

Blockade of the Tumor Necrosis Factor-Related Apoptosis Inducing Ligand Death Receptor DR5 Prevents β -Amyloid Neurotoxicity

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We originally suggested that inhibition of tumor necrosis factor-related apoptosis inducing ligand (TRAIL) death pathway could be taken into consideration as a potential therapeutic strategy for Alzheimer's disease (AD). However, because the critical role of TRAIL in immune surveillance, the neutralization of TRAIL protein by an antibody to prevent its binding to death receptors is definitely a risky approach. Here, we demonstrated that the blockade of the TRAIL death receptor DR5 with a specific antibody completely prevented amyloid β peptide ($A\beta$) neurotoxicity in both neuronal cell line and primary cortical neurons. DR5 was demonstrated to be a key factor in TRAIL death pathway. In fact, whereas TRAIL expression was enhanced dose-dependently by concentrations of β amyloid ranging from 10 nM to 1 μ M, only the highest toxic dose of $A\beta$ (25 μ M) induced the increased expression of DR5 and neuronal cell death. In addition, the increased expression of DR5 receptor after β amyloid treatment was sustained by p53 transcriptional activity, as demonstrated by the data showing that the p53 inhibitor Pifithrin α prevented both β amyloid-induced DR5 induction and cell death. These data suggest a sequential activation of p53 and DR5 upon β amyloid exposure. Further insight into the key role of DR5 in AD was suggested by data showing a significant increase of DR5 receptor in cortical slices of AD brain. Thus, these findings may give intracellular TRAIL pathway a role in AD pathophysiology, making DR5 receptor a possible candidate as a pharmacological target.

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INTRODUCTION

One of the more accredited candidates involved in etiopathology of Alzheimer's disease (AD) is thought to be the amyloid β peptide ($A\beta$), which is the major component of the extracellular deposition named senile plaques (Mori *et al*, 1992; LeVine, 2004; Heppner *et al*, 2004). Senile plaques, together with intracellular depositions of neurofibrillary tangle, are the two anatomo-pathological hall markers that characterize this disease. $A\beta$ is a 39–43 amino-acid length peptide derived from the amyloidogenic processing of the larger amyloid precursor protein (APP) (Haas *et al*, 1992; Seubert *et al*, 1992). The central role of $A\beta$ in AD is sustained by the discovery of APP mutation in some familiar cases of the disease (Selkoe, 1996; Kowalska,

2003). This mutations have been demonstrated to be associated with an elevated production of $A\beta$ peptide 1–40 and 1–42, which have the tendency to aggregate to form final insoluble fibrils (Dewachter *et al*, 2000; Lewis *et al*, 2004; Helpner *et al*, 2004). The result coming from genetic studies and *in vitro* and *in vivo* experiments on animal models of AD gives $A\beta$ a neurotoxic effect (Cotman *et al*, 1992; Gray and Patel, 1995; Oster-Granite *et al*, 1996; Moechars *et al*, 1999; Ekinci *et al*, 2000; Peng *et al*, 2002; Tamagno *et al*, 2003; Rosales-Corral *et al*, 2004).

It is well demonstrated that $A\beta$ causes different intracellular effects, involving different pathways that can proceed either separately or sequentially. We have recently shown that one of the members of the TNF (tumor necrosis factor)- α family, the TNF-related apoptosis inducing ligand (TRAIL), contributes substantially to amyloid-induced neurotoxicity in human SH-SY5Y neuronal cell line (Cantarella *et al*, 2003). TRAIL is a novel peptide molecule belonging to the TNF family, whose main role is to induce programmed cell death in tumor cell lines from various tissue origins (Pitti *et al*, 1996). TRAIL binds to five specific receptors of the TNF/NGF family (Pan *et al*, 1997a; Walczak

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et al, 1997). However, only two of them, DR4 and DR5, have an intracellular death domain, which appears essential for triggering cell death processes (Pan *et al*, 1997a; Walczak *et al*, 1997). Although the decoy receptors TRAIL-R3 and -R4 act as a dominant negative of TRAIL proapoptotic effects (Pan *et al*, 1997b; Degli-Esposti *et al*, 1997; Sheridan *et al*, 1997), a fifth receptor, named osteoprotegerin, appears to be involved in bone remodelling (Emery *et al*, 1998).

The contribution of TRAIL to $A\beta$ neurotoxicity was demonstrated by data showing that anti-TRAIL-neutralizing monoclonal antibody protects neuronal SH-SY5Y cells from $A\beta$ neurotoxicity (Cantarella *et al*, 2003). Furthermore, we recently showed TRAIL-positive cells in the brain of AD patients. TRAIL staining was localized in the cerebral cortex of human brain slices, often in the proximity of Congo Red-positive amyloid plaques (Uberty *et al*, 2004). Thus, these findings may give intracellular TRAIL pathway a role in AD pathophysiology, and indicate TRAIL peptide as a suitable target for pharmacological intervention. However, given the key role of TRAIL in providing and maintaining immune surveillance, questions have been raised regarding the potential usefulness of an antibody anti-TRAIL in AD therapy. Thus, we addressed our study toward another possible candidate belonging to the TRAIL pathway, the TRAIL DR5 receptor. Interestingly enough, TRAIL DR5 receptor is weakly expressed in human brain, and it is not detectable in other human organs (Ichikawa *et al*, 2001). In contrast, many human cancer tissues reacted positively to anti-DR5 antibody. In the brain, DR5 receptor is substantially expressed in neurons and oligodendrocytes, suggesting that the exposure to TRAIL could potentially induce apoptosis in these cells (Dorr *et al*, 2002). Consistent with this hypothesis, recombinant TRAIL was found to induce apoptosis in human neuroblastoma cell line (Milani *et al*, 2003), rat cortical neurons and in human brain slices (Nitsch *et al*, 2000). In this study, we investigated the involvement of TRAIL death receptor DR5 in $A\beta$ -mediated apoptosis in both human neuronal cell line and mouse cortical neurons. Furthermore, we characterized the expression of the DR5 receptor in human brain derived from an AD case.

