

Transforming Growth Factor β 2 Is a Neuronal Death-Inducing Ligand for Amyloid- β Precursor Protein

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APP, amyloid β precursor protein, is linked to the onset of Alzheimer's disease (AD). We have here found that transforming growth factor β 2 (TGF β 2), but not TGF β 1, binds to APP. The binding affinity of TGF β 2 to APP is lower than the binding affinity of TGF β 2 to the TGF β receptor. On binding to APP, TGF β 2 activates an APP-mediated death pathway via heterotrimeric G protein G_o, c-Jun N-terminal kinase, NADPH oxidase, and caspase 3 and/or related caspases. Overall degrees of TGF β 2-induced death are larger in cells expressing a familial AD-related mutant APP than in those expressing wild-type APP. Consequently, superphysiological concentrations of TGF β 2 induce neuronal death in primary cortical neurons, whose one allele of the APP gene is knocked in with the V642I mutation. Combined with the finding indicated by several earlier reports that both neural and glial expression of TGF β 2 was upregulated in AD brains, it is speculated that TGF β 2 may contribute to the development of AD-related neuronal cell death.

Transforming growth factor β s (TGF β s) have been implicated in a broad diversity of biological activities, including cell growth, cell death, cell differentiation, inflammation, and immunological reactions, by modifying the expression of specific sets of target genes (29, 30, 45). Three isoforms of TGF β s, TGF β 1, TGF β 2, and TGF β 3, bind to the constitutive active serine/threonine kinase TGF β receptor II (TGF β RII). Upon ligand binding, the type I TGF β receptor (TGF β RI) is recruited into a receptor signaling complex, and kinase activity of TGF β RI is activated by TGF β RII-mediated phosphorylation. The receptor complex then activates signaling cascades to target genes by phosphorylating Smad family transcription factors.

It has been generally accepted that functions of TGF β family members may vary depending on cellular status and cell types. In neuronal tissues, it is clear that TGF β s play a neurotrophic role in some situations (5, 19, 27, 37), while they elicit cell-death-inducing effects in other situations (20, 42).

Accumulated evidence has revealed clear differences in biochemical and biological characteristics of TGF β isoforms, although they share 71 to 76% identity in their amino acid sequence. It is especially noted that TGF β 2 has a lower affinity to the type II receptor than TGF β 1 and TGF β 3 (7, 28). In contrast, TGF β 2 has a higher affinity to the type III TGF β receptor, which does not have the kinase domain and is considered to help TGF β to bind to the type II receptor (29). It has also been reported that the TGF β isoforms have their selective actions in certain systems. For example, TGF β 1 and TGF β 3, but not TGF β 2, strongly inhibit the growth of some glial cells (16).

Alzheimer's disease (AD), the most prevalent neurodegenerative disease, is characterized by three major pathological manifestations: neuronal loss, intracellular neurofibrillary tangles, and extracellular senile plaques. The major constituent of the plaques is amyloid β (A β), cleaved off from the transmembrane amyloid β precursor protein (APP) (33). Formation and accumulation of A β has been implicated in the development of AD (4, 8, 13, 44, 46). The removal of A β by anti-A β antibody from the brain improves the memory impairment of some AD model mice, indicating that upregulated A β s contribute to the progressive memory impairment in vivo (11).

It has been suggested that TGF β 1 may be involved in the onset of AD. TGF β 1 enhanced the generation of A β in transgenic mice that constitutively overexpressed familial AD (FAD)-linked mutant APPs (21, 47). TGF β 1 also was shown to enhance the expression of APP in vitro (1, 12).

In addition, expression of TGF β 2 has been reported to increase in the FAD brain (10, 23, 36). Flanders et al. reported that the expression of TGF β 2 was markedly enhanced in glial cells as well as in neurons bearing neurofibrillary tangles in AD brains (10). Enzyme-linked immunosolvent assays also indicated that TGF β 2 levels were threefold higher in homogenates of AD brains than in those of controls. Despite these foregoing clinical findings, the biological significance of the upregulation of TGF β 2 in AD brains remains unknown.

APP structurally resembles a single transmembrane receptor (17). Multiple groups have found that overexpression of FAD-associated mutant APPs induces neuronal cell death by triggering intracellular death signals (14, 25, 31, 48, 49, 50). In addition, it has been shown that binding of an anti-APP antibody to APP or the artificial dimerization of the intracytoplasmic domain of APP triggers neuronal cell death mediated by a heterotrimeric G protein, G_o, c-Jun N-terminal kinase (JNK), NADPH oxidase, and caspase 3 and/or related caspases (15, 39, 43). All these findings suggest that APP may be a putative

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receptor for a cell-death-inducing ligand(s), but direct evidence supporting this hypothesis has not been provided. In this regard, it is noted that TGF β 2 was shown to bind to the extracellular domain of APP (3), suggesting that TGF β 2 may be involved in APP-mediated cellular function.

We here demonstrate that TGF β 2 is a putative neuronal death-inducing ligand for APP. Our data suggest that TGF β 2 may be involved in the pathogenesis of AD-related neuronal loss.

MATERIALS AND METHODS

Cell lines, genes, recombinant proteins, and antibodies. Neurohybrid F11 cells and F11/EcR cells were as described previously (14, 15). The wild-type APP (wtAPP), wtAPP lacking domain 20 (wtAPP Δ 20), wtAPP Δ 19, V642I-APP (the isoleucine mutation at valine 642 in APP695), and NL-APP (the asparagine/leucine mutation at Lys595/Met596 in APP695) cDNAs in the pcDNA3 vector were all as described previously (14, 15, 43). The APP isoform denoted as APP in this study is APP695. Carboxyl-terminally V5-tagged mouse amyloid precursor-like protein 2 (APLP2) cDNA in the pcDNA3.1/GS vector and horseradish peroxidase (HRP)-conjugated anti-V5 antibody were purchased from Invitrogen (Carlsbad, CA). The cDNA fragment encoding most parts of the extracellular domain of mouse APP695 (APP-ED) corresponding to amino acids 1 to 590, fused in frame to the cDNA encoding the Fc region of human immunoglobulin G (IgG) (26), was inserted into the pEF-BOS plasmid (32) and named pEF-APP-ED/Fc. The cDNA fragment encoding most parts of the extracellular domains of human ciliary neurotrophic factor receptor corresponding to amino acids 1 to 390 (CNTFR-ED), fused in frame to the cDNA encoding the Fc region of human IgG and C-terminally tagged with 6 \times histidine, was inserted into the pcDNA3 plasmid and named pcDNA3-CNTFR-ED/Fc. The vector encoding the extracellular domain and the transmembrane domain of the epidermal growth factor receptor (EGFR) fused with the cytoplasmic domain of mouse APP was as described previously (15). The human TGF β receptor II expression vector was a kind gift of Xuedong Liu (Baylor College of Medicine). Lipofectamine, N2 supplement, and PLUS reagent were from Invitrogen (Carlsbad, CA). Recombinant human TGF β 1, TGF β 2, and TGF β 3 were from R&D Systems (Minneapolis, MN) and PeproTech EC Ltd. (London, United Kingdom). Recombinant human TGF α and an anti-TGF β 2 neutralizing polyclonal antibody were from R&D Systems. Antibodies to TGF β 1, TGF β 2, TGF β 3, and TGF β R2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 22C11 anti-APP antibody was from Chemicon (Temecula, CA). The His-tagged recombinant soluble APP α corresponding to amino acids 1 to 612 of APP695 was purchased from Sigma (St. Louis, MO). Other materials were all commercially available.

Transfection procedure, cell death assay, and cell viability assay. The transient transfection procedures were as described previously (14, 15). At 24 h after transfection, F11 cells or F11/EcR cells were treated with recombinant TGF β 1, TGF β 2, TGF β 3, or TGF α in serum-free Ham's F-12 medium with N2 supplement. Transfection efficiency in these protocols has been determined to be invariably around 70%. At 72 h after transfection, the trypan blue exclusion assay was performed as a cell death assay, and the WST-8 assay and/or calcein fluorescence assay was performed as a cell viability assay (14, 15).

Immunofluorescence-based binding assays. (i) Method 1. F11 cells (7×10^4 cells/well in 6-well plates) were replated onto 96-well plates (7×10^3 cells/well) at 24 h after transfection with the indicated amounts of wtAPP- or mutant APP-encoding plasmids or the TGF β R2-encoding plasmid. At 36 h after transfection, cells were combined with the indicated amounts of TGF β 1 or TGF β 2. After incubation for 6 h, they were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min, followed by incubation at room temperature with antibody to TGF β 1 (1:50) or TGF β 2 (1:50) in PBS with 1% bovine serum albumin (BSA) for 2 h. After being washed with PBS three times, cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody (Sigma) (1:150) in PBS with 1% BSA. After being washed three times with PBS, the immunofluorescence intensity was measured (excitation, 485 nm; emission, 535 nm) with a spectrofluorometer (Wallac 1420 ARVox Multi Label Counter). Cells not combined with TGF β s were immunostained with FITC-conjugated anti-rabbit IgG antibody without treatment with antibodies to TGF β s (indicated as "none"). This procedure was used in all binding assays except for the one shown in the experiments depicted in Fig. 2.

(ii) Method 2. In the experiments shown in Fig. 2, F11 cells (7×10^4 cells/well in 6-well plates) were replated onto 96-well plates coated with poly-L-lysine (7×10^3 cells/well) at 24 h after transfection with the indicated amounts of the

wtAPP-encoding vector or the TGF β R2-encoding vector. At 36 h after transfection, cells were combined with the indicated amounts of FITC (purchased from Sigma)-labeled TGF β 1 or TGF β 2 in the presence or the absence of the indicated amounts of unlabeled TGF β 1 or TGF β 2. To keep the total FITC amounts in wells constant, proper amounts of free FITC were added. After 6 h of incubation, they were washed with PBS three times. The immunofluorescence intensity was then measured (excitation, 485 nm; emission, 535 nm) with a spectrofluorometer (Wallac 1420 ARVox Multi Label Counter).

