Transforming Growth Factor-β1 Potentiates Amyloid-β Generation in Astrocytes and in Transgenic Mice*

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The proteolytic cleavage of the amyloid precursor protein (APP) leads to the production of Aβ with a large amount of the 40-amino acid variant, Aβ40, and to a lesser extent of the 42-amino acid variant, Aβ42. In Alzheimer’s disease, this amyloidogenesis can lead to the formation of amyloid deposits within the cerebral parenchyma and vascular walls (1, 2).

Many studies have identified increased levels of a variety of cytokines in patients developing chronic neurodegenerative disorders such as AD (3–8). Among these factors, recent reports have associated transforming growth factor-β1 (TGF-β1), a potent immunosuppressive cytokine, with AD. First, recent data suggest that a genetic polymorphism of the TGF-β1 gene may be associated with a higher risk to develop AD (9). Second, post-mortem brain tissue analyses of AD patients show an increased expression of TGF-β1 correlated with the degree of cerebral amyloid angiopathy (8). Third, 16-month-old transgenic mice overexpressing TGF-β1 in astrocytes elicit Aβ deposition (8). In the same study, these authors generated biogenic mice expressing both human APP and TGF-β1. In these hAPP/TGF-β1 mice, Aβ deposits were observed after 3 months of age compared with TGF-β1 mice, suggesting that TGF-β1 would be able to influence APP metabolism or processing. In addition to its amyloidigenic effects (8), TGF-β1 was recently associated with Aβ clearance from the brain parenchyma to the cerebral blood vasculature in aged hAPP/TGF-β1 mice by activated microglia (10).

Despite this dual effect of TGF-β1 in the amyloid plaque metabolism, the mechanism(s) by which TGF-β1 promotes the production of Aβ remained to be elucidated.

EXPERIMENTAL PROCEDURES

Semiquantitative Reverse Transcription-PCR (RT-PCR)—Total RNAs were prepared using either the RNAxel extraction kit (Eurobio, Paris, France) or RNAeasy extraction columns (Qiagen, Courtaboeuf, France). Samples (1 μg) of total mRNA were transcribed into cDNA. cDNA libraries (1 μl from 20 μl) were amplified by PCR with oligonucleotides for β-actin, APP770, APP695, APP654, and APP751 (respectively 539, 242, 222, and 401 bp of PCR products). All PCRs were performed in the linear range of amplification by performing multicycle amplification to reach half of the saturation curve. The β-actin oligonucleotides were: sense, 5′-GCACTAATTCGACAGACTATCCATGCTGTCCCTG-3′, 500–536 bp; and antisense, 5′-GCTGGTGCCTGCCTGAGTC-3′, 861–1104 bp. The APP751 oligonucleotides were: sense, 5′-CTCACCCATCGTCGG-3′, 848–886 bp; and antisense, 5′-CTGGTGCCTGCCTGAGTC-3′, 861–1104 bp. The APP751 primers were: sense, 5′-CTCACTCTGAGTCGG-3′, 848–886 bp; and antisense, 5′-CTTCAGGAATAGTTGGAGACCTGCTGCACACCAC-CC-3′, 1028–1068 bp. Human APP primers were: hAPP751 antisense, 5′-CTCTCTTTCATTCACTCACTATAAATG-3′, 1070–1092 bp; hAPP751 antisense, 5′-GCTGTAGATTTTGCAGTCTGTCCTGAC-CCACACCACACCACCC-3′, 1171–1200 bp; and hAPP sense, 5′-GCACTTACGAGAAGGCACACCC-3′, 925–945 bp. TGF-β1 sense was 5′-GCTGGTATTTAAGGA-3′, 250–266 bp; and antisense, 5′-GCTGGTATTTAAGGA-3′, 250–266 bp. TGF-β1 transgene sense was 5′-GCTGGTATTTAAGGA-3′, 250–266 bp; and antisense, 5′-GCTGGTATTTAAGGA-3′, 250–266 bp. The APP751 transgene antisense was 5′-GAGCCTTAACTTCCACACCCATCGTCGG-3′, corresponding to the 1689–1708 porcine TGF-β1 sequence; TGF-β1 transgene antisense was 5′-GCAGCTTAACTTCCACACCCATCGTCGG-3′.
GTGGAGA-G-3’, 340 bp. The conditions of amplification were 30 s at 95 °C, 30 s at 58 °C, and 1 min at 72 °C for 30 cycles, corresponding to the 50% of the saturation curve of the PCR product.

**Nuclear Extracts**—Nuclear extracts were prepared from control and TGF-β1-treated astrocytes and neurons. Cells were harvested 1 h after treatment and processed as described previously (14).

**Electrophoretic Mobility Shift Assay**—Oligonucleotides were end labeled with [γ-32P]ATP using the Klenow fragment of DNA polymerase. Binding reactions, containing 10 μg of nuclear extracts and 2 ng of labeled oligonucleotides, were performed for 20 min at 37 °C in appropriate binding buffer (14). The sequence of the double stranded oligonucleotide used as probe was: 5’-GGGAGACCCGCGGGC-3’. Protein-DNA complexes were resolved in 5% polyacrylamide gels containing 0.5 × TBE.

**Primary Cell Cultures**—Mouse cortical cultures of neurons were prepared from 14–15-day-old embryos as described previously (15). After 3 days in vitro, neurons were treated with 10 μM cytosine arabinoside to inhibit the proliferation of astrocytes. Experiments were realized on pure neuronal cultures (> 98% of microtuble-associated protein-2-positive cells) after 12–14 days in vitro.