MATERIALS AND METHODS

Cell Culture

Neuroblastoma cell line SH-SY5Y was routinely cultured in 1:1 Ham's F12:Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 50 μ g/ml penicillin, and 100 μ g/ml streptomycin and was kept at 37°C in a humidified 5% CO₂/95% air. For differentiation, cultures were seeded at approximately 10⁵ cells/dish and retinoic acid was added to a final concentration of 10 μ M. The medium was changed every day and cultures were allowed to differentiate for 2 weeks.

Primary Cultures of Mouse Cortical Neurons

Fifteen embryonic day mice were taken with Caesarean section from anesthetized pregnant dams. C57/BL6 mice were purchased from Charles River, Italy. Cerebral cortices were isolated and dissociated by manual dispersion with a fire-polished Pasteur pipette. Cells were plated at a density of 1.5 \times 10⁵ cells/cm². Culture dishes were coated with

10 μ g/ml poly-L-lysine. The cells were plated in Neurobasal medium (Invitrogen Corporation) supplemented with 2% B27 (Invitrogen Corporation), 0.5 mM L-glutamine, and 50 U/ml penicillin/streptomycin (Invitrogen Corporation). Three days after plating, 50% of the medium was changed with fresh medium and subsequently 50% of the medium was changed twice a week, until 11 days *in vitro*.

Human Tissues

Brain tissues were removed for diagnostic purposes during autopsy from three AD patients and from one cognitive normal, age-matched individual deceased owing to non-neurological disease. One AD case was a female died at the age of 78 years with a 5-year history of sporadic AD. The other two cases were siblings (II-3, age of death 65 years, disease duration 7 years; II-5, age of death 56 years, disease duration 6 years) affected by a familiar form of AD caused by PS2 M239I mutation (Zatti *et al*, 2004). The neuropathological diagnosis of AD was performed in accordance with CERAD recommendations (Mirra *et al*, 1991) by the local neuropathology unit at the Department of Pathology.

Handling of β -Amyloid Peptide

$A\beta_{1-42}$ and $A\beta_{25-35}$ were purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). Different lots of the peptides were dissolved in sterile, doubly distilled water at a concentration of 2.5 mM and stored at -20°C. $A\beta_{1-42}$ stock solutions were kept for 1 week in a 37°C incubator so as to allow aggregation and, therefore, toxicity. $A\beta_{25-35}$ was used immediately after suspension (Pike *et al*, 1993). All peptides were tested at graded concentrations (range: 10 nM–10 μ M) for evaluation of cell toxicity.

Evaluation of Cell Viability

5 \times 10³ cells/well were plated in 96-well plates and grown to 70–80% confluence in complete DMEM containing 10 μ M retinoic acid. Differentiated cultures were incubated for 48 h with 25 μ M β AP_{25–35}, or β AP_{1–42} in DMEM with 1% serum at 37°C. These agents were performed alone or in combination with Pifithrin α (PIF) (200 nM), an inhibiting DR5 antibody (1 μ g/ml; Alexis Biochemicals, San Diego, CA) or an isotype-matched control (IgG₁ control) (1 μ g/ml; Alexis Biochemicals, San Diego, CA). Cell viability was evaluated 48 h after the addition of the cytotoxic agent to the media by measuring lactate dehydrogenase (LDH) activity in the culture-conditioned media. LDH activity was measured spectrophotometrically in the culture media, following nicotinamide adenine dinucleotide (reduced form) oxidation at 340 nm. Total LDH activity was defined as the sum of intracellular and extracellular LDH activity. Cytotoxicity was evaluated as percentage of total LDH activity. Each experiment was performed in triplicate and repeated at least twice.

Evaluation of Apoptosis

Terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling (TUNEL) was performed using the kit purchased by Roche Molecular Biochemicals according to the manufacturer's instructions. The TUNEL-positive cells

were counted in eight different fields for each well taken from experiments that were run in triplicate.

For May-Grunwald-Giemsa's staining, cells were washed in phosphate-buffered saline (PBS) solution, fixed with May-Grunwald's staining containing methanol for 3 min, then washed with a 200 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer (pH 7.4) for additional 3 min. Cells were then stained (3 min) with Giemsa, and air-dried after three/four rapid washes in water. Cells were then analyzed under fluorescence microscope with $\times 40$ objective and photographed.

Immunofluorescence

Cells were fixed in methanol for 5 min, raised in PBS and then incubated overnight with a polyclonal anti-TRAIL (Alexis) used at 1:500 dilution, a monoclonal anti-p53 antibody (Ab 8, Neomarker) used at 1:300 dilution, or anti-DR5 (Alexis) used at 1:400 dilution. CyTM₃-conjugated secondary antibodies were used. Slides were mounted with moviol and were examined using a fluorescence microscope.