FITC labeling of TGF β 1 and TGF β 2. A mixture of 80 μ l of 10 μ M recombinant human TGF β 1 or TGF β 2 in 0.1 M Tris-HCl (pH 9.0) and 80 μ l of 0.1 M Tris-HCl (pH 9.0) containing 100 μ M FITC was incubated at 4 $^{\circ}$ C in the dark. After labeling for 18 h, FITC-labeled TGF β s were purified by elution with ZipTip silica columns (Millipore, Tokyo, Japan) and were lyophilized according to the manufacture's instruction. Protein concentrations of each FITC-labeled TGF β were measured using a bicinchoninic acid protein assay kit (Pierce).

Scatchard analysis. Using binding data, we estimated amounts of free (unbound) TGF β 2 in each assay. When the increase of immunofluorescence intensity numbers corresponding to the amount of bound TGF β 2 is much smaller than the increase of the amount of added TGF β 2, we are able to estimate the amount of free TGF β 2 with very small errors. For example, based on the data that the immunofluorescence intensity numbers by treatment with 1 pM and 10 pM TGF β 2 are 90 and 180 arbitrary units, we are able to estimate that the amount of free TGF β 2 at 10 pM TGF β 2 is 80 to 100% of the amount of total TGF β 2. K_d was estimated with the software Cricket Graph III J.

Adenovirus vector-mediated expression. The system of a replication-deficient adenovirus vector and adenoviruses encoding LacZ and V642I-APP were described previously (34). The cosmids for wtAPP were constructed by inserting the full-length wtAPP into the *Sma*I site of pAxCatw.

Primary cortical neurons and cell viability assay. Primary cortical neurons, obtained from embryonic day 14 (E14) ICR mice or V642I-APP knock-in mice, were seeded in poly-L-lysine-coated 24-well plates (Sumitomo Bakelite) at 1.25×10^5 cells/well in Neuron medium (Sumitomo Bakelite) (14, 34). Purity of neurons by this method was >98%. After incubation for 3 days, the culture medium was replaced with Dulbecco's modified Eagle medium containing N2 supplement. If indicated, on day 3 in vitro (DIV3), primary cortical neurons (PCNs) were infected by indicated multiplicities of infection (MOIs) of LacZ-, wtAPP-, or V642I-APP-encoded adenoviruses. On DIV4, indicated concentrations of TGF β 1, TGF β 2, or TGF β 3 were added, and cell viability was assessed by WST-8 assay and/or calcein fluorescence assay at 72 h after the onset of treatment.

Coimmunoprecipitation analysis. F11 cells (7×10^4 cells/well in 6-well plates) were transfected with the pEF-APP-ED/Fc or pcDNA3-CNTFR-ED/Fc plasmid. At 24 h after transfection, cells were cultured in serum-free Ham's F-12 with N2 supplement. At 72 h after the onset of transfection, cultured-conditioned media were collected. Five-hundred microliters of the conditioned medium, 20 pmol of TGF β 2 or TGF β 1 (final concentration, 40 nM), and 40 μ l of a 1:1 slurry of Protein G-Sepharose 4B were mixed and rotated at 4 $^{\circ}$ C overnight before immunoblot analysis.

Immunoblot analysis. Cell lysates (10 to 20 μ g/lane) or pulled-down precipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and separated proteins were transferred onto polyvinylidene difluoride membranes as described previously (14). Visualization of the immunoreactive bands was performed by ECL (Amersham Pharmacia Biotech, Uppsala, Sweden). Densitometrical analysis of immunological signals were performed with NIH Image, version 1.62, software.

V642I-APP knock-in mice. The targeted introduction of the V642I mutation in the mouse APP gene was performed as described previously (18). PCNs were prepared from 14-day-old embryos generated by crossing a heterozygous male mouse with a heterozygous female mouse. To identify homozygous, heterozygous, and wild-type PCNs, PCR analysis was performed as described previously (18).

Real-time PCR. We performed real-time PCR to assess the amount of endogenous mRNA. Cells were harvested for the RNA extraction with an ISOGEN reagent (Nippon Gene, Toyama, Japan) followed by real-time PCR. The first-strand cDNAs were synthesized using SensiScript reverse transcriptase (QIAGEN) with 0.5 μ g total RNA. Real-time PCR analysis was performed using a QuantiTect SYBR Green PCR kit (QIAGEN), followed by analysis with an ABI PRISM 7700 (Applied Biosystems, Foster City, CA). We made sets of a sense primer and an antisense primer as follows: 5'-CACGCTACTTCTCTCAA G-3' and 5'-CTCTGTCTTCATCAGCTGGC-3' for mouse PAI-1 and 5'-TCC ACCACCTGTTGCTGTA-3' and 5'-ACCACAGTCCATGCCATCAC-3' for human and mouse G3PDH. Data analysis was performed using Sequence Detection System software, version 1.9.1 (Applied Biosystems). To adjust the expression level of each mRNA, G3PDH mRNA was used as an internal control.

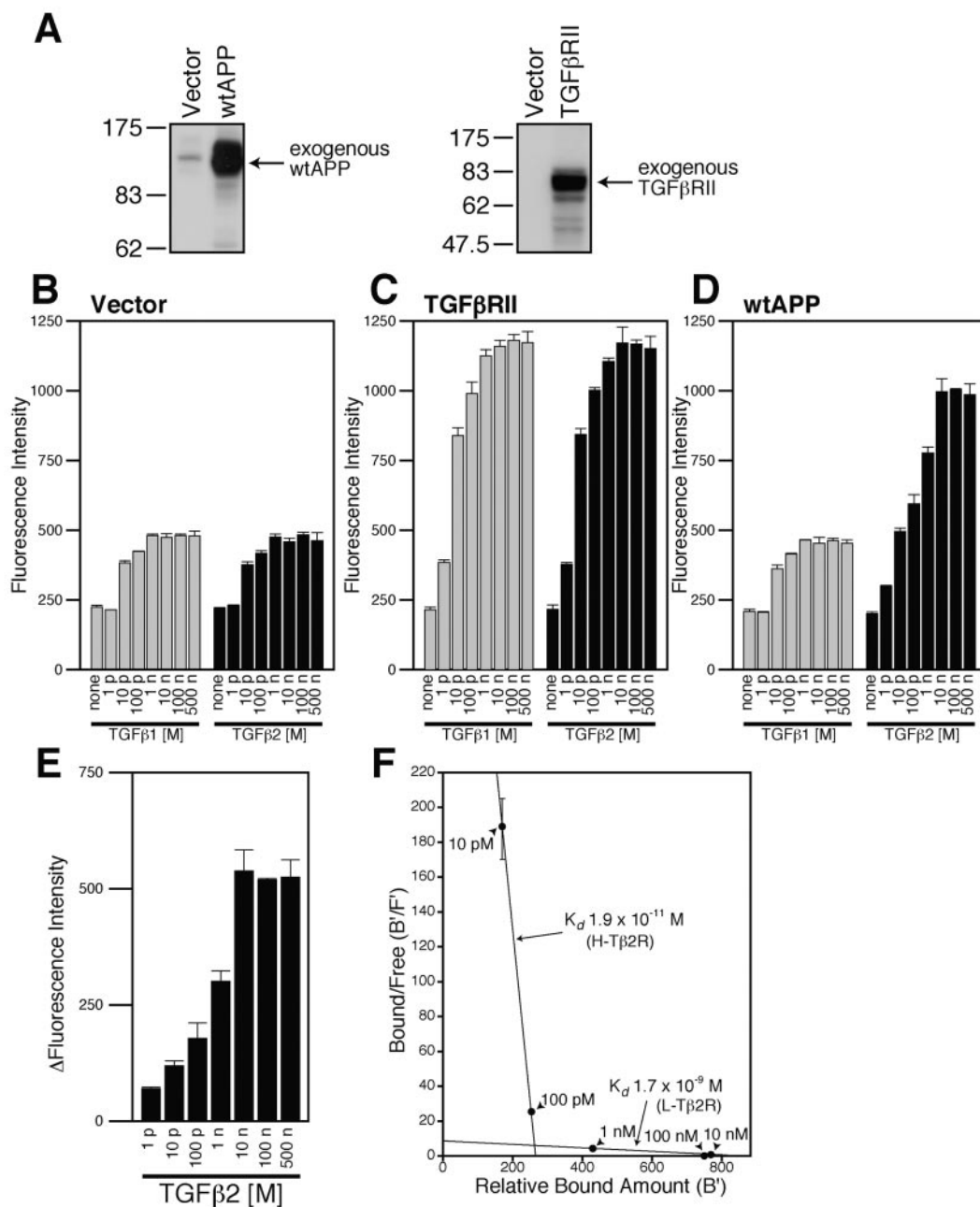


FIG. 1. Overexpression of wtAPP induces the expression of TGFβ2-specific receptors. (A) F11 cells (7×10^4 cells/well in 6-well plates) were transfected with 0.5 μ g of the pcDNA3 vector, pcDNA3-TGFβRII, or pcDNA3-wtAPP. Cell lysates (10 μ g in each lane) were immunoblotted with antibody to APP (22C11) or TGFβRII. (B to D) F11 cells (7×10^4 cells/well in 6-well plates) were transfected with 0.25 μ g of the pcDNA3 vector (B), pcDNA3-TGFβRII (C), or pcDNA3-wtAPP (D). At 36 h after transfection, the cells were combined with the indicated concentrations of TGFβ1 or TGFβ2. Immunofluorescence-based binding assays were performed as described in Materials and Methods. (E) The mean of immunofluorescence intensity numbers representing the association between TGFβ2 and F11 cells for each concentration of TGFβ2 shown in panel B were subtracted from immunofluorescence intensity numbers representing the association between TGFβ2 and F11 cells for the same concentration of TGFβ2 shown in panel D. Resulting intensity numbers were considered to correspond to the association between TGFβ2 and wtAPP-induced TGFβ2-specific receptors. (F) A simulated Scatchard plot of the association between TGFβ2 and wtAPP-induced TGFβ2-specific receptors based on the analysis shown in Table 1. A vertical bar for each point indicates an estimated range of bound/free numbers. p, pico; n, nano; B', relative amount of bound TGFβ2; F', estimated amount of free TGFβ2.

