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Neutralization of TRAIL death pathway protects human neuronal cell line from β-amyloid toxicity

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Abstract

Here we report that a novel member of the TNF-α family, TNF-related apoptosis-inducing ligand (TRAIL), contributes substantially to amyloid-induced neurotoxicity in human SH-SY5Y neuronal cell line. Involvement of TRAIL in the amyloid-induced cell death is supported by cDNA array, Northern blot, and Western blot data, demonstrating increased TRAIL expression after treatment of the cells with a neurotoxic fragment of amyloid protein (βAP). TRAIL was also found to be released in the culture media after βAP treatment with a time-course overlapping to contents of the intracellular protein. Contribution of TRAIL to βAP neurotoxicity is demonstrated by data showing that TRAIL-neutralizing monoclonal antibody protects neuronal SH-SY5Y cells from βAP neurotoxicity. Moreover, exposure of neuronal SH-SY5Y cells to TRAIL leads to cell death, indicating that this substance *per se* is endowed with neurotoxic properties. We also found that, similarly to βAP and TRAIL, activation of the death-domain adaptor protein FADD results in neuronal cell death. Lack of FADD function, by overexpression of its dominant negative, rescued cells from either TRAIL- or βAP-induced neurotoxicity, supporting the hypothesis that these three molecules share common intracellular pathways. Finally, we found that βAP strongly activated caspase-8, and the cell-permeable, selective caspase-8 inhibitor z-IETD-FMK prevents both βAP- and TRAIL-induced neurotoxicity. In view of TRAIL's potency in inducing neuronal death, and its role as mediator of βAP, it is plausible to hypothesize that TRAIL can be regarded as a molecule that provides substantial contribution to βAP-dependent cell death, which takes part in the progression of the neurodegenerative process and related chronic inflammatory response.

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Keywords: human; Alzheimer's disease; immune system; cytokines; DNA microchip array; apoptosis; SH-SY5Y neuroblastoma cell line

Abbreviations: AD, Alzheimer's diseaseβAP, β-amyloid protein; CNS, central nervous system; FADD, death-domain adaptor protein; GADPH, glyceraldehyde-3-phosphate dehydrogenase; MG, May–Grunwald–Giemsa's; MTT, methylthiazoletetrazolium; TNF, tumour necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand

Introduction

Alzheimer's disease (AD), the most common cause of dementia, is characterized by prominent, progressive neuronal loss associated with deposit of amyloid fibrils and inflammation in some areas of the central nervous system (CNS). [1] A number of factors have been thought to contribute redundantly to pathogenesis of AD, including unbalanced calcium homeostasis, cell-cycle proteins, excitatory amino acids, as well as DNA damage.[2–5] However, deposit of β-amyloid protein (βAP) in the brain parenchyma appears as the crucial factor in the onset of AD,[6] although mechanisms underlying βAP effects are far from elucidation, thus needing further investigation. For example, it has been hypothesized that proinflammatory cytokines, such as interleukin-1 and tumour necrosis factor-α (TNF-α), could partly promote neurodegenerative processes depending upon βAP.[7,8]

TNF-related apoptosis-inducing ligand (TRAIL) is a novel peptide molecule belonging to the TNF family whose main role is to induce programmed cell death in tumour cell lines from various tissue origins.[9] TRAIL binds to five specific receptors of the TNF/NGF family.[10,11] However, only two of them, DR4 and DR5, have an intracellular death domain, which appears to be essential for triggering cell death processes.[10,11] Whereas the decoy receptors TRAIL-R3 and -R4 act as a dominant-negative of TRAIL proapoptotic effects,[12–14] a fifth receptor, named osteoprotegerin, appears to be exclusively involved in bone remodelling.[15] Binding of TRAIL to DR4 and DR5 is followed by recruitment of the caspase-8 cascade, including the adaptor Fas-associated death-domain (FADD) protein.[16] In fact, TRAIL displays potent proapoptotic effects on a number of tumour cell lines,[17] whereas, with regard to normal tissues, such a property appears restricted to hepatocytes[18] and brain cells.[19] Interestingly, despite increased expression of TRAIL being described in ischaemic mouse brain,[20] there is no evidence for its presence in the adult human brain.[21] However, death-domain and TRAIL decoy receptors are distributed in the human CNS.[21]

So far, no information is available on the possible role of TRAIL and its receptors in neurodegenerative processes. In this study we evaluated the relationships between βAP and TRAIL, by means of apoptosis gene expression analysis,

performed in the differentiated human neuroblastoma cell line SH-SY5Y exposed to βAP. Furthermore, after assessment of gene expression, we studied the involvement of caspase 8 as a possible mechanism underlying TRAIL-associated neurotoxicity.

Results

βAP_{25-35} peptide induces apoptosis of SH-SY5Y cells

This study was carried out using human SH-SY5Y neuroblastoma cells differentiated in neuronal-like cells by treatment with retinoic acid for 1 week. After differentiation, these cells display neuron-like morphology and express markers of mature neurons.[23–25] Exposure of these cells to βAP_{25-35}, βAP_{1-40}, or βAP_{1-42} peptide resulted in concentration- and time-dependent cell death as determined by MTT assay (data not shown).