Western Blot Analysis

Cells were harvested in 100 μl of lysis buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 5 mM ethylene diaminetetraacetic acid, 1 mM phenyl-methyl-sulfonyl-fluoride, 0.5 $\mu\text{g}/\mu\text{l}$ leupeptin, 5 $\mu\text{g}/\mu\text{l}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ pepstatin. The samples were sonicated and centrifuged at 15 000g for 30 min at 4°C. The resulting supernatants were isolated and the protein content was determined by a conventional method (BCA protein assay Kit, Pierce, Rockford, IL). Fifteen micrograms of total protein were electrophoresed on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to nitrocellulose paper (Schleicher and Schuell, Dassel, Germany). Filters were incubated at room temperature overnight with anti-TRAIL (the same used for immunohistochemistry), anti-p53 (the same used for immunohistochemistry), anti-DR5 (C-20 Santa Cruz Biotechnology), or anti-tubulin (Ab3, Neo Markers) antibody in 3% nonfat dried milk (Sigma). The secondary antibodies (Santa Cruz Biotechnology) and a chemiluminescence blotting substrate kit (Boehringer, Mannheim, Germany) were used for immunodetection. Evaluation of immunoreactivity was performed on immunoblots by densitometric analysis using a KLB 2222-020 Ultra Scan XL laser densitometer at a wavelength of 633 nm.

Statistical Evaluation

Results in cell viability and densitometric analysis of the immunoblots are given as mean \pm standard error mean values. Statistical significance of differences was determined by mean values of the analysis of variance, following Student's test. Significance was accepted for a *p*-value of <0.05 .

RESULTS

TRAIL Expression is Necessary but Nonsufficient to Mediate $\text{A}\beta$ Neurotoxicity

As previously described, high, neurotoxic doses of $\text{A}\beta_{25-35}$ caused an increase expression of TRAIL that was followed by apoptosis (Cantarella *et al*, 2003). Here, we investigate

whether lower doses of $\text{A}\beta$ can modulate TRAIL expression on neuronal cell line SH-SY5Y. To that purpose, human SH-SY5Y neuroblastoma cells were differentiated with retinoic acid for 1 week and then challenged with increasing concentrations of $\text{A}\beta_{25-35}$, ranging from 1 nM to 25 μM , to evaluate cell death and TRAIL protein expression. Interestingly, low concentration of $\text{A}\beta_{25-35}$ (10 nM) was able to induce a significant increase of TRAIL expression and this effect proportionally enhanced with the increase of $\text{A}\beta$ doses (Figure 1, panels a and b).

Cell death was characterized by May-Grunwald-Giemsa's staining to visualize the late phase of apoptosis and by measurement of extracellular LDH release. In line with previous observations (Mattson *et al*, 1992; Weiss *et al*, 1994), the neurotoxic effects induced by $\text{A}\beta_{25-35}$ were found to be concentration-dependent, being undetectable at 10 nM. Very few scattered apoptotic nuclei with a reduction of cell viability of about 10% were present at 1 μM concentration, whereas the highest dose of 25 μM induced about 40% of cell death with evident sign of apoptosis (Figure 1, panels c and d). Altogether, these data raised the question of why, after exposure of the cells to low concentrations of $\text{A}\beta$, the increased expression of TRAIL was not followed by apoptosis. Because TRAIL toxicity is mediated by its death receptors, we examined the expression of the death receptor DR5 after different $\text{A}\beta$ concentrations. Figure 2, panel a shows a representative Western blot analysis performed on neuronal cells, carried out with anti-TRAIL and anti-DR5 antibodies. In line with the immunofluorescence data reported above, TRAIL protein levels increased progressively with the increase of $\text{A}\beta$ doses. On the other hand, DR5 expression was virtually absent in untreated cells and as well as in the cells exposed to 10 nM or 1 μM $\text{A}\beta$. The DR5 protein levels significantly increased only with toxic concentration (25 μM) of $\text{A}\beta$ peptide (Figure 2, panel a). In this set of experiments, we also examined the expression of the tumor suppressor p53, because it is well recognized to be involved in $\text{A}\beta$ neurotoxicity and to be a transcriptional inducer of the DR5 gene (Wu *et al*, 1997). Like DR5, the p53 protein levels appeared significantly enhanced only at the toxic concentration of $\text{A}\beta$ (Figure 2, panel a). The quantitative analysis of immunoblot experiment performed on three different cell culture preparations confirmed the immunofluorescence data showing that $\text{A}\beta$ promotes a progressive concentration-dependent increase of TRAIL, but only at the proapoptotic concentration increases of DR5 and p53 expression (Figure 2, panel b). These data suggest a critical role of the death receptor DR5 in TRAIL-mediated $\text{A}\beta$ neurotoxicity.

DR5 Expression is Mediated by p53 in Human Neuronal Cell Line and Mouse Cortical Neurons

Neuronal cell lines were preincubated with 200 nM PIF, a compound known to inhibit p53 activity and then exposed to 25 μM $\text{A}\beta_{25-35}$ or to 10 μM $\text{A}\beta_{1-42}$. At 8 h after the addition of $\text{A}\beta$ to the medium, the cells were processed for immunofluorescence analysis carried out with anti-DR5 and anti-p53 antibodies.