TABLE 1. Estimation of amounts of free TGFβ2 in binding assays^a

TGFβ2 concn	Total TGFβ2 amt (fmol)	Mean change in fluorescence intensity (arbitrary units)	Relative bound amt (B')	Estimated free amt (fmol) (F')	B'/F'
1 pM	0.1	70	100	0.0–0.1	
10 pM	1	119	170	0.83–1	170–205
100 pM	10	178	254	9.75–10	25.4–26.0
1 nM	100	301	430	99.6–100	≈4.32
10 nM	1,000	538	769	999–1,000	≈0.77
100 nM	10,000	519	750	9,999–10,000	≈0.075

^a TGFβ2 concentrations and mean change in fluorescence intensity numbers were derived from Fig. 1E. Relative amounts of bound TGFβ2 (B') were calculated under the condition that the amount of bound TGFβ2 was 100 when the TGFβ2 concentration was 1 pM.

Statistical analyses. All cell death experiments, TGFβ binding assays, cell viability experiments, and real-time PCR experiments were done with $n = 3$ (n is number of determinations). All values in the experiments are means \pm standard deviations. Statistical analyses were carried out with one-way analysis of variance followed by a post hoc test (Fisher's protected least significant difference test). $P < 0.05$ was assessed as significant.

RESULTS

Overexpression of APP in F11 cells results in the appearance of TGFβ2-specific receptors. Based on findings that expression of TGFβ2 is upregulated in AD brains (10, 23, 36) and TGFβ2 binds to the extracellular domain of APP (3), we hypothesized that TGFβ2 displays some biological activities by binding to APP. To address this possibility, we first tried to look at the association between TGFβ2 and APP on the cell surface, using F11 neurohybrid cells. To this end, we developed immunofluorescence-based binding assays as described in Materials and Methods, using F11 cells or F11 cells that transiently overexpress wild-type APP (wtAPP) or TGFβ receptor type II (TGFβRII) (see the expression of wtAPP and TGFβRII in Fig. 1A). Overexpression levels of APP were roughly estimated to be 1 to 2, 3 to 5, 6 to 10, or 10 to 20 times more than the endogenous APP level when F11 cells were transfected with 0.1, 0.25, 0.5, or 1.0 μg of pcDNA3 expression vectors encoding APPs. Unexpectedly, these assays have turned out to be excellent in reproducibility. Errors generated in these assays were minimal, although it has been generally thought that the procedures to enhance signals with antibodies increase errors.

First of all, to test the feasibility of these assays, we looked at the association between TGFβs and F11 cells or F11 cells ectopically overexpressing TGFβRII. As shown in Fig. 1B, treatment with either TGFβ1 or TGFβ2 increased the fluorescence intensity in a dose-dependent fashion. The pattern and the extent of the TGFβ-dose-dependent increase in the fluorescence intensity by treatment with TGFβ1 were very similar to those by treatment with TGFβ2. It also is noted that the increase in the fluorescence intensity reached a plateau at 1 nM TGFβ, suggesting that the increase in the fluorescence intensity represented the saturable association between TGFβ1 or TGFβ2 and endogenous TGFβ-binding proteins, including the TGFβ receptor. In addition, 50% effective concentrations of the association between TGFβ1 or TGFβ2 and F11 cells appeared to be around 10 pM (Fig. 1B). Considering that the K_d for the association between TGFβ1 or TGFβ2 and the TGFβ receptor was estimated to be 5 to 50 pM, this number is quite reasonable. Combined with an additional find-

ing that the increase in the fluorescence intensity by TGFβ treatment was enhanced by overexpression of TGFβRII (Fig. 1C), we have concluded that the association between TGFβ1 or TGFβ2 and the receptors is faithfully examined by the binding assay.

We then looked at the association between TGFβ1 or TGFβ2 and F11 cells overexpressing wtAPP. As shown in Fig. 1D, overexpression of wtAPP upregulated the association between TGFβ2 and F11 cells but not the association between TGFβ1 and F11 cells. It is especially noted that the association between TGFβ2 and F11 cells overexpressing wtAPP reached a plateau at a TGFβ2 concentration of 10 nM (Fig. 1D, right panel), indicating that this binding was also saturated. Similar

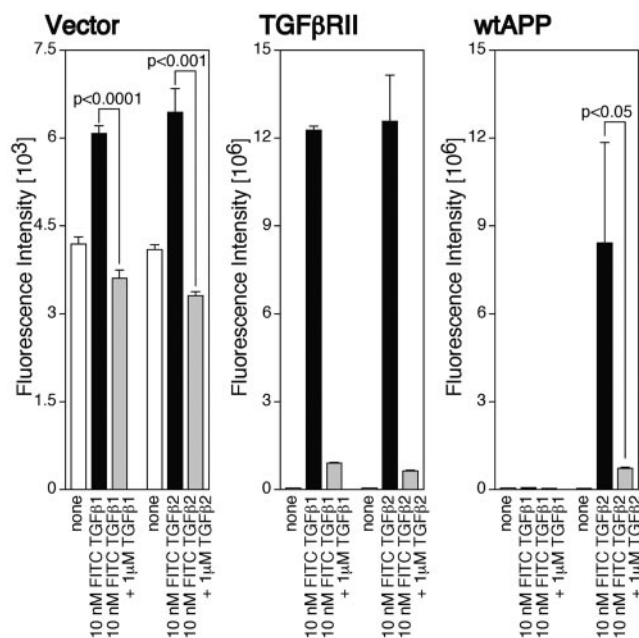


FIG. 2. Specific association between TGFβ2 and F11 cells overexpressing wtAPP. F11 cells (7×10^4 cells/well in 6-well plates) were transfected with 0.25 μg of the TGFβRII-encoding plasmid or the wtAPP-encoding plasmid and were replated into 96-well plates coated with poly-L-lysine (7×10^3 cells/well) at 24 h after transfection. At 36 h after transfection, cells were combined with 10 nM FITC-labeled TGFβ1 or TGFβ2 in the presence or the absence of 1 μM unlabeled TGFβ1 or TGFβ2. To keep the total FITC amounts constant, proper amounts of free FITC were added. After incubation for 6 h, they were washed with PBS three times. The immunofluorescence intensity was measured (excitation, 485 nm; emission, 535 nm) with a spectrofluorometer (Wallac 1420 ARVOSx multilabel counter).