βAP_{25-35}-induced apoptosis of SH-SY5Y cells was further evaluated by monitoring morphological changes and levels of externalized phosphatidylserine (PS) in the cell membrane surface. Exposure of differentiated SH-SY5Y neuroblastoma cells to 25 μM βAP_{25-35} induced early changes in PS asymmetry, as revealed by the appearance of Annexin V immunoreactivity. As shown in Figure 1, Annexin V fluorescence staining appeared already 30–60 min after βAP exposure, it was widely spread after 4 h, and disappeared 8 h later (Figure 1, upper panels). Some scattered and condensed nuclei, as well as cells with blebbing plasma membrane (MG staining), appeared 24 h after βAP_{25-35} treatment (Figure 1, middle panels). Quantitative analysis of the results shows that the majority (55%) of apoptotic cells was detectable 72 h following the addition of the neurotoxic peptide (Figure 1, lower panels). Consistent with previous data,[26] the present results provide further evidence that apoptosis is the main mechanism by which βAP_{25-35} triggers neurotoxicity in human derived neurotypic cell lines.

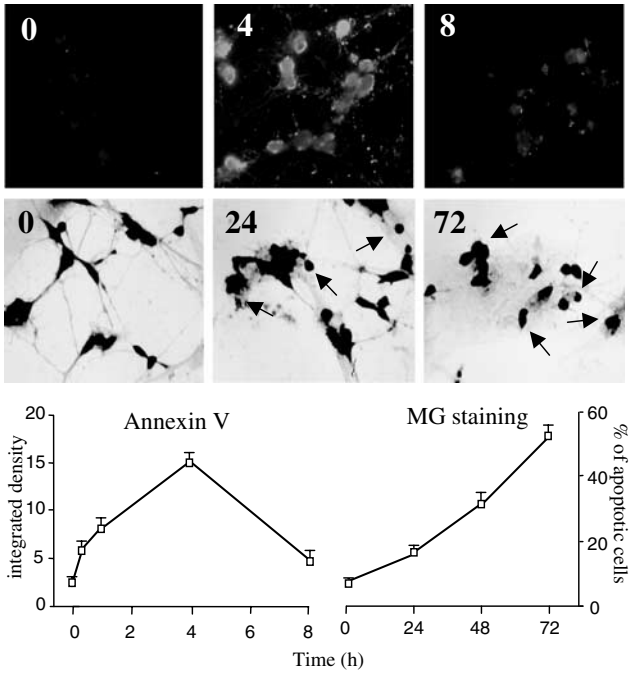

Figure 1 βAP-induced apoptosis of neuronal SH-SY5Y cell line. Representative images of cells exposed for different periods of time to 25 μM βAP_{25-35} and subjected to Annexin V (upper panels) and MG (middle panels) staining. Numbers inserted into the figures indicate time (h) of treatment. Lower panel: quantitative analysis of Annexin V fluorescent staining (left) and apoptotic cells (right). Data were presented as the mean $\pm$ S.E.M. of at least three separate experiments from two separate culture preparations. Arrows indicate condensed nuclei

Induction of TRAIL and DR5 by βAP

In order to detect candidate genes involved in βAP_{25-35}-induced apoptosis, a battery of 23 genes, known to be involved in the apoptotic process, were simultaneously analysed by cDNA expression array. To this end, cells were exposed to 25 μM βAP_{25-35} for different periods of time (1, 6, and 22 h), and mRNA was isolated and processed for cDNA array. Then, only those genes showing changes in their level of expression (either increased or decreased) by at least two-fold following treatment of cells with βAP were included in the analysis.

Results indicate that TRAIL and DR5 genes were upregulated consistently, and both of them in a significant manner, in cells treated with the neurotoxic peptide. Particularly, TRAIL mRNA levels increased by four-fold 6 h after βAP_{25-35} treatment and then decreased to basal value 22 h later. In a similar fashion, βAP_{25-35} exposure caused a transient increase of DR5 mRNA levels, which reached its peak (about two-fold) 6 h after treatment (Figure 2a).

The increased expression of TRAIL and DR5 mRNA levels revealed by the cDNA array hybridization technique was subsequently validated by Northern blot analysis (Figure 2b). Densitometry analysis of the hybridization signals and comparative ratio with GADPH data indicated that TRAIL and DR5 mRNA levels increased transiently, reaching a peak 6 h after βAP_{25-35} exposure (Figure 2b).

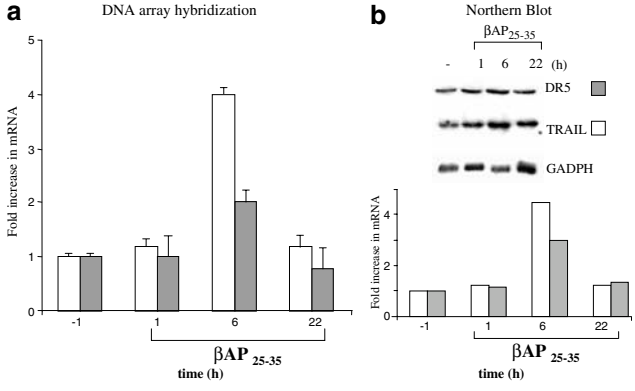

Figure 2 Induction of TRAIL (open bars) and DR5 (closed bars) mRNA after βAP_{25-35} exposure, as revealed by cDNA microarray (**a**) and Northern blot (**b**) analysis. mRNA was isolated from SH-SY5Y cells exposed for different periods of time, as indicated, to 25 μM βAP_{25-35}. Quantitative analysis was performed normalizing the sample towards GADPH mRNA levels. The results were expressed as fold increased with respect to the basal condition in untreated samples. All data are the mean $\pm$ S.E.M. from three different experiments run in duplicate. The upper panel of **b** shows representative Northern blot data