Both $\text{A}\beta_{25-35}$ and $\text{A}\beta_{1-42}$ induced a significant increase of DR5 immunoreactivity that was prevented by the

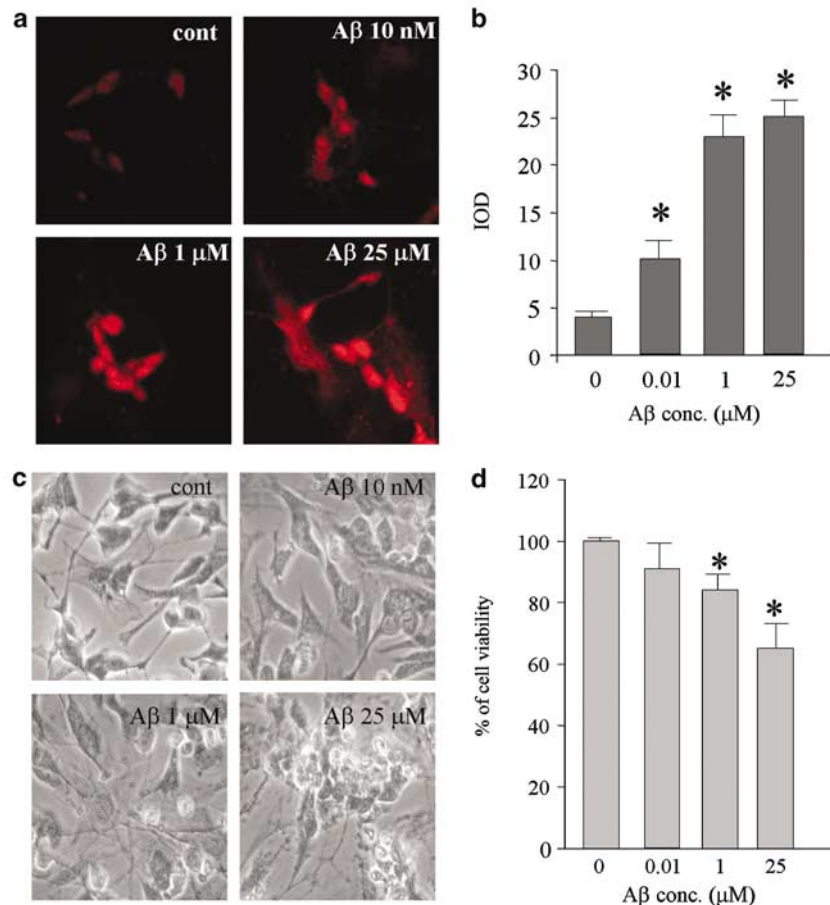


Figure 1 Expression of TRAIL after exposure to increasing concentrations of $A\beta_{25-35}$. Cells were treated with different $A\beta$ concentration and then immunofluorescence analysis with polyclonal anti-TRAIL antibody and cell viability were performed. (a) Representative immunofluorescence carried out with anti-TRAIL antibody. (b) Quantitative analysis of TRAIL immunofluorescence staining. Values are expressed as integrate optical density (IOD) and represent means \pm SEM of at least three different experiments from three separate cell preparations. * $p < 0.01$ vs the corresponding control values. (c) May-Grunwald-Giemsa's staining that visualized the late phase of apoptosis following concentration curve of $A\beta$. Scale bar = 50 μ m. (d) LDH measurement following concentration curve of $A\beta$. Data were expressed as percentage of cell viability, and represent means \pm SEM of at least three different experiments and are from three separate cell preparations. * $p < 0.01$ vs the corresponding control values.

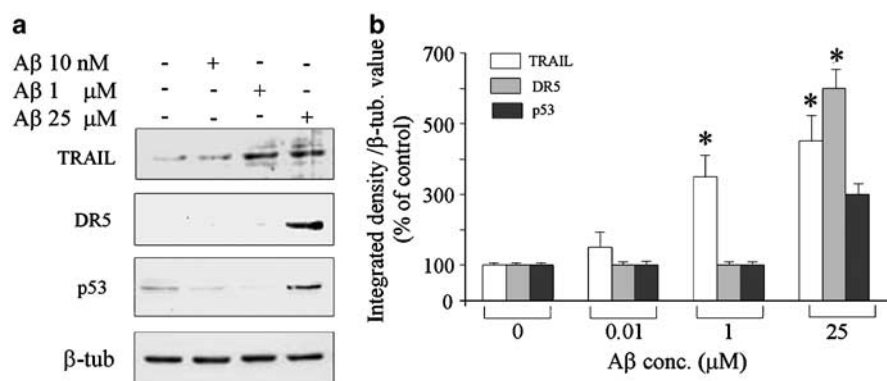


Figure 2 Expression of TRAIL, DR5, and $p53$ after exposure to increasing concentrations of $A\beta_{25-35}$. Cells were treated with different $A\beta$ concentrations and then a Western blot analysis was carried out with polyclonal anti-TRAIL, polyclonal anti-DR5, monoclonal anti- $p53$, or monoclonal anti- β tubulin (β tub) antibodies was performed. (a) Representative immunoblot. (b) Densitometric analysis of immunoblots performed with anti-TRAIL (open bar), anti-DR5 (grey bar), or anti- $p53$ (black bar) antibodies. Values are expressed as integrated density of respective antibody signal over β tubulin value and represent means \pm SEM of at least three different experiments from three separate cell preparations. * $p < 0.01$ vs the corresponding control values.

preincubation with PIF (Figure 3, panel a). Also, $p53$ immunostaining was found enhanced after $A\beta_{25-35}$ and $A\beta_{1-42}$ treatments, and the presence of PIF did not alter $A\beta$ -induced increase of $p53$ immunoreactivity (Figure 3, panel

b). These results are consistent with those of Culmsee *et al* (2001), who suggest that the mechanism of action of PIF is the inhibition of $p53$ nuclear translocation and prevention of its DNA-binding activity, rather than the downregulation