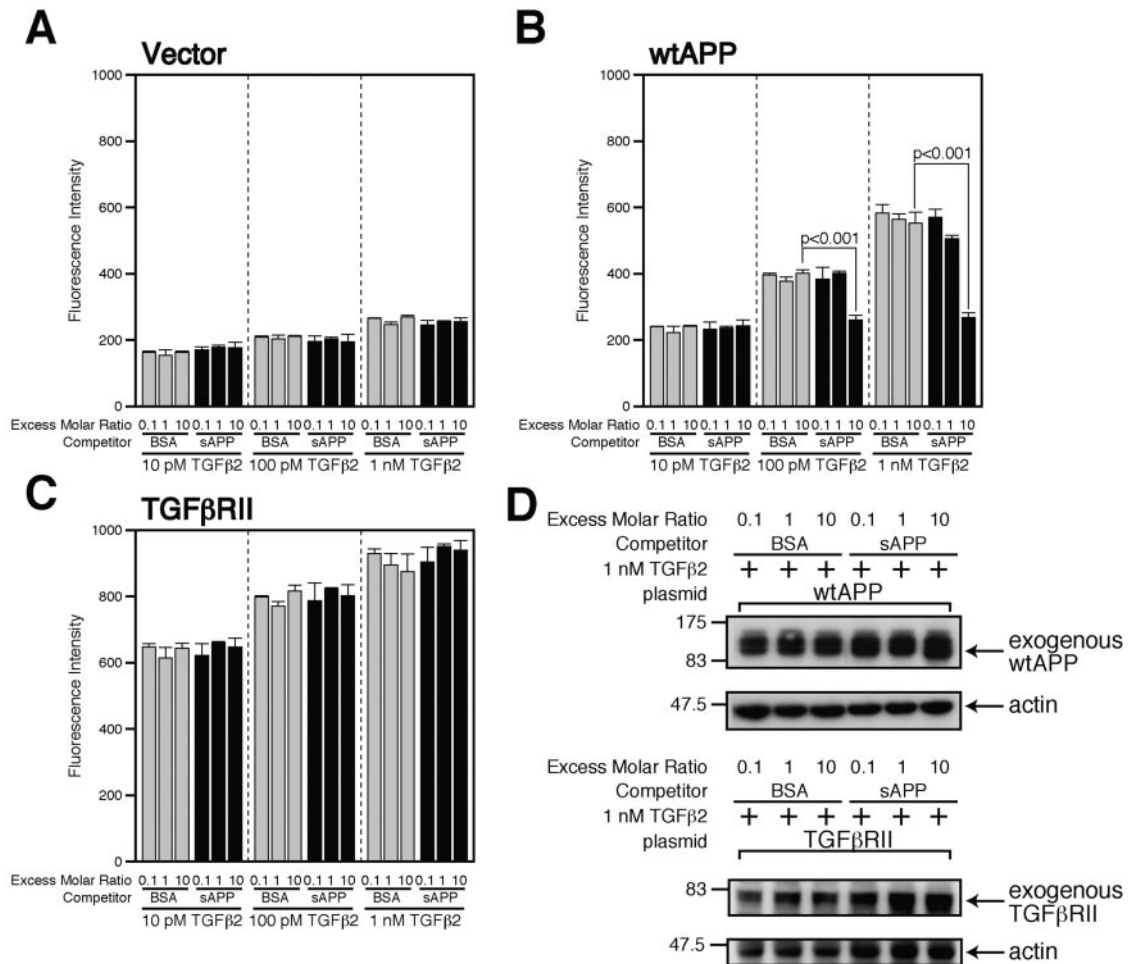


FIG. 3. Soluble APP antagonized TGFβ2-induced death. (A to C) F11 cells (7×10^4 cells/well in 6-well plates) were transfected with 0.25 μ g of the pcDNA3 vector (A), pcDNA3-wtAPP (B), or pcDNA3-TGFβRII (C). At 36 h after transfection, cells were combined with the indicated concentrations of TGFβ1 or TGFβ2 in the presence of the indicated amounts of sAPP or BSA as a competitor. Immunofluorescence-based binding assays were performed as described in Materials and Methods. (D) F11 cells (7×10^4 cells/well in 6-well plates) were transfected with 0.25 μ g of pcDNA3-wtAPP (upper panel) or pcDNA3-TGFβRII (lower panel). At 36 h after transfection, cells were combined with 1 nM TGFβ2 in the presence of 10 nM sAPP or BSA. Cell lysates (10 μ g in each lane) were immunoblotted with antibody to APP (22C11) (upper) or TGFβRII (lower) as well as actin (upper and lower).

to the association between TGFβ1 and F11 cells, the association between TGFβ3 and F11 cells remained unchanged by overexpression of wtAPP (data not shown).

To evaluate the extent of the association between TGFβ2 and exogenously overexpressed APP (or APP-induced proteins) (TGFβ2 concentrations, 1 pM to 500 nM), we subtracted the mean immunofluorescence intensity numbers shown in the right panel of Fig. 1B, representing the associations between TGFβ2 and the endogenous TGFβ-binding proteins, from the mean immunofluorescence intensity numbers shown in the right panel of Fig. 1D that indicated the association between TGFβ2 and F11 cells overexpressing APP (Fig. 1E). Using these binding data, we further tried to roughly estimate amounts of free TGFβ2 in each binding assay (Table 1). When the increase of the immunofluorescence intensity numbers corresponding to the amounts of bound TGFβ2 is much smaller than the increase of the amount of added TGFβ2, we are able to estimate the amounts of free TGFβ2 with a very small error

in each binding assay. For example, if immunofluorescence intensity numbers obtained at TGFβ2 concentrations of 1 pM and 10 pM are 90 and 180 arbitrary units, we are able to estimate that 80 to 100% of added TGFβ2 remains unbound at 10 pM of TGFβ2, because bound amounts of TGFβ2 are calculated to be 20% if we hypothesize that all TGFβ2 polypeptides bind to the receptors at the TGFβ2 concentration of 1 pM. We estimated approximate amounts of free TGFβ2 at 10 pM, 100 pM, 1 nM, 10 nM, and 100 nM of TGFβ2 in this way (Table 1). Using these data, we simulated Scatchard analysis (Fig. 1F) and found that APP overexpression seemed to induce two kinds of receptors specifically bound by TGFβ2. The roughly estimated K_d for the high-affinity receptor (H-Tβ2R) and the low-affinity receptor (L-Tβ2R) are 1.9×10^{-11} M and 1.7×10^{-9} M, respectively.

To confirm that the increase in the fluorescence intensity caused by treatment with TGFβ1 or TGFβ2 was really derived from the association of TGFβ1 or TGFβ2 with F11 cells, we

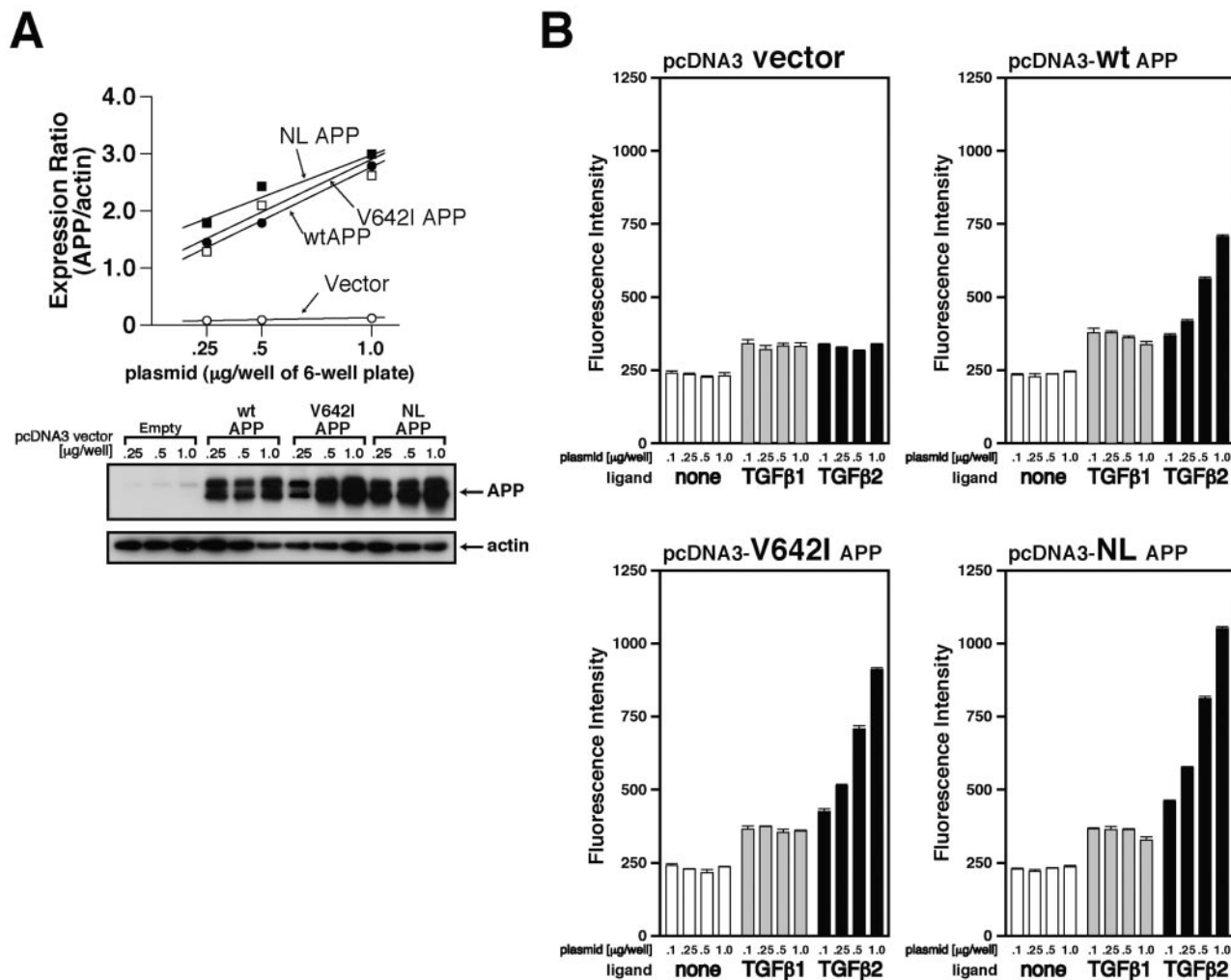


FIG. 4. TGFβ2 binds to wtAPP and FAD-related APP mutants. (A) F11 cells were transfected with stepwise-increasing amounts (0.25, 0.5, and 1.0 μg) of the pcDNA3 vector, pcDNA3-wtAPP, pcDNA3-V642I-APP, or pcDNA3-NL-APP. Cell lysates (10 μg in each lane) were immunoblotted with antibody to APP (22C11) or actin. These experiments were simultaneously performed. (B) Immunofluorescence-based binding assays were performed as described in Materials and Methods. The concentration of added TGFβs was 100 pM.

further examined whether the addition of an excess amount of unlabeled TGFβ1 or TGFβ2 inhibited the binding between FITC-labeled TGFβ1 or TGFβ2 and F11 cells (Fig. 2). To this end, we developed another immunofluorescence-based binding assay using FITC-labeled TGFβ1 or TGFβ2 instead of unlabeled TGFβ1 or TGFβ2 as the ligand. To increase sensitivity in detection of the binding, the fixation procedure with paraformaldehyde was omitted and attachment of F11 cells to culture dishes was enhanced by precoating with poly-L-lysine as shown in Materials and Methods. Using this binding assay, we again demonstrated that treatment with 10 nM FITC-TGFβ1 as well as FITC-TGFβ2 significantly increased the immunofluorescence intensity in F11 cells (Fig. 2, vector). As expected, the addition of an excess amount of unlabeled TGFβ1 or TGFβ2 completely suppressed the increase in the immunofluorescence intensity. Furthermore, overexpression of TGFβRII markedly increased the immunofluorescence intensity (Fig. 2, TGFβRII). Addition of an excess amount of unlabeled TGFβ1 or TGFβ2 again almost completely suppressed the increase in

the immunofluorescence intensity. In accordance with data shown in Fig. 1D, treatment with 10 nM FITC-TGFβ2, but not 10 nM FITC-TGFβ1, increased the immunofluorescence intensity in F11 cells overexpressing wtAPP (Fig. 2, wtAPP). The

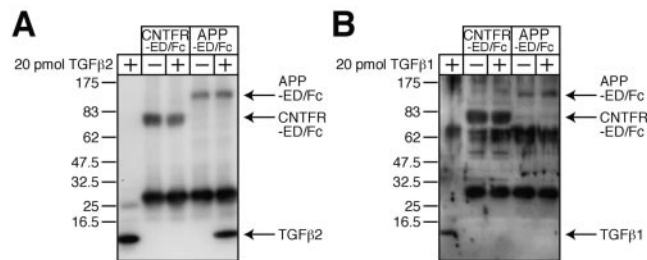


FIG. 5. TGFβ2 binds to the extracellular domain of APP. (A and B) Twenty picomoles of recombinant TGFβ2 (A) or TGFβ1 (B) (final concentration, 40 nM) was mixed with APP-ED/Fc protein or CNTFR-ED/Fc protein for coimmunoprecipitation analysis. Immunoprecipitates were immunoblotted with antibodies to TGFβ1, TGFβ2, 6× histidine (to detect CNTFR/Fc), and APP.