Murine cortical cultures of astrocytes were prepared from 1–3 day postnatal mice (15). Experiments were performed on confluent cultured cortical astrocytes after 10 days in vitro. Primary cultures of cortical astrocytes were washed three times in PBS each 3 days in vitro to prevent the adhesion of microglial cells onto the monolayer of astrocytes until use (10–12 days in vitro). This protocol leads to fewer than 1% of CD11b-positive cells.

Primary cultures of neurons and astrocytes were established from brain tissues of therapeutically aborted human brain fetuses after 10 weeks of gestation. The protocol for tissues so obtained complied with institutional and national guidelines.

**Transgenic Animals**—All transgenic mice were of BALBc × SJL background and heterozygous for the respective transgene. Nontransgenic littermates served as controls. Glial fibrillary acidic protein (GFAP)-TGF-β1 line T65 has been generated similarly to lines 64 and 115, which have been described previously (16). Briefly, a 1.35-kb porcine TGF-β1 cDNA was inserted into the first exon of a modified mouse promotor (17). This cDNA had to allow to allow expressed TGF-β1 to be functional once released into the extracellular space. Homozygous TGF-β1 transgenic mice develop communicating hydrocephalus (16); however, for this study we used heterozygous TGF-β1 mice, which do not complicate this description. Identification of transgenic mice was performed by analysis of tail genomic DNA and from cerebral total RNAs.

**Western Blotting Experiments**—Cells were harvested in a lysis solution containing 50 mM Tris-HCl (pH 7.6), 1% Nonidet P-40 (Sigma), 150 mM NaCl, 2 mM EDTA, with 100 μM phenylmethylsulfonyl fluoride in the presence of a protease inhibitor mixture (Sigma). Equally conditioned media were harvested in the presence of 100 μM phenylmethylsulfonyl fluoride and 5% Triton-X 100 (10-fold). Electrophoreses were done on 8% SDS-polyacrylamide Tris-glycine gels or 16.5% Tris-Tricine gels containing 8M urea. Gels were transferred to a polyvinylidene difluoride membrane (PolyScreen®, PerkinElmer Life Sciences), membranes were blocked in non-fat milk and probed with appropriate antisera. Blots were finally developed with an enhanced chemiluminescence Western blotting detection system (PerkinElmer Life Sciences).

**Antibodies**—The monoclonal mouse antibody 22C11 (1:1,000) (Roche Applied Science) to residues 66–81 of APP<sub>770</sub> was used to identify either membrane-bound protein or soluble derivatives. The following primary antibodies were used: R7 (1:1,000) against KPI-APP proteins (11), BI (1:100) against human APP (12), sAPP-β (19), FCAS340 (1:1,000) against human Aβ40 (20), FCAS3542 (1:1,000) against human Aβ42, sc-146 (1:200) against mouse and human mTGF-β1 (Santa Cruz Biotechnology Inc.), OX42 (1:100) against CD11b (kindly provided by Dr. Anna-Maria Planas), and anti-GFAP antibody (1:100) and M4040 (1:100) against actin (Sigma). R1742 and R1743 were used to raise antibodies to synthetic Aβ1-42 and Aβ1-40, and tested against synthetic Aβ1-40 and Aβ1-42 peptides (Sigma).

**Double Fluorescent Immunocytochemistry**—Cultured murine cortical astrocytes were gently washed in PBS and fixed with ice-cold 4% paraformaldehyde. Cells were washed, and nonadherent sites were blocked in PBS plus 4% bovine serum albumin and 0.1% Tween 20 (Sigma) for 1 h. The primary antibody were raised overnight at 4 °C with the primary antibody raised against GFAP in PBS, 1% BSA, and 0.1% Tween 20. Cells were then washed and incubated for 1 h with the respective secondary Alexa Fluor<sup>®</sup> 488-conjugated antibody (Molecular Probes). Thereafter, astrocytes were incubated with the OX42 monoclonal antibody as described above. The appropriate secondary biotin-conjugated antibody was used, and antibody-antigen complexes were revealed with streptavidin Alexa Fluor<sup>®</sup> 555-conjugate (Molecular Probes). Cells were finally counterstained with DAPI-containing PBS and 0.1% Tween 20.

**Aβ ELISAs**—Aβ peptides were captured with a monoclonal antibody raised against the N-terminal domain of Aβ (8–17) and revealed using a secondary antibody against the C-terminal extremity (40 or 42 end) of Aβ (BioSource International, Nivelles, Belgium). Either recombinant rodent or human Aβs (Calbiochem) were used as controls.

**RNA Decay Experiments**—Mouse cortical astrocytes were exposed to 10 μg/ml actinomycin D for 0–16 h after 24-h treatment with recombinant human TGF-β1 (R&D Systems Europe) before semiquantitative RT-PCR analysis. Gels were scanned and quantified by densitometry.

**Electrophoretic Mobility Shift Assay**—Cultured murine cortical astrocytes were transiently transfected with the constructs indicated using the Transfast™ Transfection Reagent (Promega) as described by the manufacturer. For each transfection experiment, sister culture dishes were used to control the efficiency of transfection using an enhanced green fluorescent protein (EGFP)-containing plasmid driven by cytomegalovirus promoter (pEGFPC1 vector) (BD Biosciences-Clontech). All transfections were performed with the empty vector (pcDNA3.1, Invitrogen) corresponding to the experimental plasmid used (pcDNA3.1-APPtre-luc).