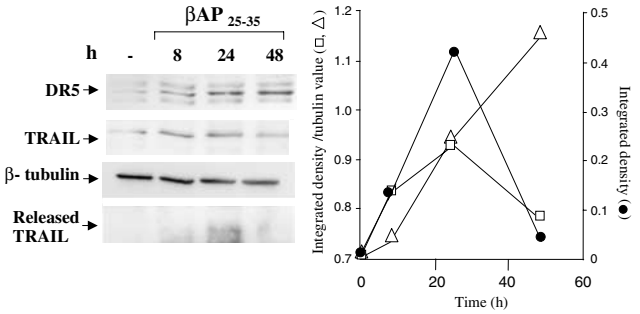

Figure 3 $\beta AP_{25–35}$ increased the expression of intracellular (black circle) and extracellular (open triangle) TRAIL and its receptor DR5 (open square). Protein extracts from cultures exposed to 25 μM $\beta AP_{25–35}$ for different time points, as indicated, were electrophoresed and immunoblotted with a polyclonal anti-DR5 or a monoclonal anti-TRAIL antibody. Conditioned medium was also processed for Western blot analysis with anti-TRAIL antibody by a double immunoprecipitation technique. The results from a representative experiment are shown in the left panel. Arrows indicate the band of the expected size. Densitometric analysis is shown in the right panel b. All data points are the means $\pm$ S.E.M. from at least three separate experiments and are expressed as integrated density by normalizing the sample values towards the corresponding β-tubulin expression levels

We then verified whether such a βAP-induced increase of TRAIL and DR5 mRNA levels was followed by increased protein synthesis. Western blot analysis of protein extracts from neuronal SH-SY5Y cells exposed to 25 μM $\beta AP_{25–35}$ for 8, 24, and 48 h was carried out by means of a monoclonal anti-TRAIL and a polyclonal anti-DR5 antiserum. Representative results are shown in Figure 3. Densitometry analysis performed for normalization of results through evaluation of the ratio to the corresponding β-tubulin data indicated that DR5 protein levels increased 24 and 48 h after the treatment. TRAIL protein levels increased transiently, and reached a peak 24 h after $\beta AP_{25–35}$ exposure (Figure 3). We also attempted to detect TRAIL in the cell culture media of SH-SY5Y cells by means of immunoprecipitation. In basal conditions, TRAIL amounts in cell culture media were below detection levels. On the other hand, substantial amounts of soluble TRAIL were detected after 8 and 24 h $\beta AP_{25–35}$ exposure (Figure 3).

These results provide evidence that exposure of human neuronal SH-SY5Y cells to $\beta AP_{25–35}$ leads to an increase of TRAIL transcription, protein synthesis, and release in the culture media.

Contribution of TRAIL to βAP neurotoxicity

To characterize possible relationships between neurotoxic effects of $\beta AP_{25–35}$ and increased expression of TRAIL, neuronal SH-SY5Y cell cultures were challenged with maximally effective concentrations of either TRAIL or $\beta AP_{25–35}$, in the presence or absence of a TRAIL-neutralizing monoclonal antibody. As shown in Figure 4a, the extent of neurotoxicity detectable 48 h following 100 ng/ml TRAIL exposure (about 25–30%) was comparable to that caused by 25 μM $\beta AP_{25–35}$ treatment. Preincubation of cells with a TRAIL-neutralizing monoclonal antibody resulted in complete reversal of the neurotoxic effect of TRAIL and, in addition, significantly reduced that of $\beta AP_{25–35}$. The specificity of the neuroprotective action elicited by the anti-TRAIL antibody was demonstrated by the lack of effects of the isotype IgG_1 control.

Morphological analysis of cells exposed to TRAIL revealed changes similar to those induced by $\beta AP_{25–35}$, in terms of both transient expression of Annexin V in the plasma membrane and appearance of scattered and condensed nuclei, as assessed by MG staining (Figure 4b). Furthermore, similarly to βAP, TRAIL induced increased expression of DR5 with no changes in DR4 protein levels. The results from a representative experiment are shown in Figure 5.