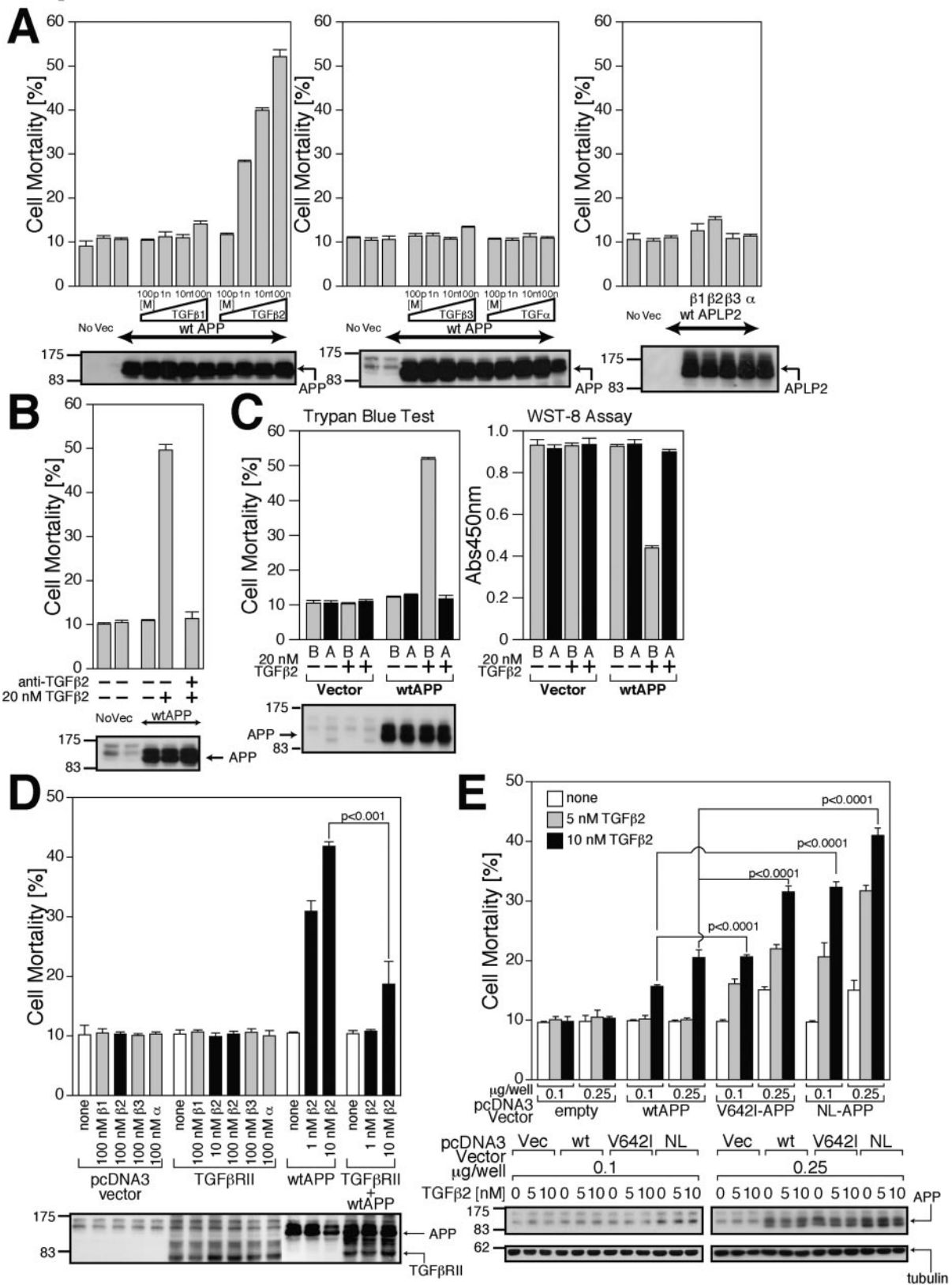


FIG. 6. TGFβ2 triggers death in F11 cells overexpressing APPs. (A) F11 cells, transfected with 0.5 μg of pcDNA3-wtAPP or pcDNA3.1/GS-mouse APLP2, were treated with 100 pM, 1 nM, 10 nM, or 100 nM of TGFβ1, TGFβ2, TGFβ3, or TGFα. Cell mortality was determined by trypan blue exclusion assays at 48 h after the onset of TGF treatment. Cell lysates (20 μg in each lane) were immunoblotted with antibody to APP or HRP-conjugated anti-V5 monoclonal antibody for APLP2. (B) The effect of anti-TGFβ2 neutralizing antibody on TGFβ2/wtAPP-induced death

increase was also inhibited by treatment with an excess amount of unlabeled TGF β 2. These findings clearly indicated that the TGF β -mediated increase in the immunofluorescence intensity in F11 cells really reflected the specific binding between TGF β s and F11 cells and confirmed that overexpression of APP in F11 cells resulted in the appearance of TGF β 2-specific receptors.

The low-affinity APP-induced TGF β 2-specific receptor is APP. To address whether the TGF β 2-specific receptor is APP itself or other unidentified proteins whose expression are induced by APP, we performed competition experiments by adding as a competitor larger amounts of soluble APP α of human APP695 (sAPP), corresponding to amino acids 1 to 612 of APP695 (the numbering follows that of Kang et al. and Santiago-Garcia et al. [17, 41]), to binding assays (Fig. 3). The addition of sAPP did not reduce the association between TGF β 2 and F11 cells (Fig. 3A) or F11 cells overexpressing TGF β RII (Fig. 3C), even when the amount of added sAPP was 10 times more than the amount of TGF β 2 (Fig. 3A and C). Expression levels of cotransfected TGF β RII were not affected by addition of sAPP (Fig. 3D, bottom panel). It is speculated that competition did not occur in these cases because TGF β 2 has a far higher affinity to TGF β RII or the TGF β receptor than to these APP-induced TGF β 2-specific receptors.

In contrast, sAPP reduced the association between TGF β 2 and F11 cells expressing wtAPP only when the concentration of added TGF β 2 was 100 pM or 1 nM (but not 10 pM TGF β 2) and the amount of added sAPP was 10 times more than the amount of TGF β 2 (Fig. 3B). What was concluded by this observation was not affected by another observation that the addition of sAPP slightly increased expression of cotransfected wtAPP (Fig. 3D, top panel), because the increase in expression of wtAPP is considered to enhance the association between TGF β 2 and F11 cells. Based on the fact that TGF β 2 bound to L-T β 2R as well as H-T β 2R at 1 nM and 10 pM while almost all TGF β 2 bound to H-T β 2R at 10 pM as shown in Fig. 1E and F, we have concluded that sAPP competes with L-T β 2R, but not with H-T β 2R, for the association with TGF β 2. This finding strongly suggests that L-T β 2R is APP itself or an APP derivative, while H-T β 2R may be a protein or proteins with a specific affinity to TGF β 2 whose expression is induced by APP.

TGF β 2 binds to wtAPP and mutant APPs. We further examined whether TGF β 2 binds to various FAD-linked mutant APPs in a similar fashion as well as whether the association between TGF β 2 and a mutant APP occurs in a manner dependent on the expression levels of APP. As shown in the top

panel of Fig. 4A, there is a good positive correlation between the amount of the transfected plasmid and protein expression of APP. Treatment with 100 pM TGF β 1 or TGF β 2 increased the immunofluorescence intensity of F11 cells which had been transfected with the empty vector by about 100 U compared to that of nontreated cells in this particular experiment (Fig. 4B, top left panel, vector). In a similar fashion, TGF β 1 treatment increased by about 100 U the fluorescence intensity of F11 cells which had been transfected with the wtAPP-coding vector independently of the APP expression level (range of plasmid amounts, 0.25 to 1.0 μ g) (Fig. 4B, top right panel, TGF β 1). In contrast, TGF β 2 treatment (100 pM) increased by more than 100 U the fluorescence intensity of F11 cells which had been transfected with the wtAPP-coding vector in a fashion dependent on the APP expression level (Fig. 4B, top right panel, TGF β 2). It maximally increased the fluorescence intensity by 500 U when F11 cells had been transfected with 1.0 μ g of the wtAPP-coding plasmid. Binding experiments using F11 cells have further indicated that the association between TGF β 2 and V642I-APP (the isoleucine mutation at valine 642 in APP695) or NL-APP (the asparagine/leucine mutation at Lys595/Met596 in APP695) occurs in a similar dose-responsive fashion (Fig. 4B, bottom panels).