Transfection efficiency was determined by counting total cells and EGFP-positive astrocytes. In our hands, we reached the ratio of 71 ± 8% of transfected astrocytes. Potential toxicity was examined by examination of the cultures under phase microscopy and quantified by measurement of the activity of the cytosolic enzyme lactate dehydrogenase released by damaged cells into the bathing medium as described previously (21). No differences were observed between untransfected and transfected cells (data not shown). Moreover, the pRL-TK values provided by the Dual-Luciferase™ Reporter Assay System did not differ from controls (data not shown).

**Reporter Gene Assay**—Two days after transfection, cells were treated and luciferase activities (firefly luciferase and Renilla luciferase) were evaluated after 1 day using the Dual Luciferase™ Reporter Assay System (Promega). Values were normalized to the Renilla luciferase activity (Promega). The Dual-Luciferase™ Reporter Assay System refers to the ratio of the signal of the two individual reporter enzymes within a single system. Typically, the “experimental” reporter (firefly luciferase) is correlated with the effect of specific experimental conditions (e.g. TGF-β1 treatment), whereas the activity of the cotransfected “control” (Renilla luciferase) reporter provides an internal control, which serves as the base line of the response. Indeed, the pRL-TK values provided by the Dual-Luciferase™ Reporter Assay System did not differ from controls (data not shown).

**APP Promoter Constructs**—The −1104/−104 fragment of the Rhesus monkey APP promoter (GenBank accession number AF076971) was provided by Dr. Lahiri (22). The −309/+104 and the −201/+104 fragments were PCR amplified, digested by KpnI and BglII restriction enzymes, and ligated into pGL-MLP. The −390/+104 luciferase vector was digested by XhoI and BglII restriction enzymes to obtain the −75/+104 fragment. The +54/+74 luciferase reporter vector was obtained by inserting into pGL-MLP a double stranded oligonucleotide corresponding to the +54/+74 region of the human APP promoter and flanked by two XhoI restriction sites.

**Densitometric Analyses**—Agarose gels or blots from three independent experiments were acquired by a CCD camera and saved as a resolution of 600 dpi for software analysis. PCR products and Western blot signals were quantified by two-dimension densitometric analysis using the OptiQuant® software (Packard Instruments Inc.). The data were expressed as mean + S.D. Statistical analyses were performed with StatView (Abacus, Berkeley, CA) by one-way variance analysis (ANOVA) followed by the Bonferroni-Dunn test or Student’s t test.

**RESULTS**

**Endogenous Increase of APP and Aβ Expression in TGF-β1 Transgenic Mice**—To understand the role of TGF-β1 in Aβ deposition, the influence of TGF-β1 on APP and Aβ expression was investigated in 6-month-old transgenic mice overexpressing TGF-β1 (line T65) through the use of semiquantitative
RT-PCR, Western blotting analysis, and Aβ ELISAs. Transgenic mice were generated by Prof. Lennart Mucke’s laboratory similarly to the previously described low expressing GFAP-TGF-β1 transgenic lines, T64 and T115 (16). These mice express large amounts of the transgene associated with enhanced expression of endogenous TGF-β1 as determined either by RT-PCR (Fig. 1A) or by immunoblotting (Fig. 1B). The membrane was reprobed to determine the expression of actin to estimate the homogeneity of proteins loaded. C, expression of APP mRNAs in the brain of 6-month-old mice overexpressing TGF-β1 in astrocytes (T65, TGF-β1) was determined by semiquantitative RT-PCR analysis. Experiments were performed in triplicate. D, densitometric quantification of brain relative expression of APP mRNA isoforms. Results are the mean ± S.D. of experiments performed in triplicate. *Asterisk, p < 0.01, Student’s t test. E, Western blotting analysis of total APP proteins in the brain of 6-month-old mice overexpressing TGF-β1 in astrocytes (T65, TGF-β1) revealed using the 22C11 antibody (n = 3). The membrane was reprobed to determine the expression of actin to estimate the homogeneity of proteins loaded. F, Aβ ELISAs in the brain of 6-month-old mice overexpressing TGF-β1 in astrocytes (T65, TGF-β1). Amounts of respective Aβ species are indicated in the left panel, and rationalized expressions are shown in the right panel. Results are the mean ± S.E. of six independent experiments. *Asterisk, p < 0.05, Student’s t test.

Fig. 1. TGF-β1 transgenic mice display enhanced Aβ generation. A, expression of the GFAP-TGF-β1 transgene, endogenous TGF-β1 mRNA in the brain of 6-month-old mice overexpressing TGF-β1 in astrocytes (T65, TGF-β1) was determined by semiquantitative RT-PCR analysis. Experiments were performed in triplicate. B, Western blotting analysis of endogenous TGF-β1 in the brain of 6-month-old mice overexpressing TGF-β1 in astrocytes (T65, TGF-β1) revealed using the sc-146 antibody (n = 3). The membrane was reprobed to determine the expression of actin to estimate the homogeneity of proteins loaded. C, expression of APP mRNAs in the brain of 6-month-old mice overexpressing TGF-β1 in astrocytes (T65, TGF-β1) was determined by semiquantitative RT-PCR analysis. Experiments were performed in triplicate. B-Actin was used as a housekeeping gene. D, densitometric quantification of brain relative expression of APP mRNA isoforms. Results are the mean ± S.D. of experiments performed in triplicate. *Asterisk, p < 0.01, Student’s t test. E, Western blotting analysis of total APP proteins in the brain of 6-month-old mice overexpressing TGF-β1 in astrocytes (T65, TGF-β1) revealed using the 22C11 antibody (n = 3). The membrane was reprobed to determine the expression of actin to estimate the homogeneity of proteins loaded. F, Aβ ELISAs in the brain of 6-month-old mice overexpressing TGF-β1 in astrocytes (T65, TGF-β1). Amounts of respective Aβ species are indicated in the left panel, and rationalized expressions are shown in the right panel. Results are the mean ± S.E. of six independent experiments. *Asterisk, p < 0.05, Student’s t test.