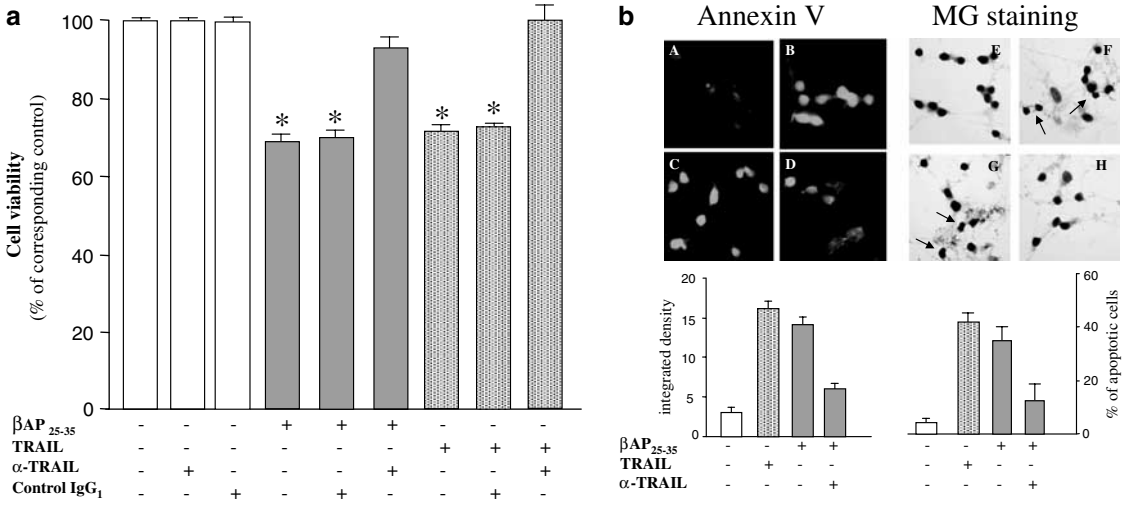

Figure 4 Effects of TRAIL and anti-TRAIL neutralizing antibody on cell viability (**a**) and morphology (**b**) In panel a: cells were exposed for 48 h to 25 μM $\beta AP_{25–35}$ or 100 ng/ml TRAIL in the presence or absence of the anti-TRAIL neutralizing antibody (α-TRAIL) or the isotype IgG_1 control, as indicated, and then processed for MTT fluorescence assay. Panel b shows representative images of cells exposed to 25 μM $\beta AP_{25–35}$ (**B**, **F**), 100 μg/ml TRAIL (**C**, **G**) in the presence (**D**, **H**) or absence (**A–C**, **E–G**) of α-TRAIL, and subjected to Annexin V (upper panel b, left) and MG (upper panel b, right) staining. Lower panel: quantitative analysis of Annexin V fluorescent staining (left) and apoptotic cells (right). Bars represent the data as the mean $\pm$ S.E.M. of at least three separate experiments from two separate culture preparations. Arrows in **F** and **G** indicate condensed nuclei. * $P < 0.05$ *versus* the corresponding control value

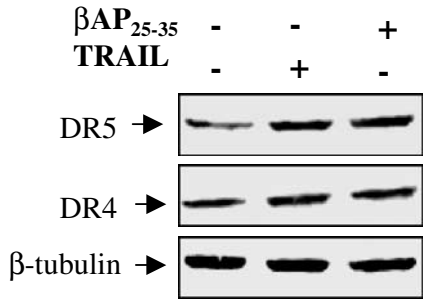

Figure 5 Selective upregulation of DR5 by βAP and TRAIL. Representative Western blot analysis of protein extracts from cells exposed for 48 h to 25 μM βAP_{25-35} or 100 ng/ml TRAIL. Protein extracts were electrophoresed and immunoblotted with a polyclonal anti-DR5 or a polyclonal anti-DR4 antibody. Arrows indicate the band of the expected size

It is well established that binding of TRAIL to its death-domain receptors leads to activation of caspase-8, with subsequent activation of caspase 3, finally resulting in apoptosis.[27] Caspase-8 activation via TRAIL occurs, in turn, through the recruitment of the downstream adaptor protein FADD.[27] For this reason, we investigated the TRAIL-related signal transduction pathway in neuronal SH-SY5Y cells following exposure to either βAP or TRAIL. The involvement of FADD in apoptosis of neuronal SH-SY5Y cells was examined by overexpressing FADD or its dominant negative version (FADD-DN) in these cells. As depicted in Figure 6, overexpression of FADD caused neurotoxic effects, with about 30% decrease of cell viability at 48 h after transfection. The extent of cell loss was comparable to that found after

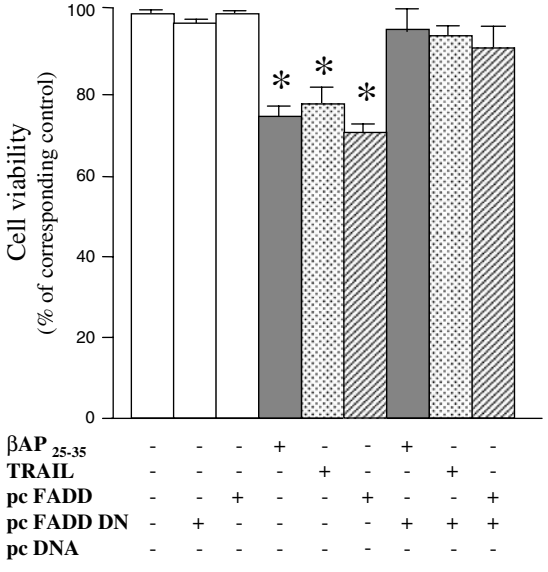

Figure 6 Loss of FADD activity suppresses βAP and TRAIL neurotoxicity. Cells were transfected with plasmid with no insert (pcDNA) , FADD (pc FADD), or FADD dominant negative (pc FADD DN) and, 48 h later, challenged with 25 μM βAP_{25-35} or 100 ng/ml TRAIL, as indicated. Cell viability was determined by MTT fluorescence staining 48 h after exposure of the cells to the neurotoxic agent. Bars represent the data as the mean $\pm$ S.E.M. of at least three separate experiments from two separate culture preparations. *$P<0.05$ *versus* the corresponding control value