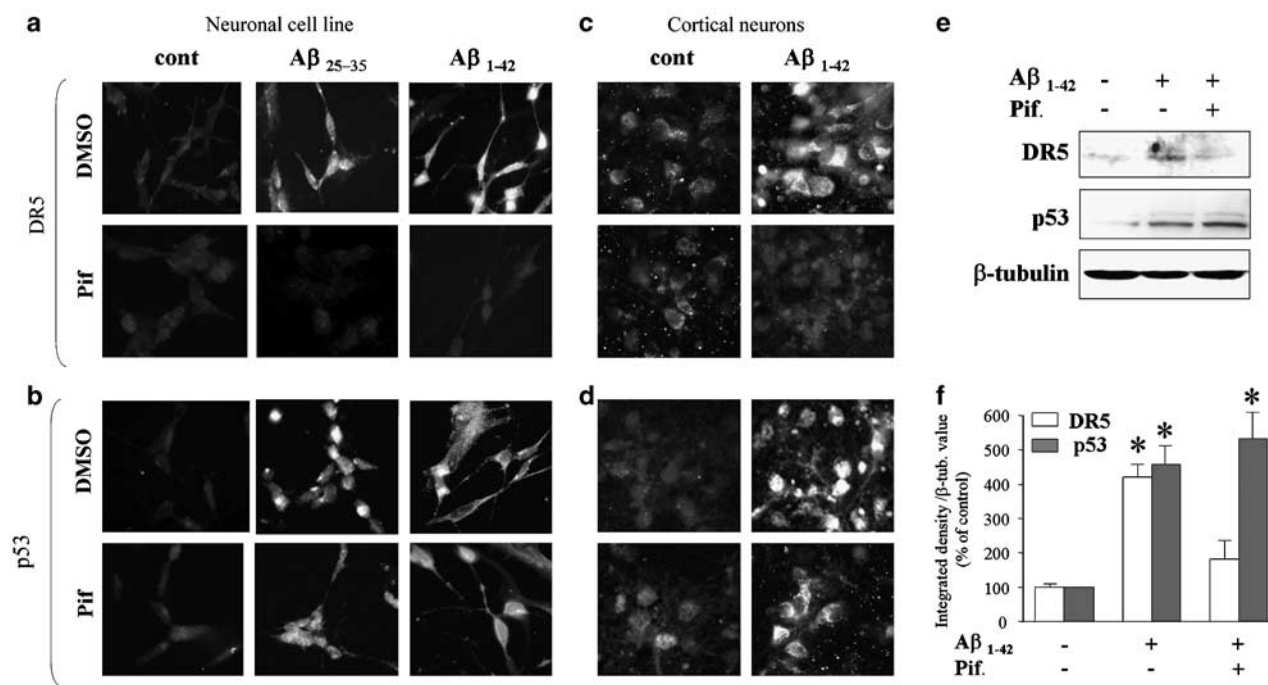


Figure 3 Effects of PIF on DR5 and *p53* expression following $A\beta$ injury. Representative immunofluorescence analysis carried out with polyclonal anti-DR5 antibody (a) and monoclonal anti-*p53* antibody (b) in neuronal cell lines treated with 25 μ M $A\beta_{25-35}$, or 10 μ M $A\beta_{1-42}$ in the absence or presence of 200 nM PIF. Representative immunofluorescence analysis carried out with polyclonal anti-DR5 antibody (c) and monoclonal anti-*p53* antibody (d) in cortical neurons treated with 10 μ M $A\beta_{1-42}$ in the absence or presence of 200 nM PIF. Scale bar = 50 μ m. (e) Representative Western blot analysis with polyclonal anti-DR5 antibody in cortical neurons treated with 10 μ M $A\beta_{1-42}$ in the absence or presence of 200 nM PIF. (f) Densitometric analysis of DR5 immunoblot performed on cells treated 10 μ M $A\beta_{1-42}$ in the absence (open bar) or presence of 200 μ M PIF (black bar). Values are expressed as integrated density of DR5 signal over β tubulin value and represent means \pm SEM of at least three different experiments from three separate cell preparations. * $p < 0.01$ vs the corresponding control values.

of newly synthesized *p53* or the inhibition of its activation by relevant kinases.

Similar results were obtained with cortical neurons exposed to 10 μ M $A\beta_{1-42}$. An intense cytoplasmic staining was observed in cortical neurons treated with $A\beta_{1-42}$ at the dose of 10 μ M and immunostained with anti-DR5 antibody. Similarly to what was found in neuronal cell line, the inhibition of *p53* activity by PIF prevented the induction of DR5 expression induced by $A\beta_{1-42}$ (Figure 3, panel c). Furthermore, many cortical neurons exposed to $A\beta_{1-42}$ accumulated *p53* protein in the nuclear compartment, whereas in some cells *p53* remained confined to the cytoplasm. Pretreatment with PIF did not modify *p53* induction induced by $A\beta_{1-42}$ (Figure 3, panel d). In support to immunofluorescence data, Western blot analysis confirmed the direct correlation between *p53* and DR5 receptor. In fact, an intense band representing DR5 protein levels was observed after $A\beta_{1-42}$ exposure. DR5 protein levels returned to basal levels after the combined treatment with PIF and $A\beta_{1-42}$. Differently, PIF did not change the increased *p53* protein levels induced by $A\beta_{1-42}$ (Figure 3, panel e). The quantitative analysis performed on three different cell culture preparations confirmed the data observed in the representative Western blot analysis. Both DR5 and *p53* protein levels were increased after treatment of the cells with $A\beta_{1-42}$. The pretreatment with PIF prevented only $A\beta_{1-42}$ -induced increase of DR5 but not *p53* expression (Figure 3, panel f).

