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The Amyloid-β Peptide Suppresses Transforming Growth Factor-β1-induced Matrix Metalloproteinase-2 Production via Smad7 Expression in Human Monocytic THP-1 Cells*

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Eun Ok Lee‡, Jihee Lee Kang§, and Young Hae Chong‡¶

From the Departments of ‡Microbiology and §Physiology, College of Medicine, Division of Molecular Biology and Neuroscience, Ewha Medical Research Institute, Ewha Womans University, 911-1, Mok-6-dong, Yangcheonku, Seoul 158-710, Korea

Accumulation of the amyloid-β (Aβ) peptide in the brain is a crucial factor in the development of Alzheimer disease. Expression of transforming growth factor-β1 (TGF-β1), an immunosuppressive cytokine, has been associated *in vivo* with Aβ accumulation in transgenic mice and recently with Aβ clearance by activated microglia, suggesting its deleterious and beneficial effects in neuronal cells. In this study, we demonstrated that TGF-β1 stimulated the production of matrix metalloproteinase-2 (MMP-2) in a time- and dose-dependent manner in a human monocytic THP-1 cell line. Notably, we found that Aβ1–42 consistently inhibited the TGF-β1-induced production of MMP-2, the endogenous gene containing Smad response elements, whereas the reverse peptide, Aβ42–1, evidenced little effect. Additionally, Aβ1–42 reduced TGF-β1-induced increase in plasminogen activator inhibitor-1 (PAI-1). This inhibitory effect of Aβ1–42 was also seen in human astroglial T98G cell line. Furthermore, Aβ1–42 significantly induced the expression of Smad7, which appears in turn to mediate the Aβ suppression of the TGF-β1-induced MMP-2 production. Indeed, Smad7 overexpression mimicked the inhibitory effect of Aβ1–42 on TGF-β1-induced MMP-2 production. Importantly, Aβ1–42 markedly suppressed the transactivation of the transfected reporter construct, p3TP-Lux, which contains TGF-β1-inducible Smad response elements. This was concomitant with a decreased MMP-2 production in TGF-β1-treated cells. Inhibition of cellular Smad7 levels via the small interference RNA method significantly ameliorated the Aβ1–42-mediated suppression of TGF-β1-inducible transcription reporter activity, thereby restoring MMP-2 induction, whereas Smad7 transfection down-regulated TGF-β1-inducible transcription reporter activity. Collectively, these data suggest that Aβ1–42 may play an important role in the negative regulation of TGF-β1-induced MMP-2 production via Smad7 expression.

Alzheimer disease (AD)[1] is a progressive neurodegenerative disorder, which is characterized by the loss of higher cognitive functions. The increased production of amyloid-β (Aβ) peptide, a 39–42 residue of the proteolytic product of amyloid precursor protein, and fibrillar Aβ deposition in dense senile plaques have been correlated with the progression of cognitive dysfunction in AD (1). Immunoreactivity to numerous inflammatory mediators has been detected in sections of AD brains including pro-inflammatory cytokines, acute phase proteins, and several proteins involved in the classical complement pathway (2, 3). In AD brains, the co-localization of a broad variety of these inflammation-related proteins and clusters of reactive microglia and astrocytes with Aβ deposits is consistent with the notion that chronic inflammation plays a significant role in the progression to enhanced neurodegeneration (2, 4). However, the precise role of these molecules in the neuropathology of AD has yet to be clarified.