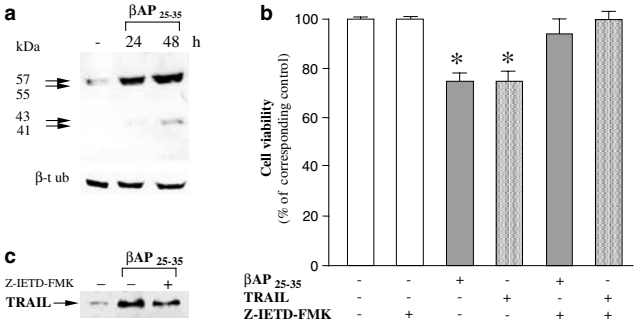

Figure 7 Inhibition of caspase 8 activity suppresses β-amyloid and TRAIL neurotoxicity *t*. Panel **a** shows representative Western blot of protein extract from cells exposed to 25 μM βAP_{25-35} for 0, 24, and 48 h, as indicated on the top of figure, immunoblotted with a monoclonal anti-TRAIL or anti-β-tubulin (β-tub) antibody. Arrows indicate the bands of the expected size, as indicated. Panel **b** shows the effects of the specific caspase inhibitor z-IETD-FMK (2 μM) in cells exposed to 25 μM βAP_{25-35} or 100 ng/ml TRAIL for 48 h. Values are expressed as percent of neurotoxicity induced by the specific neurotoxic agent. Bars represent the data as the mean $\pm$ S.E.M. of at least three separate experiments from two separate culture preparations. *$P<0.05$ *versus* the corresponding control value. Panel **c** shows a representative Western blot analysis of protein extracts from cells exposed to 100 ng/ml TRAIL for 48 h in the presence or absence of 2 μM z-IETD-FMK, as indicated

exposure of the cells to TRAIL or βAP_{25-35}. The neurotoxic effects induced by FADD, TRAIL, or βAP was not occurring in cells over-expressing the FADD-DN.

Involvement of caspase-8 in the βAP-induced apoptosis was investigated by studying cleavage of procaspase-8 by Western blot analysis carried out using an anticaspase-8 monoclonal antibody, as well as the caspase-8 inhibitor z-IETD-FMK at a concentration of 2 μM (Figure 7). In basal conditions, SH-SY-5Y cells expressed extremely low levels of procaspase-8 protein. A 24 h exposure of the cells to βAP_{25-35} resulted in a significant increase of the inactive caspase-8 isoform, which was subsequently cleaved, as indicated by the appearance of 43–41 kDa bands 48 h after the treatment (Figure 7a). Moreover, when the caspase-8 inhibitor z-IETD-FMK was added to the cultures 30 min before βAP_{25-35}, about 70 % of peptide cytotoxicity was neutralized. In addition, TRAIL-induced cytotoxicity was completely rescued by the caspase-8 inhibitor z-IETD-FMK (Figure 7b). Exposure of the cells to z-IETD-FMK did not significantly alter the capability of βAP_{25-35} to increase TRAIL protein levels. The results from a representative Western blot experiment are shown in Figure 7c.

Discussion

We report here for the first time that (i) a novel member of the TNF-α family, TRAIL, mediates neuronal death induced by βAP and (ii) inhibition of the TRAIL pathway results in neuroprotection.

Indeed, possible involvement of the TRAIL pathway in neurodegenerative processes has recently been suggested by data showing activated TRAIL in neurons undergoing ischaemia-induced apoptosis.[20] In addition, human brain cells express TRAIL receptors and are susceptible to TRAIL exposure.[19,21]

The involvement of TRAIL in the βAP-induced neurotoxic effects is supported by our cDNA array, Northen blot, and Western blot data, demonstrating increased TRAIL expression in cells treated with βAP. The increase of TRAIL mRNA levels was transient and reached a peak 6 h after treatment, whereas intracellular protein levels appeared significantly increased between 8 and 24 h after the treatment, suggesting active translational activity. Moreover, TRAIL was found to be released in the culture media after βAP treatment with a time-course overlapping to contents of the intracellular protein. These results suggest that, once synthesized, TRAIL is rapidly activated and released in the culture media. The substantial amounts of TRAIL measured in the culture media after 24 h treatment with βAP may be responsible for the increased βAP-induced DR5 expression, as suggested by the finding that TRAIL, *per se*, upregulates DR5 protein levels. Thus, subsequent activation of TRAIL and its surface death receptor DR5 may cause βAP to trigger a death program.