PIF and Anti-DR5 Blocking Peptide Prevent $A\beta$ Neurotoxicity in Neuronal Cell Line and Cortical Neurons

To give more insight into the intracellular cascades activated by $A\beta$, neuronal cell lines and cortical neurons were treated with $A\beta$ peptides in the presence or absence of PIF or the anti-DR5 blocking peptide and then they were evaluated for cell viability. Treatment of neuronal cell line with $A\beta_{25-35}$ 25 μ M resulted in a reduction of cell viability by about 40%. A dose curve with fibrillar $A\beta_{1-42}$ was also performed. The fibrillar peptide $A\beta_{1-42}$ reduced cell viability in a concentration-dependent manner (data not show). At the dose of 10 μ M $A\beta_{1-42}$ peptide cause a 65% of cell death. PIF partially but significantly rescued $A\beta_{25-35}$ and $A\beta_{1-42}$ -induced cell death (Figure 4, panel a). Interestingly, blockade of DR5 receptor with a specific anti-DR5 antibody completely prevented $A\beta$ -induced cell death. These effects were specific as treatment of the cells with a control isotype IgG1 did not affect $A\beta$ -induced reduction of cell viability (Figure 4, panel b). Similar results were obtained with cortical neurons treated with $A\beta_{1-42}$. The neurotoxic effects of $A\beta_{1-42}$ were partially abolished in cortical neurons pretreated with PIF (Figure 5, panel A). Cortical neurons were also treated with $A\beta_{1-42}$ with or without anti-DR5 antibody and 24 h later a TUNEL analysis was performed. As shown in Figure 5, panel B, few scattered TUNEL-positive cells were observed in untreated cells or in cells

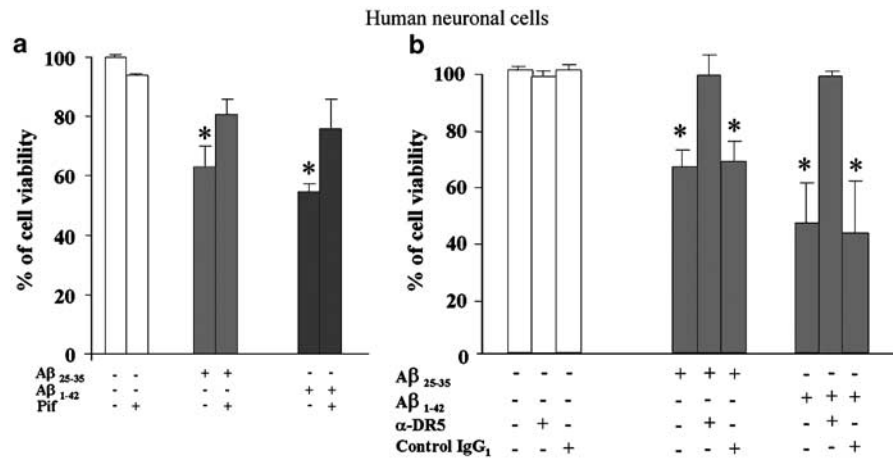


Figure 4 Effects of PIF or the DR5 blocking peptide on cell viability following $A\beta$ injuries in human neuronal cell line. LDH release was measured in the culture media of cells treated with saline (open bars), 25 μ M $A\beta_{25-35}$ (grey bars) or 10 μ M $A\beta_{1-42}$ (dark bars) in the absence or presence of 200 nM PIF (a), or the DR5 blocking peptide (1 μ g/ml) (b). IgG₁ isotype was used as control. Values represent means \pm SEM of at least three different experiments and are from three separate cell preparations. * $p < 0.01$ vs the corresponding untreated control values.

treated only with anti-DR5 antibody (panel a and b). TUNEL-positive cells were found very abundant in the sample treated with $A\beta_{1-42}$ alone (Figure 5, panel Bc), whereas the combined treatment of $A\beta$ with anti-DR5 antibody showed a pattern of cell viability similar to that found in untreated cells with very few apoptotic nuclei (Figure 5, panel Bd). These results were confirmed by LDH release measurement, showing that anti-DR5 antibody prevented the cell loss induced by $A\beta_{1-42}$ (Figure 5, panel C).

DR5 Receptor is Expressed in AD Brain

We previously found a significant expression of TRAIL protein in the brain of AD patients, suggesting an involvement of TRAIL death pathway in the neurodegenerative process of AD (Uberti et al, 2004). Different studies have also demonstrated the presence of TRAIL death receptor in human brain (Dorr et al, 2002; Nitsch et al, 2000). We had the opportunity to evaluate the expression of DR5 receptor in the brain of three AD patients. The patients showed a history of AD, as designed according to the *Diagnostic and Statistical Manual of Mental Disorders* (fourth edition) and the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association clinical criteria. Postmortem examination confirmed the diagnosis of AD because the presence of abundant Congo red-stained amyloid deposits and neurofibrillary tangles; hematoxylin-eosin staining also demonstrated marked cell loss (data not shown). Some cortical brain slices were also analyzed for DR5 expression with an anti-DR5 antibody. The DR5-like immunoreactivity was then compared with the one found in the brain obtained from an age-matched non-AD subject. As shown in a representative picture of brain slices derived from the sporadic AD patient (Figure 6), consistent and significant DR5 immunoreactivity was found in the AD brain (panel b). At high magnification, the signal was found to be confined on cytoplasmic edge with a punctate staining (panel d). Similar results were obtained on brain slices derived from the two familiar case of AD (data not shown).