TGF-β1 Potentiates Amyloid-β Generation—To determine which cell type was involved in the increased expression of APP in TGF-β1 mice, we exposed primary cultures of mouse cortical neurons or astrocytes to exogenous recombinant TGF-β1. As reported previously (23), the three APP isoforms are expressed in astrocytes with a predominant expression of APP770 and APP751, whereas cortical neurons abundantly express APP695 mRNA but only small amounts of APP770 and APP751 mRNAs (Fig. 2, A and C). Although 1 ng/ml TGF-β1 exposure failed to modify the expression pattern of APP mRNAs in cortical neurons (Fig. 2, A and B), TGF-β1 markedly
enhanced the expression of the mRNAs encoding the three isoforms of APP in astrocytes (Fig. 2, C and D).

Because it could be advanced that the up-regulation of APP observed after TGF-β1 exposure could result from the secondary activation of astrocytes by proinflammatory cytokines such as interleukin-1β released by microglia cells, we have estimated the amount of microglia in our cultures of cortical astrocytes by performing double immunocytochemical labeling against the GFAP, an astrocytic marker, and CD11b, a specific marker of microglial cells. Less than 1% of microglial contamination in our primary cultures of cortical astrocytes was evidenced (Fig. 2E).

TGF-β1 elicits its biological effects through a heteromeric complex of transmembrane serine/threonine kinase receptors, cloned as type I and type II receptors. After receptor-dependent phosphorylation, Smad2 or Smad3 interacts with the common mediator Smad4 to form a heteromeric complex that translocates to the nucleus to initiate TGF-β-dependent transcriptional activity. This complex binds DNA sequences termed Smad-binding elements that contain a minimal four-nucleotide domain AGAC also called “CAGA box” (24). Accordingly, we
Fig. 3. Regulation of APP transcription by the Smad-dependent TGF-β1 signaling pathway in astrocytes. A, efficiency of transient transfection of primary cultured astrocytes. The expression vector pEGFP-C1 encoding EGFP was transiently transfected in astrocytes before immunostaining with an antibody against the GFAP (red) and counterstaining with 1 μg/ml DAPI (blue). Overlaid images are presented in the bottom right panel. B, transient transfections of cultured murine astrocytes, with expression vectors encoding constitutively activated versions of the TGF-β family type I receptors, were performed before RT-PCR analyses as described in Fig. 2. C, relative expression of APP mRNA isoforms compared with β-actin in transiently transfected astrocytes presented in B. Dark, dark gray, and light gray bars represent the ratio obtained for APP770, APP751, and APP695 isoforms, respectively. Results are the mean ± S.D. of experiments performed in triplicate. Asterisk, p < 0.003, Sharp (different from controls or empty vector), p < 0.01, Student’s t test. D, primary cultures of mouse cortical astrocytes were transfected either with expression vector encoding the transcription factors Smad3 or Smad7 or with empty vector (pcDNA3) before RT-PCR analyses as described in Fig. 2 (n = 3). Astrocytes transfected with the empty vector were exposed to TGF-β1 at 1 ng/ml for 24 h after transfection. The letter C refers to sham wash control cultures, and T refers to TGF-β1-treated cell cultures. E, densitometric quantification of the experiments presented in D 24 h after treatment. White bars represent control astrocytes transfected with the empty vector (pcDNA3), white hatched bars represent control astrocytes transfected with the expression vector encoding Smad3, and dark hatched bars represent control astrocytes transfected with the expression vector encoding Smad7. Results are the mean of three experiments. Asterisk, p < 0.01, Sharp (different from TGF-β1-treated cells), p < 0.02, Student’s t test.
have transiently transfected astrocytes with expression vectors encoding constitutively activated versions of the TGF-β type I receptor (Alks) (25) or with an expression vector encoding the transcription factor Smad3 (Fig. 3). To validate the efficiency of transfection in our system, murine cultured astrocytes were transfected with the EGFP-C1 vector encoding EGFP using transfection efficiency (black bars). Results are the mean of three independent experiments. Asterisk, p < 0.01, Student’s t test. B. Mean ± S.D. of the luciferase activity of Mv1Lu cell line transiently transfected with luciferase reporter constructs containing the regions −309/+104, −201/+104, or −75/+104 of the Rhesus monkey APP promoter, respectively. For each condition transfected cells were treated (black bars) or not (white bars) in the presence of 1 ng/ml TGF-β1 for 24 h or cotransfected with the expression vector encoding Smad3 (hatched bars) (n = 12). Asterisk, p < 0.01, ANOVA followed by the Bonferroni-Dunn test. C. Alignment of DNA sequences of the 5’ untranslated regions of human, Rhesus monkey, mouse, and rat APP genes. Letters in bold differ from the human DNA sequence. Underlined letters indicate the minimal binding DNA sequence for Smad3. The dark square elicits the conserved sequences among the different species analyzed.