The concept that TRAIL significantly contributes to the neurotoxic effects of βAP is corroborated by other data, showing that immunoneutralization of TRAIL results in protection of neuronal SH-SY5Y cells from βAP neurotoxicity. Moreover, exposure of neuronal SH-SY5Y cells to TRAIL leads to cell death, indicating that this substance *per se* is endowed with neurotoxic properties. In this regard, it is noteworthy that exposure of SH-SY5Y cells to βAP does not induce TNFα,[28] nor does it modify FasL mRNA levels (data not shown). Thus, our data indicate that, in our experimental model, TRAIL is one of the relevant contributors to βAP neurotoxicity. However, it cannot be ruled out that in other experimental models, that is, primary culture of rat cortical neurons, additional members of the TNF-α family participate in βAP neurotoxicity, as recently found by Morishima *et al*.[29]

We then investigated the intracellular pathway involved in βAP- and TRAIL-induced neurotoxicity. It is recognized that, after stimulation by TRAIL, the apoptosis transducing receptors DR4 and DR5 homo- or heterotrimerize and then bind to downstream death-domain proteins, such as FADD, TRADD, and RIP, depending upon the cellular system used.[30]

Recruitment of FADD seems particularly crucial, since it allows activation of caspase-8, leading in turn to cell death. It should be noted that recruitment of FADD and activation of procaspase-8 are required for apoptosis induced by FasL and TNF R1 receptors.[31,32]

Our results show that, similarly to βAP and TRAIL, activation of FADD results in neuronal cell death. In addition, lack of FADD function in cells transfected with its dominant negative, rescues cells from either TRAIL- or βAP-induced neurotoxicity, supporting the hypothesis that these three molecules share common intracellular pathways.

We also demonstrated that the cell-permeable, selective caspase-8 inhibitor z-IETD-FMK prevents both βAP- and TRAIL-induced neurotoxicity. These results are consistent with previous data, indicating an involvement of both FADD and caspase-8 in βAP-induced toxicity,[33,34] and supply additional evidence that recruitment of FADD and activation of caspase-8 are crucial and common events in the cell death program triggered by either βAP or TRAIL. In addition, despite the neuroprotective effects, z-IETD-FMK did not significantly alter the capability of βAP_{25-35} to increase TRAIL expression. These results indicate that βAP-induced TRAIL expression is upstream caspase-8 activation, and further support the contribution of TRAIL in βAP-induced cell death.

Blockade of the FADD/caspase-8 pathway resulted in complete prevention of neurotoxicity induced by TRAIL, whereas βAP-induced cell death was only partially affected. These data suggest that βAP-induced neuronal death may recruit an array of candidate mediators other than TRAIL and DR5.[29,33,34]

All together, these data provide evidence that the TRAIL/DR5/FADD/caspase-8 pathway acts as an efficacious destructive effector system in the human neuronal cell line SH-SY5Y exposed to βAP. The fact that a human cell line undergoes apoptosis upon activation of the above pathways has special relevance, as different studies have revealed a substantial difference in responsiveness among human neural cells and cells from other species used in preclinical studies, in which TRAIL fails to induce neuronal cell death.[18,35–37] Although our data are apparently discrepant with the general view that cytotoxic effects of TRAIL are restricted to tumor cells, they are nevertheless corroborated by the results from Nitsch *et al*.[19] suggesting extreme care in using TRAIL in the treatment of human brain tumors, owing to its potential toxicity in normal cells.

In summary, we found that TRAIL mediates neuronal cell death induced by βAP and that the related neurotoxic effects may be neutralized by inhibiting the TRAIL pathway. Our data suggest that the inhibition of TRAIL effects represents a potential therapeutic tool for intervention in neurodegenerative diseases with prominent immunological component, such as, for example, Alzheimer's disease.

Materials and Methods

Cell culture

The human neuroblastoma cell line SH-SY5Y was routinely cultured in 1 : 1 Ham's F12 : Dulbecco modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum, 2 mM glutamine, 50 μg/ml penicillin, and 100 μg/ml streptomycin and kept at 37°C in humidified 5% CO_2/95% atmosphere. For differentiation, cultured cells were treated for 1 week with 10 μM retinoic acid.

Handling of β-amyloid peptide

βAP_{1-42}, βAP_{1-40}, and βAP_{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. Before the experiments, βAP_{1-42} and βAP_{1-40} stock solutions were kept for 1 week in a 37°C incubator so as to allow aggregation and, therefore, toxicity. βAP_{25-35} was toxic soon after solubilization.[22] All peptides were tested at graded concentrations (range: 5–60 μM) for evaluation of cell toxicity.

Evaluation of cell viability

A total of 5×10^3 cells/well were plated in 96-well plates and grown to 70–80% confluence in complete DMEM containing 10 μM retinoic acid. Differentiated cultures were incubated for 48 h with 25 μM βAP_{25-35} or 100 ng/ml TRAIL and its Potentiator (1.5 μg/ml; Potentiator enhanced

TRAIL-induced cell death (Upstate Biotechnology, Lake Placid, NY, USA), either alone or with the caspase-8 inhibitor z-IETD-FMK (2 μM; Alexis Biochemicals, San Diego, CA, USA), a TRAIL-neutralizing monoclonal antibody or an isotype-matched control (IgG_1 control) (1 μg/ml; Alexis Biochemicals, San Diego, CA, USA), in serum-free DMEM at 37°C. The caspase inhibitor as well as the Trail-neutralizing monoclonal antibody or IgG_1 control were added 30 min before the neurotoxic agent. At the end of the treatment, cells were incubated with 1 mg/ml MTT (Sigma-Aldrich, Milan, Italy) in fresh medium for 4 h at 37°C. Then, a mixture of isopropanol and 1N HCl (24 : 1, v/v) was added to dissolve resulting formazan crystals. Dye absorbance in viable cells was measured at 570 nm. Each experiment was performed in triplicate and repeated at least twice. Statistical significance of differences was calculated by one-way analysis of variance (ANOVA) followed by Student's *t*-test. Significance was accepted for a *P*-value <0.05.