Transforming growth factor-β1 (TGF-β1), a potent immunosuppressive cytokine found in neurite plaques, has been implicated recently as a cofactor for AD progression, largely due to its ability to promote perivascular inflammation and/or amyloid deposition (5, 6). Postmortem brain tissue analyses of AD patients show increased TGF-β1 expression, which can be closely correlated with the degree of cerebral amyloid angiopathy (CAA) (7), a major pathological feature of AD and related disorders (8). A genetic polymorphism of the TGF-β1 gene may be associated with a higher risk of developing AD (9). In contrast to its amyloidogenic effect, TGF-β1 may also exert a more complex role since TGF-β1 facilitates increased Aβ clearance from the brain parenchyma to the cerebral blood vasculature following the activation of parenchymal microglial cells and also induces plaque burden reduction in TGF-β1/human amyloid precursor protein bigenic mouse brains compared with human amyloid precursor protein mice (10). The TGF-βs have also been shown to protect neuronal cell cultures from Aβ- and glutamate-induced neurotoxicity by up-regulating either anti-apoptotic (Bcl-2, Bcl-XL) or calcium-stabilizing factors (calbindin) (11, 12). Thus, in the Aβ function of neuronal cells, TGF-βs apparently exert both deleterious and beneficial effects via regional and/or cell type-specific TGF-β isoforms and receptor expression (7, 10–13).

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¶ To whom correspondence should be addressed: Dept. of Microbiology, College of Medicine, Ewha Woman's University, 911-1, Mok-6-dong, Yangcheonku, Seoul 158-710, Korea. Tel.: 822-02-2650-5739; Fax: 822-02-2653-8891; E-mail: younghae@ewha.ac.kr.

[1] The abbreviations used are: AD, Alzheimer disease; Aβ, amyloid-β; TGF-β1, transforming growth factor-β1; SBEs, Smad-binding elements; TNF-α, tumor necrosis factor-α; MMP, matrix metalloproteinase; CAA, cerebral amyloid angiopathy; PAI-1, plasminogen activator inhibitor-1; PA, plasminogen activator; PK, protein kinase; TK, thymidine kinase; MAPK, mitogen-activated protein kinase; MEK1; MAPK kinase kinase, JNK, c-jun-N-terminal kinase; JNKI, JNK inhibitor; ERK, extracellular signal-regulated kinase; siRNA, small interfering RNA.

The TGF-β receptors are transmembrane serine/threonine kinases that propagate signals downstream (14–16). TGF-β binding induces the phosphorylation of the TGF-β type I receptor by the TGF-β type II receptor, the essential process in TGF-β signaling. Smads associate with these activated TGF-β receptors and play a crucial role in TGF-β signal transduction. Smad2 and Smad3 are direct substrates of the TGF-β receptor kinase, and they interact with a common partner, Smad4. Smad4-containing heterometric Smad complexes then translocate from the cytoplasm into the nucleus where they function as transcriptional regulators. Thus, the activation of gene transcription via Smad protein binding to Smad-binding elements (SBEs) represents terminal event in TGF-β signaling (17, 18). In contrast to the receptor-activated Smads, inhibitory Smad7 binds stably to TGF-β receptors and interferes with the ligand-induced phosphorylation of both Smad2 and Smad3 (14–16). Importantly, Smad7 is considered to function as a negative regulator of the TGF-β/Smad signaling cascade and abnormal Smad7 expression is implicated in human disease (19, 20).

Increased levels of matrix metalloproteinases (MMPs) have been observed in the plasma and the proximity of extracellular amyloid plaques in the brain tissues from the AD patients (21, 22). Of important interest, pathogenic Aβ (Dutch variant Aβ), possibly associated with CAA, was shown to stimulate the expression and activation of MMP-2. Thus, these activities may contribute to the loss of vessel wall integrity in CAA, resulting in hemorrhagic stroke (23). In contrast, earlier studies showed that MMPs are involved in Aβ degradation (24, 25). These findings together suggest a critical role of MMPs in the pathophysiology of AD.

Despite extensive studies on the dual effects of TGF-βs in amyloid plaque metabolism, the role of Aβ in the modulation of TGF-β function has remained enigmatic. Because dysregulation of TGF-β function is also implicated in chronic inflammation, leading to enhanced neurodegeneration, it is important to uncover any synergistic or antagonistic modulations of TGF-β-mediated effects elicited by Aβ to generate effective therapeutic strategies involving TGF-β and Aβ in AD pathology. Furthermore, few studies try to understand how TGF-β1 affects MMP production and how Aβ may modulate this process at the molecular level.

