a possible link between FAD-associated mutant APP and cholesteryl-esters in the activation of cell death.

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