Evaluation of apoptosis

A total of 5×10^5 cells were plated onto 10 μg/ml poly-L-lysine-coated glass coverslips and cultured as described above. At different time (30 min to 8 h) following βAP_{25-35} exposure or treatment with 100 ng/ml TRAIL together with its Potentiator (1.5 μg/ml), culture media were replaced with an Annexin V staining solution (Alexis Biochemicals, San Diego, CA, USA) (25 μl/ml of Annexin V-FITC in the binding buffer containing 10 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$). After 20 min of incubation at room temperature, the cells were washed twice in phosphate-buffered solution (PBS) and fixed with 4% paraformaldehyde for 30 min, followed by two additional washes. Cells were then observed under a fluorescence microscope. About 50–100 cells in at least three randomly-selected microscopic fields from duplicate cultures in at least two different experiments were scored for fluorescent intensity using Pro-plus Image analysis software.

The latest stage of apoptosis, characterized by nuclear condensation and cytoplasmic blebbing, was evaluated 24, 48, and 72 h following exposure to cytotoxic agents using the May–Grunwald–Giemsa's (MG) staining. Briefly, cells were washed in PBS solution, fixed with May–Grunwald's staining containing methanol for 3 min, and then washed with a 200 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7.4) for an additional 3 min. Cells were then stained (3 min) with Giemsa, and air-dried after three/four rapid washes in water. The percentage of apoptotic cells was measured by calculating the ratio between blebbing cells and condensed nuclei, and normal cells staining in photomicrographs of at least three representative fields from each dish. Data were presented as the mean $\pm$ S.E.M. of at least three separate culture preparations. Statistical significance of differences between the values was made by one-way analysis of variance (ANOVA) followed by Student's t test. Significance was accepted for a *P* value <0.05.

Cell transfection

A total of 5×10^3 cells/well were plated in 96-well plates and grown for 7 days with 10 μM retinoic acid. At 24 h before transfection, the medium was replaced by 10% foetal calf serum DMEM without antibiotics. The expression vectors for pcDNA3 FADD and its dominant negative (pcDNA3 FADD-DN) were kindly provided by Dr. Marcus Schuchmann (Klinikum der Johannes Gutenberg, Universitat, Mainz, Germany). The plasmid DNA (300 ng) was diluted in 25 μl of serum and antibiotic-free DMEM/well, and 0.5 μl of LF2000 reagent was diluted into 25 μl/well in the same media in a different tube and incubated for 5 min at room temperature. The diluted DNA and LF2000 reagent were then combined and incubated at room temperature for 30 min. The resulting complexes were directly added to cultures (50 μl/well) and incubated for 5 h. Transfection medium was then replaced with growth medium, containing 10% FBS without antibiotics. Cells were cultured for an additional 48 h before undergoing specific treatments.

Gene array

Human Apoptosis-1 GEArray kit, composed of 23 apoptosis-related genes as well as two house-keeping genes, actin and glyceraldehyde-3-phosphate dehydrogenase (GADPH), was used to characterize the gene expression profile of differentiated SH-SY-5Y cells after treatment with βAP_{25-35} for 0, 1, 6, and 22 h. Total RNA was isolated from cells after solubilization in guanidinium thiocyanate by phenol–chloroform extraction and precipitation. cDNA probes for array analysis were synthesized following the manufacturer's directions (SuperArray Inc, Bethesda, MD, USA). Briefly, 10 μg of each total cell RNA was used as template to generate cDNA probes in the presence of [α-^{32}P]dCTP, GEAprimer mix, GEAlabeling buffer 5 $\times$, Moloney-murine leukemia virus reverse transcriptase (50 U/μl). Probes were then hybridized to Human Apoptosis-1 GEArray nylon membranes (SuperArray Inc., Bethesda, MD, USA), containing 23 spotted human cDNAs and two house-keeping genes, overnight at 68°C. Arrays were washed using the manufacturer's protocols, and differential gene expression patterns were detected by autoradiography.