In this regard, we sought to determine the regulatory effects of Aβ on the TGF-β-induced stimulation of MMP-2 and plasminogen activator inhibitor-1 (PAI-1), endogenous genes containing SBEs (26, 27), in human monocytic THP-1 cells as a model for microglia and in astroglial T98G cells. We also assessed the effects of Aβ1–42 on the transcriptional activation of a transfected reporter gene containing TGF-β-inducible SBEs and also on Smad7 expression. We then attempted to ascertain in what way Smad7 expression was related to TGF-β-induced effect on MMP-2. To this end, we analyzed the effects of Smad7 overexpression on TGF-β1-mediated MMP-2 production and TGF-β1-induced transcription reporter activity and looked at decreases in cellular Smad7 levels via the siRNA method on the Aβ suppression of TGF-β1-inducible effects. Our data constitute evidence that TGF-β-mediated MMP-2 production primarily occurs through the activation of the Smad pathway. This study posits a novel role for Aβ as a negative regulator of TGF-β-mediated MMP-2 production via Smad7 expression.

EXPERIMENTAL PROCEDURES

Materials—Human TGF-β1, TGF-β2, TGF-β3, and anti-human TGF-β1 antibody were purchased from R&D (Minneapolis, MN). A goat polyclonal antibody against Smad7 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-human MMP-2 antibody (Ab-3) was obtained from Calbiochem. Anti-β-actin antibody and other chemicals were obtained from the Sigma. Anti-human TNF-α antibody and anti-human PAI-1 antibody were purchased from Upstate Biotechnology (Lake Placid, NY) and American Diagnostica (Greenwich, CT), respectively. LY294002, worthmanin, PD98059, SB202190, c-Jun-N-terminal kinase inhibitor (JNKI), cycloheximide, and actinomycin D were all obtained from Calbiochem. The Smad7 expression plasmid and the control pcDNA3 were kindly provided by Dr. C. H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden). The TGF-β-responsible p3TP-Lux promoter reporter plasmid and pRL-TK-*Renilla* were generous gifts from Dr. J. Massague (University of California, San Francisco, CA) and Dr. E. H. Jho (University of Seoul, Seoul, Korea), respectively.

Aβ Peptide Preparation—Aβ1–42 and Aβ42–1 were purchased from U. S. Peptide (Fullerton, CA), and stock solutions were prepared as described previously with some modifications (28). Aβ peptides were dissolved at 5 mM in dimethyl sulfoxide (Me_2SO) to be diluted next at 250 μM in double-distilled water before experiments. This preparation contains predominantly monomer and dimers with larger oligomers up to 6-mers (data not shown). These small Aβ oligomers, similar to small SDS-stable Aβ oligomers (29) isolated from AD brains (30), are the neurotoxic species, which are better correlated with synaptic loss in AD than plaques (31, 32). Aβ peptides, if used in the indicated concentrations in the presence of TGF-β1, did not significantly affect the viability of THP-1 cells when LDH release was measured. The effects of Me_2SO alone in the used range of concentrations were not detectable.