TGF β 2 binds to the extracellular domain of APP. We next examined by a coimmunoprecipitation method whether there is an association between the extracellular domain of APP (APP-ED) and TGF β 2 (Fig. 5). To this end, we constructed a plasmid coding for APP-ED fused in frame to the Fc region of human IgG1 (APP-ED/Fc) that is secreted from transfected cells into media. As a negative control, we constructed another plasmid encoding the extracellular domain of human ciliary neurotrophic factor receptor fused to the IgG1 Fc portion (CNTFR-ED/Fc). Conditioned media containing APP-ED/Fc or CNTFR-ED/Fc were then mixed with 20 pmol of TGF β 2 (concentration, 40 nM) together with Protein G-Sepharose for coimmunoprecipitation analysis, which indicated that TGF β 2 bound to APP-ED/Fc while it did not bind to CNTFR-ED/Fc (Fig. 5A). A similar binding experiment further indicated that, in contrast to TGF β 2, either TGF β 1 (Fig. 5B) or TGF β 3 (data not shown) did not coprecipitate with APP-ED/Fc or CNTFR-ED/Fc, confirming the specificity of the association between TGF β 2 and APP-ED.

TGF β 2 induces death in F11 cells overexpressing wtAPP. We then asked what the biological consequence of the association between TGF β 2 and APP was. To answer this question, we examined whether TGF β 2 treatment induces death in F11

in F11 cells. F11 cells, transfected with 0.5 μ g of pcDNA3-wtAPP, were treated with 20 nM TGF β 2 in the presence of 10 μ g/ml anti-TGF β 2 neutralizing antibody or control rabbit IgG. Cell lysates (20 μ g in each lane) were immunoblotted with antibody to APP. (C) The effect of sAPP on TGF β 2/wtAPP-induced death in F11 cells. F11 cells, transfected with 0.5 μ g of pcDNA3-wtAPP, were treated with 10 nM TGF β 2 in the presence or the absence of 100 nM of sAPP (labeled "A") or BSA (labeled "B"). Cell mortality and cell viability were determined by trypan blue exclusion assay and WST-8 assay at 48 h after the onset of TGF β 2 treatment. Cell lysates (20 μ g in each lane) were immunoblotted with antibody to APP. (D) F11 cells, transfected with 0.25 μ g of the pcDNA3 vector, pcDNA3-TGF β RII, or pcDNA3-wtAPP or 0.25 μ g of pcDNA3-TGF β RII and 0.25 μ g of pcDNA3-wtAPP, were treated with indicated concentrations of TGF β 1 (β 1), TGF β 2 (β 2), TGF β 3 (β 3), or TGF α (α). To keep the amounts of transfected vectors constant, proper amounts of the backbone vector were added. Cell mortality was determined by trypan blue exclusion assay at 48 h after the onset of TGF treatment. Cell lysates (20 μ g in each lane) were immunoblotted with antibody to APP or TGF β RII. (E) TGF β 2 induces higher grade death in F11 cells ectopically expressing FAD-related APPs. F11 cells, transfected with 0.1 or 0.25 μ g of pcDNA3-wtAPP, pcDNA3-V642I-APP, and pcDNA3-NL-APP, were treated with or without 5 or 10 nM TGF β 2. Cell mortality was determined by trypan blue exclusion assay at 48 h after the onset of TGF β 2 treatment. Cell lysates (20 μ g in each lane) were immunoblotted with antibody to APP and tubulin. Vec, vector; Abs450nm, absorbance at 450 nm.

cells transfected with 0.5 μg of the wtAPP-encoding plasmid, which was not enough to induce death in F11 cells. We then found that cell death was induced by treatment with TGF β 2 in a dose-responsive manner (Fig. 6A). In contrast, treatment with TGF β 1, TGF β 3, or TGF α did not induce death. Neither TGF β 1, TGF β 2, TGF β 3, nor TGF α induced death in F11 cells overexpressing amyloid precursor-like protein 2 (APLP2), suggesting that cell death occurred only when TGF β 2 was added in the presence of overexpressed wtAPP in F11 cells. We simultaneously recognized that 1 nM or higher concentrations of TGF β 2 were necessary for substantial induction of death (Fig. 6A). Combined with the finding that TGF β 2 concentrations of 100 pM or more are necessary for the substantial association between TGF β 2 and L-T β 2R (APP) in F11 cells while the association between TGF β 2 and H-T β 2R is saturated at 100 pM TGF β 2 (Fig. 1F), it is speculated that TGF β 2 induced death in F11 cells by binding to APP.

To confirm the involvement of TGF β 2 in neuronal cell death, we performed a neutralizing experiment using anti-TGF β 2 antibody. The addition of the neutralizing anti-TGF β 2 antibody completely abolished TGF β 2-induced neuronal cell death (Fig. 6B). In addition, we confirmed that addition of an excess amount of sAPP nullified TGF β 2-induced cell death without alteration of wtAPP expression (Fig. 6C), strongly supporting the notion that TGF β 2 induces neuronal cell death by binding to APP.

We then asked if the association between TGF β 2 and the endogenous TGF β receptor (TGF β R) affects TGF β 2-induced death mediated by wtAPP in F11 cells overexpressing wtAPP. To address this question, we actually examined whether overexpression of TGF β RII altered TGF β 2-induced neuronal cell death. As shown in Fig. 6D, TGF β 2 did not induce death in F11 cells overexpressing TGF β RII alone. Conversely, coexpression of TGF β RII markedly reduced TGF β 2-induced death in F11 cells overexpressing wtAPP (Fig. 6D), probably because overexpressed TGF β RII inhibited the association between TGF β 2 and wtAPP by preferentially trapping TGF β 2. Taken altogether, we have concluded that TGF β 2-induced death in F11 cells, mediated by APP, is not affected by the TGF β receptor-mediated signal.

TGF β 2 treatment results in higher-grade death in F11 cells expressing FAD-related mutant APPs. We further characterized TGF β 2-induced neuronal cell death from the standpoint of FAD-linked APP mutation and APP expression levels (Fig. 6E). Very low-level or low-level ectopic expression of wtAPP, V642I-APP, and NL-APP was obtained by transiently transfecting 0.1 or 0.25 μg of each expression vector in F11 cells (bottom panel). In accordance with our earlier findings (14), low-grade death was induced by low-level expression of V642I-APP or NL-APP alone but not by low-level expression of wtAPP alone (see transfection with 0.25 μg of vectors) in F11 cells (Fig. 6E). Treatment with TGF β 2 induced or accelerated death in F11 cells ectopically expressing wtAPP, V642I-APP, or NL-APP in a manner dependent on the APP expression level and the TGF β 2 concentration. Apparently, treatment with TGF β 2 resulted in higher grade cell death in F11 cells expressing V642I-APP or NL-APP than in those expressing wtAPP.

Characterization of TGF β 2-induced neuronal cell death. To obtain information about intracellular mediators for TGF β 2-

induced cell death, we performed pharmacological analysis using a cell-permeable caspase 3 and/or related caspase inhibitor, Ac-DEVD-CHO (DEVD), and an established cell-permeable antioxidant, glutathione-ethyl-ester (GEE). Both of them completely inhibited TGF β 2-induced cell death (Fig. 7A), indicating that caspase 3 and/or related caspases and reactive oxygen species (ROS) source enzyme are involved in this neuronal cell death.

We further treated cells with the pertussis toxin (PTX) to define the involvement of PTX-sensitive heterotrimeric G proteins with mitogen-activated protein kinase (MAPK) family inhibitors, including SP600125 (SP) for a JNK-specific inhibitor, SB203580 (SB) for a p38 MAPK-specific inhibitor, or PD98059 (PD) for a MEK/MAPK inhibitor to define the involvement of MEK/MAPK family proteins and with specific inhibitors of ROS source enzymes, including apocynin (APO) for an NADPH oxidase inhibitor, oxypurinol (OXY) for a xanthine oxidase inhibitor, or *N*^G-methyl-L-arginine methyl-esterhydrochloride (L-NMMA) for a nitric oxide synthase (NOS) inhibitor to specify the source enzyme of ROS (Fig. 7B). Treatment with DEVD, GEE, PTX, SP, and APO inhibited TGF β 2-induced cell death, indicating that TGF β 2-induced cell death is mediated by a PTX-sensitive heterotrimeric G protein, JNK, NADPH oxidase, and caspase 3 and/or related caspases.

Involvement of the intracellular G_o-interacting domain, but not the A β -corresponding domain, of APP in TGF β 2/APP induces cell death. To further confirm the involvement of the PTX-sensitive heterotrimeric G protein, we tried to identify the domain in the intracytoplasmic region of APP essential for TGF β 2-induced cell death (Fig. 7C). F11 cells, transfected with the full-length wtAPP, wtAPP lacking domain 20 (wtAPP Δ 20), or wtAPP lacking domain 19 (wtAPP Δ 19) (14), were treated with 20 nM TGF β 2. We found that domain 20, corresponding to the His⁶⁵⁷-Lys⁶⁷⁶ region, is essential for TGF β 2-induced cell death. This finding indicates that G_o is the signal transducer, because it was shown that G_o is functionally coupled with domain 20 of APP (14, 35, 48, 49), and A β is not involved in TGF β 2-induced cell death, because wtAPP Δ 20 does not mediate TGF β 2-induced cell death. wtAPP Δ 20, which contains the A β -corresponding region, should mediate TGF β 2-induced cell death if A β plays a major role in TGF β 2-induced cell death.

Thus, we concluded that TGF β 2/APP-induced cell death is mediated by G_o, JNK, NADPH oxidase, and caspase 3/related caspases. We confirmed that this pathway also is triggered in F11 cells expressing FAD-related APP mutants upon TGF β 2 binding (data not shown).

TGF β 2 does not induce neuronal cell death by triggering other neurotoxic signals unrelated to APP. We next tried to completely rule out the possibility that TGF β 2 indirectly enhanced APP-mediated cell death by binding to other molecules unrelated to APP, which is linked to a certain cell-death-enhancing signal. To this end, we examined the effect of TGF β 2 treatment on APP-mediated cell death by using a system expressing a fusion construct consisting of the extracellular and transmembrane domains of the epidermal growth factor receptor and the full cytoplasmic domain of APP (APP-CD hybrid) (15). Treatment with 1 nM EGF induces death in F11 cells transfected with the vector encoding APP-CD hybrid (Fig.