Expression of either Alk-5 or Alk-4 led to an enhanced transcription of all isoforms of APP, whereas Alks linked to other members of the TGF-β superfamily did not enhance APP transcription (Fig. 3, B and C). Indeed, transfecting astrocytes with Alk-3 unexpectedly lowered APP mRNA expression. Accordingly, Smad3 overexpression potentiated APP mRNA expression (Fig. 3, D and E). As expected, overexpression of Smad7, a physiological dominant negative Smad, prevented TGF-β1-induced expression of APP in cultured astrocytes (Fig. 3, D and E). Thus, TGF-β1 signaling machinery controls APP transcription in cultured astrocytes by a Smad3-dependent mechanism.

A TGF-β-responsive Element Mediates TGF-β1-dependent Expression of APP—We determined whether the induction of the APP mRNAs by TGF-β1 in astrocytes was the result of a stabilization of the transcripts. In the presence of actinomycin D, the relative amount of APP and β-actin mRNAs was reduced in a time-dependent manner. The administration of TGF-β1 failed to modify the stability of APP transcripts (Fig. 4A). Considering the TGF-β1-dependent overexpression of the three APP isoforms mRNAs, we investigated whether TGF-β1 modulates the activity of the APP promoter. Thus, different regions (−309/+104, −201/+104, and −75/+104) of the Rhesus monkey APP promoter were subcloned into the pGL3-basic luciferase reporter vector containing a MLP. When transfected in the mink lung epithelial cell line Mv1Lu, previously characterized as a TGF-β-responsive cell line (26, 27), these entire promoter constructs were activated by TGF-β1 treatment or Smad3 transfection (Fig. 4B). By performing sequence alignments

![Diagram](image-url)
FIG. 5. Smads proteins mediate TGF-β1-induced activation of the APP promoter. A, mean ± S.D. of the luciferase activity of Mv1Lu cell line transiently transfected with a luciferase reporter construct containing five copies of the +54/+74 sequence of the human APP promoter inserted 5′ of the MLP luciferase reporter gene. For each condition transfected cells were treated or not in the presence of TGF-β1 or cotransfected with Smad3 (n = 12). For each condition transfected cells were treated (black bars) or not (white bars) in the presence of 1 ng/ml TGF-β1 for 24 h or cotransfected with the expression vector encoding Smad3 (hatched bars). Asterisk, *p < 0.01, ANOVA followed by the Bonferroni-Dunn test. B, the same experiments as described in A were performed in primary cultures of murine cortical astrocytes (n = 12). Asterisk, p < 0.01, ANOVA followed by the Bonferroni-Dunn test. C, mean ± S.D. of the luciferase activity of astrocytes transiently cotransfected with a luciferase reporter construct containing five copies of the +54/+74 sequence of the human APP promoter inserted 5′ of the MLP luciferase reporter gene and with expression vectors encoding autoactivated type I TGF-β receptors (Alks). Black bars represent astrocytes expressing autoactivated Alks, and white bars represent astrocytes transfected with the empty vector (n = 12). Asterisk, *p < 0.01, Sharp (different from untreated cells), different from TGF-β1, *p < 0.01, ANOVA followed by the Bonferroni-Dunn test. D, mean ± S.D. of the luciferase activity of Mv1Lu cell line transiently transfected with a luciferase reporter construct containing five copies of the +54/+74 sequence of the human APP promoter inserted 5′ of the MLP luciferase reporter gene. For each condition transfected cells were treated (black bars) or not (white bars) in the presence of TGF-β1 or cotransfected with Smad7 (hatched bars) (n = 8). Asterisk, p < 0.01, Sharp (different from TGF-β1-treated cells), different from TGF-β1, p < 0.01, ANOVA followed by the Bonferroni-Dunn test. E, mean ± S.D. of the luciferase activity of Smad4-deficient MDA cell line transiently transfected with a luciferase reporter construct containing five copies of the +54/+74 sequence of the human APP promoter inserted 5′ of the MLP luciferase reporter gene. For each condition transfected cells were treated (black bars) or not (white bars) in the presence of TGF-β1 or cotransfected with either Smad3 (dark hatched bars) or Smad4 (gray bars) or Smad3 and Smad4 (gray hatched bars) (n = 8). Asterisk, p < 0.03, ANOVA followed by the Bonferroni-Dunn test. F, mean ± S.D. of the luciferase activity of astrocytes transiently cotransfected with a luciferase reporter construct containing the wild-type (AGAC) or mutated (ACAT) region −201/+104 of the Rhesus monkey APP promoter inserted 5′ of the MLP luciferase reporter gene. For each condition transfected cells were treated (black bars) or not (white bars) in the presence of TGF-β1 (n = 12). Asterisk, p < 0.01, ANOVA followed by the Bonferroni-Dunn test. G, immunoblot analyses of full-length APP proteins associated with the cell monolayer of cultured murine cortical astrocytes treated (T) or not (C) in the presence of TGF-β1 for 24 h were performed using the 22C11 antibody. To determine the involvement of endogenous TGF-β1 in controlling APP expression, parallel experiments were done in the presence of soluble TGF-β type II receptor (sTβRII) as a TGF-β antagonist (n = 2).
Moreover, overexpression of Smad7 led to 90% inhibition of TGF-β1-induced activation of the APP promoter (named APP TGF-β1-responsive element or APPtre) was inserted (Fig. 5, A and B). Results were similar both in Mv1Lu cells (Fig. 5A) and in primary cultures of murine astrocytes (Fig. 5B). Transfection of cultured astrocytes with expression vectors encoding Alk-4 and Alk-5 resulted in a marked increase in luciferase activities confirming the necessity of TGF-β receptor activation to induce its response (Fig. 5C). Moreover, overexpression of Smad7 led to 90% inhibition of TGF-β1-induced activation of the APPtre reporter construct (Fig. 5D). In MDA-MB468 cells deficient for endogenous Smad4 expression (28, 29), TGF-β1 or Smad3 had no effect, whereas transfection of Smad4 rescued TGF-β1-induced activation of the APPtre reporter construct (Fig. 5E). These data demonstrate that a Smad3-Smad4 complex is necessary to mediate the TGF-β1-induced transcription of APP.