Cell Culture and Treatment for Preparation of Conditioned Media—THP-1 (a mononuclear cell line of human origin) and T98G (the human astroglial cell line) were obtained from ATCC (Manassas, VA) and cultured in RPMI 1640 medium (Sigma). THP-1 has been widely used as a model of human monocytes/macrophages or microglia, not only because of its functional and morphological similarities including its capacity to perform signal transduction pathways but also because of functional differences in the metabolisms of rodent and human microglial cells, as was described previously (33). Confluent cells suspended in serum-free RPMI 1640 media supplemented with glucose (0.5%) were seeded into 96-well culture plates ($\sim 1 \times 10^5$/well) and incubated for 2 h at 37 °C before experimental manipulation. The cells were then treated with TGF-β1 and/or Aβ peptides as indicated. To determine the effects of specific inhibition of TGF-β1-induced responses, cells were pretreated for 30 min with various chemical inhibitors at the concentrations specified followed by incubation with TGF-β1 and/or Aβ peptides. After incubation for the indicated periods, conditioned media were collected for subsequent analysis by gelatin zymography and cells were lysed for Western blot. To confirm TGF-β1-mediated MMP-2 production, TGF-β1 was preincubated with anti-TGF-β1-neutralizing antibody, anti-TNF-α-neutralizing antibody, or preimmune IgG for 30 min at room temperature at the indicated concentrations before the addition to the cell cultures. None of the drugs at the concentrations used affected cell viability.

Analyses of Gelatinolytic Zymography—The gelatinolytic activities in the cell-free supernatants, normalized for equal amounts of protein, were determined by zymography with gelatin according to previously published methods (33). Clear bands on the zymograms were quantitated by densitometric scanning to determine the intensity of MMP-2 activity. The arbitrary densitometric units were expressed or converted to a fold measurement of the response of the vehicle-treated controls for each individual experiment.

Smad7 Overexpression—THP-1 cells grown in 96-well plates were transiently transfected with Lipofectamine 2000 (Invitrogen) and with 0.2 μg/well of either Smad7 expression plasmid or control pcDNA3 (19). After 24 h of transfection, cells were starved for 2 h in serum-free RPMI 1640 medium and then treated with TGF-β1 as indicated. Western blot analysis and zymography were performed to assess Smad7 expression and MMP-2 gelatinolytic activities as described below.

Transient Transfection and Luciferase Reporter Assay—THP-1 cells grown in 96-well plates were transiently transfected with Lipofectamine 2000 and with 0.2 μg/well TGF-β-responsive luciferase reporter construct and plasmid p3TP-Lux containing TGF-β-inducible SBEs (34, 35). Also, 0.01 μg/well pRL-TK-*Renilla* was co-transfected to normalize for variations in transfection efficiency between assays (36, 37). After 4 h of transfection, the cells were treated with TGF-β1 and/or Aβ peptides in serum-free RPMI 1640 medium for 4 h at the indicated concentrations. In some experiments, to measure the effect of Smad7 depletion, cells were transfected with either synthetic siRNA for Smad7 or nonspecific control pool for 24 h, as described below, prior to transfection with p3TP-Lux. The effect of Smad7 overexpression was also analyzed by transfecting either Smad7 expression plasmid or control pcDNA3 for 24 h, as described above, prior to transfection with p3TP-Lux. Firefly and *Renilla* luciferase activities were measured in either duplicate or triplicate and normalized using a dual luciferase reporter

assay system (Promega, Madison, WI) as described previously (37). Luciferase activity values were normalized with respect to protein concentrations, and the results are presented as fold increases.

siRNA Studies—Synthetic siRNA for Smad7 and nonspecific control pool were purchased from Cellogenetics (Gaithersburg, MD), and transfection of the RNA oligonucleotide was performed using Lipofectamine 2000. THP-1 cells were treated with Lipofectamine 2000 (mock transfection), siRNA, or nonspecific RNA pool at the concentrations indicated. After 24 h of transfection, cells were starved for 2 h and then treated with TGF-β1 and/or Aβ peptides as indicated. Western blot analysis and zymography were performed to measure cellular Smad7 depletion and MMP-2 gelatinolytic activities as described above.