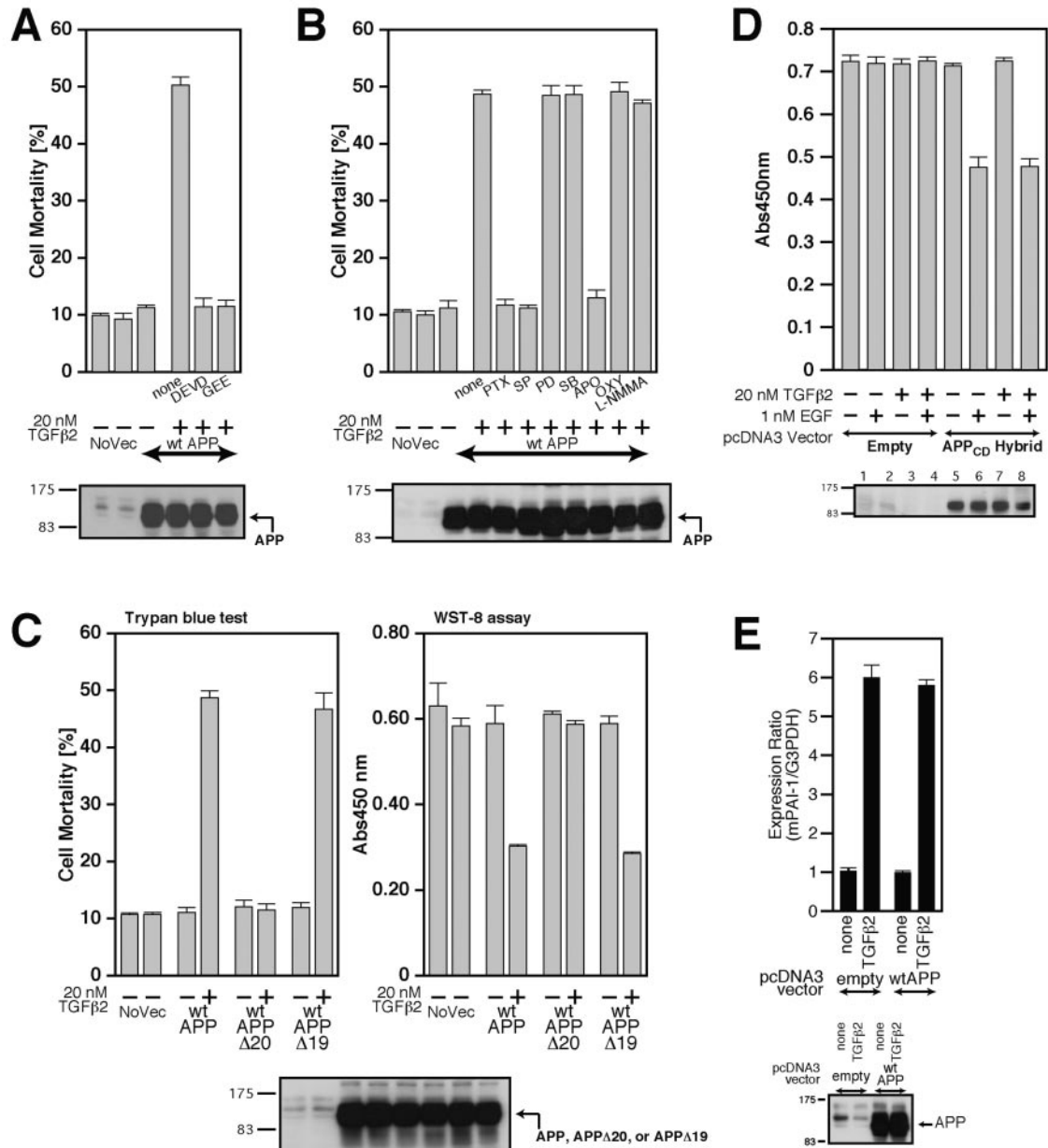
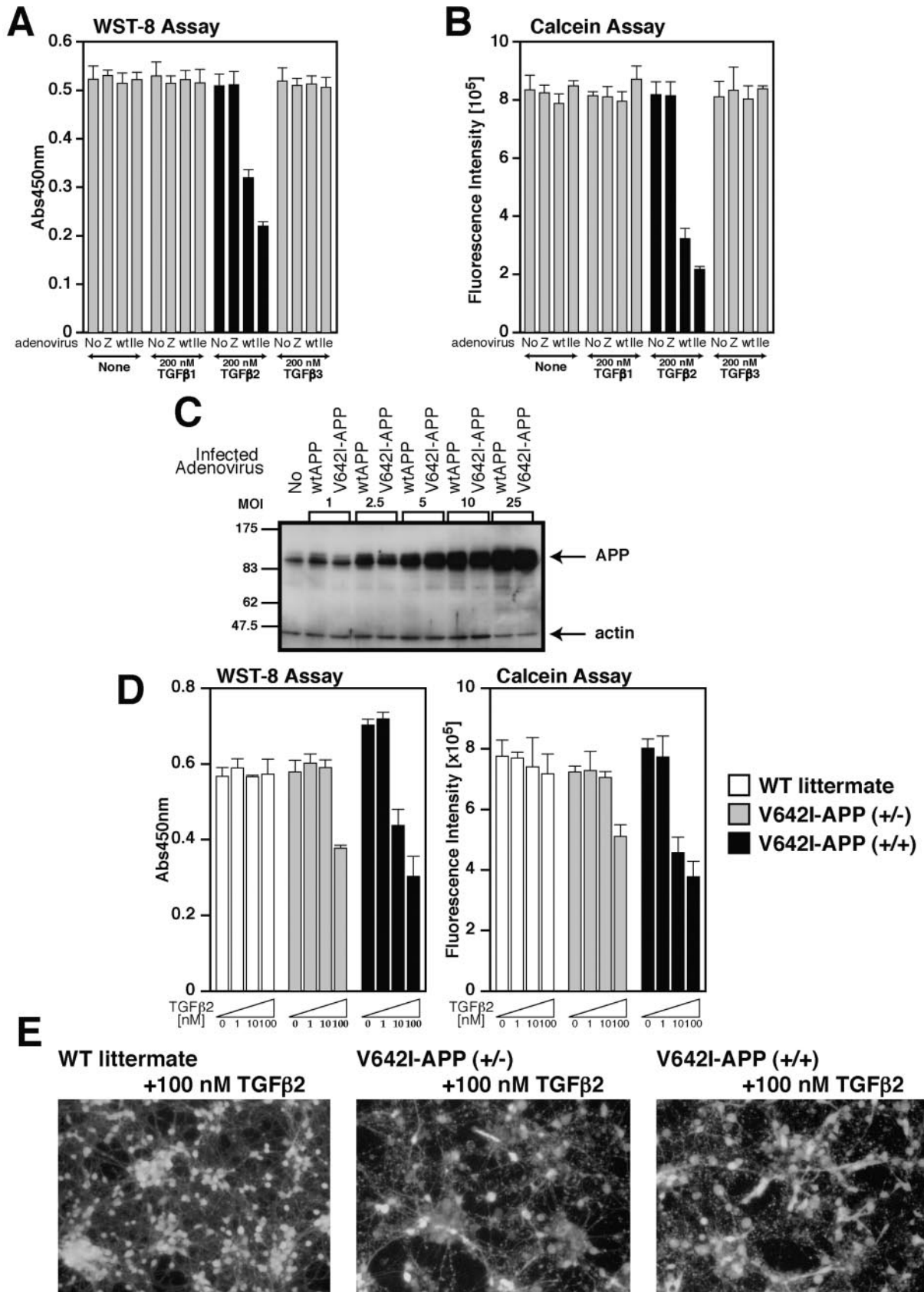


FIG. 7. Characterization of TGFβ2-triggered death. (A and B) F11 cells, seeded on 6-well plates at 7×10^4 cells/well, were transfected with $0.5 \mu\text{g}$ of pcDNA3-wtAPP and then treated with 20 nM TGFβ2 in the presence or the absence of either 100 μM Ac-DEVD-CHO (DEVD), 1 mM GEE (A), 1 μg/ml PTX, 1 μM SP600125 (SP), 50 μM PD98059 (PD), 20 μM SB203580 (SB), 300 μM apocynin (APO), 100 μM oxypurinol (OXY), or 1 mM L-NMMA (B). Cell lysates (20 μg in each lane) were submitted to immunoblot analysis with 22C11 for APP. (C) F11 cells, seeded on 6-well plates at 7×10^4 cells/well, were transfected with $0.5 \mu\text{g}$ of pcDNA3-wtAPP, pcDNA3-wtAPPΔ20, or pcDNA3-wtAPPΔ19 and then treated with or without 20 nM TGFβ2. Cell mortality (trypan blue exclusion assay) and cell viability (WST-8 assay) were determined at 48 h. (D) TGFβ2 treatment does not enhance EGFR-ED+TM/APP-CD (labeled APP_{CD}-Hybrid)-mediated death of F11 cells induced by treatment with 1 nM EGF. F11 cells, transfected with 1.0 μg of pcDNA3-EGFR-ED+TM/APP-CD or the backbone pcDNA3 vector, were treated with 1 nM EGF together with or without 20 nM TGFβ2. Cell viability was determined by WST-8 assays at 48 h after the onset of TGFβ2 treatment. Cell lysates (20 μg in each lane) were immunoblotted with antibody to EGFR to detect the APP_{CD} hybrid. (E) Enforced expression of wtAPP did not result in the enhancement of the TGFβ2-induced activation of plasminogen activator inhibitor-1 (PAI-1) mRNA expression. F11 cells, transfected with $0.5 \mu\text{g}$ of the pcDNA3 vector or pcDNA3-wtAPP, were treated with 100 pM of TGFβ2 at 24 h after transfection. After incubation for 48 h, they were harvested for real-time PCR-based determination of mRNA amounts. NoVec, no vector; Abs450nm, absorbance at 450 nm; mPAI-1, mouse PAI-1.