Finally, we mutated the AGAC sequence required for Smad binding to the DNA into an ACAT sequence, previously reported to abolish Smad binding to the DNA (14), within the −204/+104 gene promoter construct (Fig. 5F). This construct also provides evidences of the influence of this element in a similar context to the full-length APP promoter. Results obtained by Dual-Luciferase® Reporter Assay System clearly indicate that this double site mutation abolishes the ability of this construct to respond to TGF-β1 transcription and to Smad3 transfection. Thus, our findings demonstrate the absolute necessity of this sequence for TGF-β1-induced transcriptional activity of the APP promoter.

To characterize further the involvement of TGF-β1 in the control of APP expression in cultured astrocytes, we have performed experiments using a soluble TGF-β type II receptor as a TGF-β antagonist (30, 31). Addition of the TGF-β receptor in the media of cultured astrocytes prevented TGF-β1-induced APP overexpression. Moreover, it is interesting to note that treatment in the presence of the soluble TGF-β type II receptor also decreased the basal expression of APP, confirming the presence of endogenous TGF-β1 in our model of cultured astrocytes as demonstrated previously (32). Thus, endogenous TGF-β1 regulates APP basal expression and can enhance APP transcription.

To determine whether astrocytes and neurons were TGF-β1-responsive, we performed an electrophoretic mobility shift assay using astrocytic and neuronal nuclear extracts in an attempt to characterize the DNA binding activity on the TGF-β-responsive APP sequence containing the previously characterized TGF-β-responsive AGAC sequence (14). Although TGF-β1 failed to induce formation of complexes in cultured neurons, increased binding complexes were observed in TGF-β1-treated astrocytes (Fig. 6, center lanes). To confirm the specificity of the assay, a 50-fold excess of unlabeled probe was added to nuclear extracts from TGF-β1-treated cells, which totally prevented the formation of complexes (Fig. 6, right lanes). These data provide evidence that primary cultured mature cortical neurons (14 days in vitro) are not capable of mediating Smad-dependent TGF-β1 signaling in the APP promoter.

Aβ Production Is Enhanced by TGF-β1 Signaling—As observed at the mRNA level, TGF-β1 failed to modify the expression of either full-length or derivatives of APP in cortical neurons (Fig. 7). In contrast, immunoblotting performed from cell extracts of murine astrocytes showed that membrane-bound APP proteins were increased markedly in the presence of TGF-β1 over 24–72 h, whereas actin remained unchanged (Fig. 7, A and B). Using the R7 antiseraum targeted against the KPI domain of KPI-APPs, i.e. APP770 and APP751, we confirmed the overexpression of APP770 and APP751 at the membrane of astrocytes stimulated by TGF-β1 (Fig. 7, A and B). Furthermore, we evidenced that the release of sAPP derivatives was increased in the media of TGF-β1-treated astrocytes (Fig. 7C). Similarly, we showed that sAPP-β was increased in the conditioned media of astrocytes treated by TGF-β1 (for 24 or 72 h) (Fig. 7C). Because β-secretase activity is necessary for Aβ production, incubation with TGF-β1 for 24 or 72 h induced a marked secretion of the 4-kDa Aβ species in the bathing medium of cultured astrocytes (Fig. 7D). Moreover, our results revealed that the overexpression of Smad3 led to an accumulation of Aβ as obtained previously after TGF-β1 treatment (Fig. 7E). In contrast, overexpression of Smad7 prevented the TGF-β1-induced accumulation of Aβ (Fig. 7E). Using FCA3340 and FCA3542 antisera raised against Aβ40 or Aβ42, we observed an accumulation of Aβ40 and Aβ42 under TGF-β1 treatment, whereas Aβ was not detected in control conditions (Fig. 7F). Because we were unable to detect basal secretion of astrocytic Aβ using immunoblotting (Fig. 7, D–F), we have finally estimated Aβ release by quantitative ELISAs and confirmed a −2-fold elevation of Aβ loads (Fig. 7G). This 2-fold increase in Aβ secretion (×1.9 for Aβ42 and ×1.8 for Aβ40) can be directly associated with the 2-fold up-regulation of astrocytic APP transcription. Nevertheless, we cannot exclude the possibility of indirect effects of TGF-β1 on either the APP processing or the Aβ degradation. Taken overall, these experiments evidence that TGF-β1 promotes Aβ generation in astrocytes.