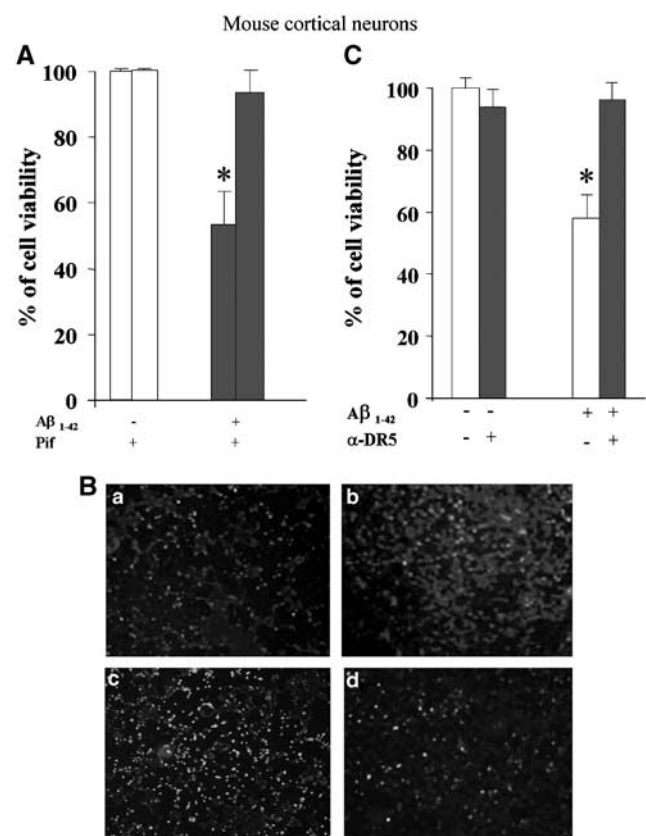


Figure 5 Effects of PIF or the DR5 blocking peptide on cell viability after $A\beta$ injuries in primary cortical neurons. LDH release was measured in the culture media of cells treated with saline (open bars) or 10 μ M $A\beta_{1-42}$ (dark bars) in the absence or presence of 200 nM PIF (A), or the DR5 blocking peptide (1 μ g/ml) (C). Values represent means \pm SEM of at least three different experiments and are from three separate cell preparations. (B) TUNEL analysis of cortical neurons untreated (a), treated with the DR5 blocking peptide antibody (1 μ g/ml) alone (b), 10 μ M $A\beta_{1-42}$ (c) or 10 μ M $A\beta_{1-42}$ plus the DR5 blocking peptide (1 μ g/ml) (d). * $p < 0.01$ vs the corresponding control values.

In brain slices from the control subjects, DR5 immunoreactivity was indeed found although at very low levels (panels a and c).

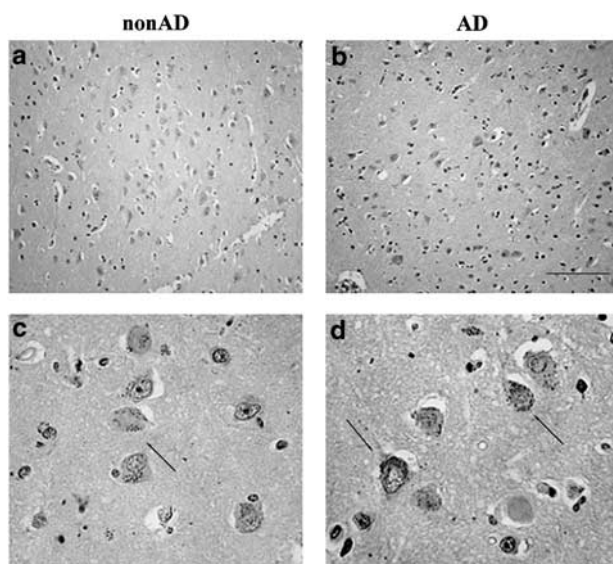


Figure 6 Expression of DR5 receptor in AD human brain cortex. Representative images of cerebral cortex from one non-AD (a, c) and one AD (b, d) subjects immunostained with polyclonal anti-DR5 antibody. Panels c and d represent high magnification of non-AD and AD cortical slices, respectively. Arrows indicate DR5 immunoreactivity. Scale bar = 500 μ m.

DISCUSSION

It is well recognized that AD is a multifactorial disease in which many events occurring either separately or sequentially can contribute to the neurodegenerative process. In this scenario, $A\beta$ seems to play a pivotal role. However, up to now, the mechanism behind $A\beta$ -induced neurodegeneration is still controversial (Yankner *et al*, 1989; Geula *et al*, 1998). The difficulty in understanding the $A\beta$ effects is sustained also by the fact that this toxic peptide can activate simultaneously different pathways that proceed separately or converge together, and ultimately all lead to neuronal death. Identification of a preferential pathway could definitely be useful to design a new therapeutic drug to slow the progression of AD pathology.

Here, we show that the exposure of differentiated SH-SY5Y neuronal cells or primary cortical neurons to $A\beta$ peptide results in the induction of TRAIL, its death receptor DR5, and *p53*. A dose-response study of the changes in TRAIL, *p53*, and DR5 expression demonstrated that $A\beta$ displays concentration-related effects characterized by the sequential involvement of the three factors. In particular, low, nontoxic concentrations of $A\beta$ (nanomolar range) induced the expression of TRAIL without changing DR5 and *p53* protein levels. High, neurotoxic concentrations of $A\beta$ (micromolar range) caused an increased expression of all three proteins examined. Thus, in our experimental model, TRAIL was a necessary but not sufficient factor to induce cell death upon $A\beta$ exposure.