7D). This cell death progresses via G_{α} , c-Jun N-terminal kinase, NADPH oxidase, and caspase 3 and/or related caspases (15). In this cell death system, the addition of TGFβ2 did not enhance EGF-induced cell death (Fig. 7D), confirming that TGFβ2 does not increase APP-mediated cell death by activat-

ing another cell-death-enhancing signal through receptors other than APP.

Expression of APP does not enhance the TGFβ2-induced TGFβ receptor-mediated signal. We also tried to exclude the possibility that enforced expression of APP proteins increases



the TGF β 2-induced TGF β receptor-mediated signal by modifying the function of various signal transducers. To address this issue, we examined with real-time PCR how TGF β 2-induced upregulation of expression of plasminogen activator inhibitor 1 (PAI-1) mRNA (24) is modified by the expression of wtAPP. PAI-1 is a representative target of TGF β -induced TGF β receptor-mediated signals. Consequently, we found that enforced expression of wtAPP did not alter TGF β 2-induced upregulation of PAI-1 mRNA, indicating that overexpression of APP does not affect the TGF β 2-induced TGF β receptor-mediated signal (Fig. 7E). This experiment additionally suggests that H-T β 2R is not TGF β receptor type III (TGF β RIII) or an alternatively spliced TGF β RII, TGF β RII-B, both of which have special affinities to TGF β 2 (40), because upregulation of these proteins should enhance the TGF β 2-induced TGF β receptor-mediated signals.

TGF β 2 induces death in primary cortical neurons. We then asked whether TGF β 2 induces death in primary cortical neurons (PCNs), cells that are more physiologically postmitotic neuronal than F11 cells. To express APP proteins efficiently, we used an adenovirus-mediated expression system. In our earlier study, we showed that adenovirus-mediated overexpression of V642I-APP induces death in PCNs (34). In order to minimize death in PCNs induced by expression of V642I-APP itself, we selected a multiplicity of infection (MOI) of 5, a very low MOI, for adenoviral infection. The overexpression levels of APPs at the MOI of 5 were estimated to be five times higher than the endogenous APP expression level. In contrast to the fact that lipofection-based overexpression of V642I-APP alone induces low-grade death in F11 cells (Fig. 6E) (14), adenovirus-mediated overexpression of V642I-APP and wtAPP at this MOI did not induce death in PCNs. At 24 h after infection with the control LacZ-encoding virus, the wtAPP-encoding virus, or the V642I-APP-encoding virus, we treated cells with 200 nM TGF β 1, TGF β 2, or TGF β 3. We found that TGF β 2, but neither TGF β 1 nor TGF β 3, reduced cell viability of PCNs that adenovirally overexpressed wtAPP or V642I-APP (Fig. 8A and B). Expression levels of wtAPP and V642I-APP at each MOI were similar (Fig. 8C). Note that higher grade cell death was induced in PCNs ectopically expressing V642I-APP than in those expressing wtAPP.

TGF β 2 induces death in PCNs prepared from mice knocked in with the V642I-APP mutation. We next tested whether TGF β 2 induces death in PCNs even in the absence of ectopic overexpression of APP. To this end, we used "V642I-APP knock-in mice," mice that contain a V642I-APP mutant allele (or alleles) under the physiological promoter (18). The protein expression level of V642I-APP is equal to that of wtAPP in vivo, as described in our earlier study (18). We prepared PCNs from V642I-APP^{+/+} (homozygous), V642I-APP^{+/-} (heterozy-

gous), and wild-type littermate mice. Treatment with TGF β 2 at concentrations of 1 nM or less did not decrease viability in any type of PCN. Treatment with 10 nM TGF β 2 decreased viability in PCNs from the homozygous mouse but not in those from the heterozygous mouse or in those from the wild-type littermate mouse. Treatment with 100 nM TGF β 2 decreased viability in PCNs from both homozygous and heterozygous mice but not in those from the wild-type littermate mouse (Fig. 8D and E).

DISCUSSION

This study has shown that TGF β 2, but not TGF β 1 or TGF β 3, acts as a ligand for APP. It has revealed a novel function unique to TGF β 2. TGF β 2 triggers the APP-mediated cell death cascade and induces neuronal cell death. It is especially noted that TGF β 2 induces death in PCNs without ectopic overexpression of APP, prepared from heterozygous V642I-APP knock-in mice whose single allele contains the V642I-APP gene under the physiological APP promoter (Fig. 8D and E), leading to the speculation that various FAD-related point mutations contribute to the development of neuronal cell death in the presence of superphysiological concentrations of TGF β 2 by increasing the sensitivity of APP to TGF β 2-induced cell death in vivo.

At present, however, we do not have direct evidence that TGF β 2 induces neuronal cell death through APP in vivo. We need to add 10 nM TGF β 2 or more, which is supposed to be 100-fold higher than the levels observed in normal sera and cerebrospinal fluids, in order to induce neuronal cell death in vitro. However, it is natural that physiological concentrations of cell-death-inducing ligands are far lower than the effective concentrations, because death should not occur easily. We have found that TGF β 2 is substantially secreted from neurons as well as from glial cells (Y. Hashimoto, M. Nawa, and M. Matsuoka, unpublished observations). This finding implies that TGF β 2, secreted by neurons and glial cells, binds to and affects neurons by the autocrine mechanism in cooperation with the paracrine mechanism. In this situation, it is possible that the local TGF β 2 concentration in the fluid around neurons increases to a level sufficient for induction of neuronal cell death in vivo when expression of TGF β 2 is enhanced. In AD brains, it has been reported that upregulated expression of TGF β 2 is seen not only in glial cells but also in neurons themselves (6, 9, 10, 23, 36, 38). In agreement with this finding, we have recently found that toxic A β 42 upregulates expression of TGF β 2 (Y. Hashimoto, M. Nawa, and M. Matsuoka, unpublished), supporting the idea that expression of TGF β 2 is upregulated in AD brains.

It has been reported that some cytokines, such as tumor

FIG. 8. TGF β 2 triggers death in primary cortical neurons. (A and B) On day 3 in vitro (DIV3), PCNs were infected by LacZ (Z)-, wtAPP (wt)-, or V642I-APP (Ile)-encoding adenoviruses at an MOI of 5. On DIV4, PCNs were treated with 200 nM TGF β 1, TGF β 2, or TGF β 3. At 72 h after the onset of TGF β treatment, cell viability was measured by WST-8 assay (A) and calcein assay (B). (C) wtAPP and V642I-APP were adenovirally overexpressed in PCNs at various MOIs. PCN lysates were subjected to immunoblot analysis with antibody to APP or actin. (D) PCNs derived from homozygous or heterozygous V642I-APP knock-in mice at E14 or wild-type littermate mice at E14 were seeded on poly-L-lysine-coated 96-well plates at 5×10^4 cells/well. On DIV4, they were treated with 0, 1, 10, and 100 nM TGF β 2. At 72 h after the onset of TGF β treatment, cell viability was measured by WST-8 assay (left panel) and calcein assay (right panel). (E) Representative fluorescent microscopic views of calcein acetoxy-methyl ester-stained PCN shown in panel D. Abs450nm, absorbance at 450 nm; WT, wild type.

necrosis factor α , interleukin-1 β , and gamma interferon, stimulate γ -secretase-mediated cleavage of APP (22). Similarly, TGF β 2 may stimulate the γ -secretase activity. If this is the case, concentrations of toxic A β and the amount of the APP intracellular domain (AICD) may get sufficiently upregulated by TGF β 2-mediated putative stimulation of the γ -secretase activity to induce neuronal death. We did not accurately test whether TGF β 2 treatment increases γ -secretase activity in our cell death system. However, we estimated that concentrations of toxic A β 42 secreted in the culture media were at the level of <1 μ M by immunoblot analysis (Y. Hashimoto and M. Matsuoka, unpublished data), which is far less than the concentration necessary for induction of death in PCNs or F11 cells. It also is indicated in Fig. 7C that wtAPP Δ 19, but not wtAPP Δ 20, can mediate TGF β 2-induced cell death. Considering that wtAPP Δ 19 lacks the 19-amino-acid-long cytoplasmic domain essential for the transcriptional activity of AICD while wtAPP Δ 20 contains the full extracellular domain as well as the full transmembrane domain of APP from which toxic A β is generated (14), we can speculate that neither A β nor AICD is involved in TGF β 2-induced cell death.

Most sporadic AD patients do not have APP mutations. However, this study indicates that TGF β 2 induces death in neuronal cells without APP mutations only when they overexpress wtAPP. Therefore, an important issue to be addressed is whether TGF β 2 is involved in the development of AD without APP mutations. Currently, we do not have evidence directly confirming or disproving that expression of APP is upregulated in brains of AD patients in vivo. In contrast to such sporadic AD patients, APP has been shown to be overexpressed in patients with Down's syndrome, who develop AD at a higher incidence rate (2), supporting the possibility that TGF β 2-induced death signals mediated by overexpressed wtAPP contribute to the onset of AD associated with Down's patients.

In summary, we provide evidence that superphysiological concentrations of TGF β 2, whose expression has been demonstrated to be upregulated in AD brains, induces neuronal cell death by triggering a cell death signal through APP. The findings shown in this study may contribute to the further clarification of the precise mechanism of AD-related neuronal cell death.

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