TGF-β1 Promotes Aβ In Cultured Human Astrocytes—Because rodents do not display amyloid deposits, we performed a
FIG. 7. TGF-β1 potentiates sAPP-β and Aβ accumulation in the conditioned media of astrocytes. **A**, immunoblot analyses of full-length APP proteins associated with the cell monolayer of cultured murine cortical neurons or astrocytes treated (T) or not (C) in the presence of TGF-β1 for 24 h were performed using either the 22C11 antibody or the R7 antiserum. Note that only small amounts of KPI-APPs are detected in neurons. The same immunoblot was reprobed with actin antibody as control. Experiments were performed in triplicate. **B**, densitometric quantification of the experiments presented in **A** 24 h after treatment. White bars represent sham wash control cells, and dark bars represent TGF-β1-treated cells. Results are the mean of three experiments. Asterisk, p < 0.01, Student’s t test. **C**, immunoblot analyses of total sAPP and sAPP-β from conditioned media of cultured murine cortical neurons or astrocytes treated (T) or not (C) in the presence of TGF-β1 for 24 h were performed using either the 22C11 antibody or the 192wt antiserum. The membrane was stained by naphthol blue to confirm the homogeneity of loaded amounts of proteins (data not shown). Experiments were performed in triplicate. **D**, conditioned media of astrocytes treated in the presence of 1 ng/ml TGF-β1 for 72 h were concentrated as described under “Experimental Procedures,” and Western blot analyses were performed using the R600 polyclonal antiserum raised against the N terminus (residues 1–10) of the Aβ peptide. Experiments were performed in triplicate. **E**, Western analysis of Aβ was performed using the R600 antiserum from concentrated conditioned media of primary cultures of cortical astrocytes previously transfected either with the expression vector encoding Smad3 or the expression vector encoding Smad7 or with the empty vector in the presence or not of TGF-β1 for 24 h. Asterisks indicate nonspecific labeling. **F**, to discriminate Aβ species, Western analyses of Aβ were performed from concentrated conditioned media of astrocytes incubated with 1 ng/ml TGF-β1 for 72 h and probed with a polyclonal antiserum raised against Aβ1-40, FCA3340, or Aβ1-42, FCA3542. Asterisks indicate nonspecific labeling. The experiments were performed in triplicate. **G**, in parallel, Aβ ELISAs were realized from the same extracts used for immunoblotting experiments. White bars correspond to untreated astrocytes and dark bars to TGF-β1 treated astrocytes. Asterisk, p < 0.001, Student’s t test.

set of experiments in primary cultures of human astrocytes and neurons. As observed previously in murine cultures, TGF-β1 treatment failed to influence either transcription or accumulation of APP derivatives in cultured human neurons (Fig. 8). However, the addition of exogenous TGF-β1 to human astrocytes induced an increased expression of APP (Fig. 8A) as well as the activation of the APP-trc luciferase reporter vector (Fig. 8B). These transcriptional data were confirmed at the protein level. Although TGF-β1 failed to influence APP expression in neurons, it increased the amounts of full-length APP and sAPP-β associated with either the plasma membrane or the conditioned media of cultured human astrocytes (Fig. 8, C and D). Moreover, TGF-β1 (Fig. 8E) or transfection with the Smad3 vector (Fig. 8F), respectively, led to a 1.5–2.5 increase in Aβ content in the conditioned media of human astrocytes. Finally, Aβ ELISAs (n = 8) confirmed a significant enhanced generation of Aβ42 (7.32 ± 0.59 in controls versus 10.01 ± 0.97 pg/mg in treated cells; p = 0.02) and Aβ40 (43.26 ± 3.77 in controls versus 74.13 ± 5.31 pg/mg in treated cells; p = 0.001) in human astrocytes after TGF-β1 treatment. Interestingly, the Aβ42:Aβ40 ratio decreased (0.169 ± 0.045 in controls versus 0.135 ± 0.018 pg/mg in treated cells; p = 0.04) in human treated cells.
TGF-β1 Potentiates Amyloid-β Generation

Aβ accumulation into amyloid plaques in the brain is one of the two histopathological hallmarks of AD. The process that regulates the deposition of Aβ in the brain is still under investigation. A better understanding of the mechanism leading to Aβ production would facilitate the development of treatments for AD. We document here that the overexpression of the anti-inflammatory cytokine TGF-β1 in transgenic mice induces higher expression of endogenous APP isoforms and increased Aβ generation in cerebral tissues. Furthermore, we demonstrate that exogenous TGF-β1 enhances APP synthesis in astrocytes and leads to Aβ generation in vitro.

Although neurons are known to be the major source of Aβ in AD (33–35), the contribution of astrocytes to amyloidogenic processes has never been clearly established. Studies have suggested that cultured astrocytes could generate modest amounts of Aβ compared with neurons (36–39). Although TGF-β1 induced the overexpression of APP in astrocytes by involving a TGF-β-responsive element, we observed no effect of TGF-β1 in primary cultures of mature neurons (14 days in vitro). This absence of a TGF-β1 response is puzzling but has been described previously (40). In addition, contradictory data report the neuronal expression of type II TGF-β receptor (32, 41), which binds the ligand and then activates the TGF-β signaling intracellular pathway. Finally, these data are in agreement with a previous study (42), demonstrating that although TGF-β induced transcription of plasminogen activator inhibitor-1 in cultured astrocytes, it failed to mediate this response in cultured neurons. To understand better why TGF-β1 did not activate APP transcription in mature cultured neurons (14 days in vitro), we have performed an electrophoretic mobility shift assay and observed that TGF-β1 is unable to activate the Smad pathway in mature neurons.