In this model, DR5 receptor appears to play a critical role as its expression was found to increase only after exposure to the highest, neurotoxic concentration of $A\beta$. It has been shown that TRAIL binds to five specific receptors belonging to the TNF/NGF family (Pan *et al*, 1997a; Walczak *et al*, 1997). However, only two of them, DR4 and DR5, have an

intracellular death domain, which appears essential for triggering cell death processes (Pan *et al*, 1997a; Walczak *et al*, 1997). In mice only DR5 receptor has been identified (Wu *et al*, 1999). This receptor is a homolog of human DR5 and DR4 and binds TRAIL with an affinity similar to the one displayed by human DR4 and DR5 receptors. In this study, DR4 was not taken into consideration because we previously demonstrated that in differentiated SH-SY5Y cells, $A\beta_{25-35}$ as well as TRAIL synthetic peptide did not alter the DR4 expression (Cantarella *et al*, 2003). The most convincing result indicating the crucial role of DR5 in $A\beta$ neurotoxicity comes from the experiments showing that the DR5 blocking peptide completely prevents $A\beta$ -induced neurotoxicity in both neuronal cell line and cortical neurons.

The earlier biochemical event following the engagement of DRs receptors by their ligand is the recruitment of protein towards the intracellular death domain of the receptor to form a structure known as the death-inducing signalling complex (DISC) (Kischkel *et al*, 1995). DISC includes the recruitment of FADD and the activation of pro-caspase 8 or pro-caspase 10. Caspase 8 activated the pro-caspase 3 and in turn they lead to apoptosis. Furthermore, caspase 8 can induce the cleavage of bid, which translocates to the mitochondria where it activates bax and bak, providing a mechanism for crosstalk between the DRs and the apoptotic intrinsic pathway.

The correlation between DR5 and *p53* comes from the identification of a subtractive hybridization screen for *p53*-target genes indicating DR5 gene as one of them. We found that the increased expression of DR5 after $A\beta$ was prevented by PIF, a compound that inhibited *p53* activity. *p53* is a pleiotropic transcription factor that plays an important role in determining cell fate under certain conditions (see review Almog and Rotter, 1997). *p53* is constitutively present in many cell types including neurons and is upregulated and activated via phosphorylation following various insults including excitotoxicity, ischemic injury, ionizing radiation, and oxidative stress (Crumrine *et al*, 1994; Sakhi *et al*, 1997; Uberti *et al*, 1998; Johnson *et al*, 1998; Inamura *et al*, 2000). $A\beta$ -induced cytotoxicity is also demonstrated to involve *p53* in primary cortical neurons (Copani *et al*, 2001) and different neuronal cell lines (Misiti *et al*, 2005; Matsumoto *et al*, 2006). In line with these latter studies, our data obtained with PIF demonstrated that $A\beta$ neurotoxicity is mediated by *p53* in both neuronal cell line and cortical neurons. In fact, inhibition of *p53* activation prevented the $A\beta$ -induced neurotoxicity. PIF is a synthetic inhibitor of *p53* and was shown to protect against radiation, excitotoxicity, and, as here demonstrated, against $A\beta$ toxicity (Culmsee *et al*, 2001). The postulated mechanism of PIF activity involved the inhibition of *p53* nuclear translocation and prevention of its binding to specific DNA sites and it is not related to downregulation of newly synthesized *p53* or inhibition of *p53* activation by relevant kinases (Culmsee *et al*, 2001). In line with these evidences, we have shown that PIF did not alter $A\beta$ -induced increase of *p53* expression whereas it prevented the increased expression of DR5.

p53 promotes cellular apoptosis through transactivation of various pro-apoptotic genes that can act via an intrinsic pathway involving the mitochondria activation or an extrinsic pathway requiring activation of death receptors.

Although the extrinsic pathway and the intrinsic pathway for apoptosis are capable of operating independently, accumulated evidence suggests that a crosstalk between the two pathways exists in cells (for a review see Green, 1998). The results obtained with anti-DR5 antibody indicated that the blocking of DR5 receptor completely prevented $A\beta$ -induced neurotoxicity in neuronal cultures. These data suggest that p53-mediated apoptosis may occur by a preferential activation of death receptors pathway. We cannot exclude the involvement of intrinsic apoptotic pathway in $A\beta$ neurotoxicity. Indeed, our data suggest that the death receptor pathway, through the activation of caspase 8, can also activate the intrinsic pathway through the cleavage of bid. The truncated Bid then translocates to mitochondria and triggers cytochrome *c* release (Li et al, 1998; Luo et al, 1998). It has been proposed that Bid regulates cytochrome *c* release by inducing Bax translocation into mitochondria. In this regard, cells lacking Bax were resistant to TRAIL-induced apoptosis (Deng and Wu, 2000).

Finally, this study demonstrated a significant expression of DR5 receptor in AD brain. The expression of apoptosis-mediating TRAIL receptors indicates that brain cells, including neurons, are potentially susceptible to TRAIL. Although it is unknown whether or not other TRAIL receptors are modulated in AD pathology, the upregulation of TRAIL death receptor DR5 together with the presence of TRAIL in AD brain (Uberti et al, 2004) generate an appropriate environment to execute a cell death program. A possible role of TRAIL pathway in neurodegenerative diseases was also suggested by Martin-Villalba et al (1999) who showed that, after middle cerebral artery occlusion in adult rats, TRAIL is expressed in the apoptotic areas of the postischemic brain. Furthermore, TRAIL was found to be one of the major apoptotic factors inducing neuronal death in a murine model of HIV central nervous system infection (Miura et al, 2003; Ryan et al, 2004).

In conclusion, these data suggest a role of TRAIL death pathway in $A\beta$ neurotoxicity and propose DR5 receptor as a possible target for the development of neuroprotective drugs in those neuropathologies in which TRAIL pathway plays a relevant role.

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