Probe synthesis and Northern blot analysis

The cDNA probes of the selected genes, respectively human TRAIL, its DR5 receptor, and the GADPH, were synthesized by RT–PCR. For first-strand cDNA synthesis, 1 μg of total RNA was reverse-transcribed using 25 μg/ml oligo $(dT)_{12-18}$ primer in a final volume of 20 μl, in the presence of 200 units of M-MLV reverse transcriptase (Invitrogen, San Giuliano Milanese, Italy). The reaction was carried out at 37°C for 1 h and heated at 95°C for 10 min, and subsequently for 5 min at 4°C. PCR was performed in a total volume of 100 μl, containing 5 μl of the cDNA , 5 pmol of each upstream and downstream primer, and 1.8 units of Taq polymerase (Invitrogen, San Giuliano Milanese, Italy). The cycle program was as follows; (a) for human TRAIL and human DR5 primers, it consisted of 35 runs of denaturation at 95°C for 45 s, annealing at 60°C for 1 min, and elongation at 72°C for 1 min; (b) for human GADPH primers, it consisted of 25 runs of denaturation at 95°C for 1 min, annealing at 56°C for 1 min, and elongation at 72°C for 1 min. The cycle program was preceded by an initial denaturation at 95°C for 3 min and followed by a final extension at 72°C for 5 min. PCR products were analysed by 1.0% agarose gel electrophoresis and visualized with ethidium bromide. The following RNA transcripts were detected via amplification of the corresponding cDNAs: the human TRAIL using a primer pair composed of the sense primer 5′-CAGGATCATGGC-TATGATGGAGGTC-3′ and the antisense primer 5′-GCTGTTCA-TACTCTCTTCGTCATTG-3′; the human DR5 using a primer pair composed of the sense primer 5′-GGGAGCCGCTCATGAGGAAGTT-3′ and the antisense primer 5′-CTGGGTGATGTTGGATGGGAGAGT-3′; the human GADPH using the primer set composed of the sense primer 5′-CCACCCATGGCAAATTCCATG-3′ and the antisense primer 5′-TCTA-GACGGCAGGTCAGGTCCACC-3′. The resulting cDNAs were purified by High Pure PCR product (Boehringer, Mannheim, Germany) according to the manufacturer's protocol. Then, probes were labeled with [^{32}P]dCTP by random primer labelling (Invitrogen). Northern hybridization was performed according to standard protocols. A total of 20 μg of total RNA from differentiated SH-SY-5Y after treatment with βAP_{25-35} at time

points 0, 1, 6, and 22 h was electrophoresed on a formaldehyde-containing 1.2% agarose gel. The RNA was transferred to a nylon membrane for hybridization with ^{32}P-labelled cDNA probes. The hybridized RNA was detected by autoradiography.

Data analysis

Scanlyser software developed by Micheal Eisen at Lawrence Berkeley National Laboratory and GEArray analyser software by SuperArray Inc. were used to analyse the gene spots and process data. First, X-ray film recorded array images were converted into raw data files using scanlyser software. The data files were then processed with GEArray analyser software. Background subtraction was performed normalizing the data to the negative control (bacterial plasmid pUC18). The gene expression levels in the different samples were then normalized to the house-keeping gene expression level of each array. The results were expressed as fold increase compared to basal conditions in untreated samples. The data were presented as the mean $\pm$ S.E.M. of two different experiments in duplicate. Statistical significance of differences between the values was made by one-way analysis of variance (ANOVA) followed by Student's *t*-test.

Western blot analysis

Cells were harvested in 100 μl of lysis buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 5 mM EDTA, 1 mM phenyl methyl sulphonyl fluoride, 0.5 μg/μl leupeptin, 5 μg/μl aprotinin, 1 μg/ml pepstatin. The samples were sonicated and centrifuged at 15 000 $\times$ *g* for 30 min at 4°C. The resulting supernatants were isolated and protein content was determined by a conventional method (BCA protein assay kit, Pierce, Rockford, IL, USA). Protein extracts (15 μg) underwent electrophoresis on 12% SDS–PAGE, and transferred to nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Filters were incubated at room temperature overnight with polyclonal anti-DR5, anti-DR4 (Alexis Biochemicals, San Diego, CA, USA), monoclonal anti-TRAIL (Alexis Biochemicals, San Diego, CA, USA), anticaspase-8 (Cell Signaling Technology Inc., USA), or anti-tubulin (Ab3 Neo Markers) antibody in 5% nonfat dried milk (Sigma). Secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a chemiluminescence blotting substrate kit (Boehringer, Mannheim, Germany) were used for immunodetection. Densitometric analysis was performed on immunoblots by using a KLB 2222-020 Ultra Scan XL laser densitometer at a wavelength of 633 nm. To detect TRAIL protein in the conditioned media, supernatants were collected, freeze-dried and solved in RIPA buffer (10 mM Tris, pH 8, 140 mM NaCl, 1 mM sodium orthovanadate, 1 mM phenyl methyl sulphonyl fluoride, 10% SDS, 1% Triton X-100) supplemented with protease inhibitors (0.5 μg/μl leupeptin, 5 μg/μl aprotinin, 1 μg/ml pepstatin). A volume of 50 ml RIPA buffer was incubated with 2 μg/ml anti-TRAIL antibody for 2 h at 4°C, followed by 2 h incubation with 25 μl of protein A-Sepharose. After three washes with RIPA buffer, the complex TRAIL/antibody anti-TRAIL/A-Sepharose was resolved in 20 μl loading buffer (50 mM Tris, pH 6,8, 100 mM dithiothreitol, 2% SDS, 0,1% bromophenol blue, 10% glycerol), underwent electrophoresis on 15 % SDS–PAGE and immunoblotted with the monoclonal anti-TRAIL antibody.

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