However, an astrocytic gliosis is always observed in brains of 0.01, ANOVA followed by the Bonferroni-Dunn test. C, immunoblot analysis of full-length APP proteins associated with the cell monolayer of cultured human cortical neurons or astrocytes treated (T) or not (C) in the presence of TGF-β1 for 24 h using the R7 antibody. The same immunoblot was reprobed with actin antibody as control. Experiments were performed in triplicate. The histogram illustrates densitometric quantification of the experiments presented in C 24 h after treatment. White bars represent sham wash control cells, and dark bars represent TGF-β1-treated cells. Results are the mean of three experiments. Asterisk, p < 0.01, Student’s t test. D, immunoblot analysis of sAPP-β was performed from the conditioned media of cultured human cortical neurons or astrocytes treated (T) or not (C) in the presence of TGF-β1 for 24 h using the R7 antibody. The same immunoblot was reprobed with the R600 antiserum from concentrated conditioned media of primary cultures of cortical astrocytes previously transfected with the expression vector encoding Smad3 or with the empty vector in the presence or not of TGF-β1 for 24 h. The amounts of respective Aβ species are indicated in the left panel, and rationalized expressions are shown in the right panel. Results are the mean ± S.E. of eight independent experiments. Asterisk, p < 0.05, Student’s t test.

DISCUSSION

Aβ accumulation into amyloid plaques in the brain is one of the two histopathological hallmarks of AD. The process that regulates the deposition of Aβ in the brain is still under investigation. A better understanding of the mechanism leading to Aβ production would facilitate the development of treatments for AD. We document here that the overexpression of the anti-inflammatory cytokine TGF-β1 in transgenic mice induces higher expression of endogenous APP isoforms and increased Aβ generation in cerebral tissues. Furthermore, we demonstrate that exogenous TGF-β1 enhances APP synthesis in astrocytes and leads to Aβ generation in vitro.

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patients with AD (43). These data led us to reconsider the participation of astrocytes in the amyloidogenic process. Indeed, recent work performed from transgenic mice (TG2576) exhibiting amyloid plaques evidenced that astrocyte-derived Aβ participate in plaque formation and maturation at later stages than neuronal Aβ (44). Here, we demonstrate that TGF-β1 potentiates Aβ production in astrocytes.

Our data are in agreement with previous findings demonstrating that the expression of TGF-β1 in the brain parenchyma induces cerebrovascular and meningeal Aβ deposition at 12–18 months of age in TGF-β1 transgenic mice (line T64 or T115) or at 2–3 months of age in hAPP/TGF-β1 biogenic expressing the human APP and TGF-β1 (line T64 or T115) (8). In addition to its proamyloidogenic effect, TGF-β1 may exert a more complex role because 12–15-month-old hAPP/TGF-β1 double transgenic mice (10) displayed plaque burden reduction associated with increased microglia activation and increased clearance of Aβ compared with hAPP mice. In the present study, we report that 6-month-old transgenic mice overexpressing TGF-β1 (line T65) display increased endogenous APP expression and Aβ production in vivo. Moreover, we postulate that this effect is sustained by a transcriptional activation of APP in both murine and human astrocytes. Although AD cannot be equated with a simple unique gene deregulation, this, to our knowledge, the only transgenic animal overexpressing a brain. Nevertheless, we cannot exclude the possibility of independent effect of TGF-β1 on either the APP processing or the Aβ degradation. These data are strengthened by several publications. First, TGF-β1 immunoreactivity has been found within the plaques of AD (5). Second, increased TGF-β2 levels in reactive astrocytes are associated with AD (7). Third, aged transgenic mice containing the Swedish double mutation of APP695 display TGF-β1 immunoreactive astrocytes found in close proximity to Aβ deposits (44). We demonstrate that TGF-β1-induced APP overexpression leads to an enhanced Aβ production. Similarly, the increase in APP expression because of duplication of the 21 chromosome in Down’s syndrome results in an overproduction of Aβ peptides leading to the appearance of AD-type brain lesions with associated microgliosis and astroglisis (45).

In vitro, an overexpression of APP mRNAs induced by TGF-β1 has been reported previously in astrocytes or in astrocytoma cell line (46, 47). Amara et al. (47) have described an increased half-life of the APP transcripts induced by TGF-β1. By performing mRNA decay experiments after 24 h of TGF-β1 treatment, we demonstrated that in our hands the half-life of APP mRNA was not stabilized by a TGF-β1 treatment. In contrast, we evidenced that TGF-β1-induced up-regulation of APP mRNA expression involved the activation of a TGF-β1-responsive element within the +54/+74 region of the APP promoter. Additional data provided evidences that the 5′-untranslated region of the APP promoter is crucial for driving APP expression. Indeed, the −75/+104 region has been shown to mediate up to 40% of the promoter activity compared with the full-length promoter-driven activity (−7900/+104) (48).

Overall, these data reveal a molecular mechanism through which TGF-β1 promotes Aβ generation and underline the critical role that astrocytes could hold in AD pathogenesis.

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