Western Blot Analysis—Cytoplasmic fractions were prepared as described previously (38), and whole cell lysates were prepared by protein extraction, using a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS supplemented with protease inhibitors and phosphatase inhibitors as described above. For Western blot analysis, equal amounts of protein (~30 μg) were subjected to reducing SDS-PAGE. After electroblotting and blocking, blots were then probed at room temperature for 3 h with the primary antibody and incubated for 1 h with the specific secondary antibody conjugated with horseradish peroxidase. Proteins were visualized using an ECL Western blotting detection system (Amersham Biosciences). After the antibodies were stripped by incubating the membranes with stripping buffer (62.5 mM Tris-HCl, pH 6.7, 2% SDS, 100 mM 2-mercaptoethanol) at 50 °C for 30 min, they were processed for re-labeling with β-actin monoclonal antibody (1:2000, Sigma).

Statistical Analysis—All of the values were expressed as means ± S.E. Student's *t* tests were used for unpaired results to evaluate differences between groups. Differences in *p* values of <0.05 were considered to be significant.

RESULTS

Induction of MMP-2 by TGF-β1—TGF-β1 is a pleiotropic inflammatory mediator with diverse immunomodulatory properties (39). TGF-β1 may affect monocyte chemotaxis and migration into tissues by enhancing the monocytic expression of MMPs. MMP-2 is released from the cells as inactive precursor (72 kDa of pro-MMP-2), which is activated by MT1-MMP (MMP-14) (40). To assess the potential involvement of MMP-2 in the Aβ modulation of TGF-β1 function, we examined TGF-β1-induced MMP-2 production in monocytic THP-1 cells because MMP-2 contains TGF-β-inducible SBE domains in its promoter regions (17, 26). MMP-2 activity was determined by gelatin zymography, and the specificity of MMP-2 protein was verified by Western blot analysis as a 68-kDa intermediate-active MMP-2 (Fig. 1, *E*), both of which were recently described (41). However, 62 kDa of full-active MMP-2 and inactive MMP-2 (72 kDa of pro-MMP-2) were not seen on the zymograms. The treatment of THP-1 cells with TGF-β1 at a concentration of 10 ng/ml induced MMP-2 expression in a time-dependent manner. MMP-2 levels began to increase at 12 h and reached peak 40 h after treatment (Fig. 1, *A*). This induction of MMP-2 by TGF-β1 in THP-1 also occurred in a dose-dependent manner (Fig. 1, *B*). Maximal induction of MMP-2 was found to occur at a concentration of 20 ng/ml TGF-β1. Increases in TGF-β1 concentration beyond this level had no further effect on MMP-2 levels (data not shown). In contrast, TGF-β1 treatment had only a minimal effect on MMP-9 activity in THP-1 cells. Similar results were obtained using TGF-β2 and TGF-β3. These results confirm the presence of the three functional TGF-β receptors (Fig. 1, *C*). This TGF-β-mediated MMP-2 production was clearly specific, because only anti-TGF-β1-neutralizing antibody was able to reverse the process (Fig. 1, *D*). Moreover, MMP-2 induction was consistently inhibited when *de novo* mRNA expression and protein synthesis were inhibited by actinomycin and cycloheximide, respectively, which indicates that TGF-β-mediated MMP-2 induction is dependent on both transcriptional and translational activities.

MMP-2 Induction by TGF-β1 Is Dependent on Intracellular Smad Signaling—To unravel the mechanism by which TGF-β1 induces MMP-2 expression in THP-1 cells, we investigated the results of blocking various signaling pathways of TGF-β1 on MMP-2 expression since TGF-β1 has been shown to activate several distinctive signaling pathways, such as Smad2, p38 MAPK, and Akt (also known as protein kinase B) (42, 43). However, LY294002 and worthmanin, inhibitors of phosphatidylinositol 3-kinase, an activator of Akt, evidenced only minimal effects on TGF-β1-mediated MMP-2 induction (Fig. 2, *A*). Likewise, SB202190, a specific inhibitor of p38 MAPK, and PD98059, known to selectively block the activity of MAPK kinase kinase (MEK1), an activator of ERKs, as well as an inhibitor of JNK MAPK (JNKI), also failed to abolish TGF-β1-induced MMP-2 production. On the evidence presented in pre-

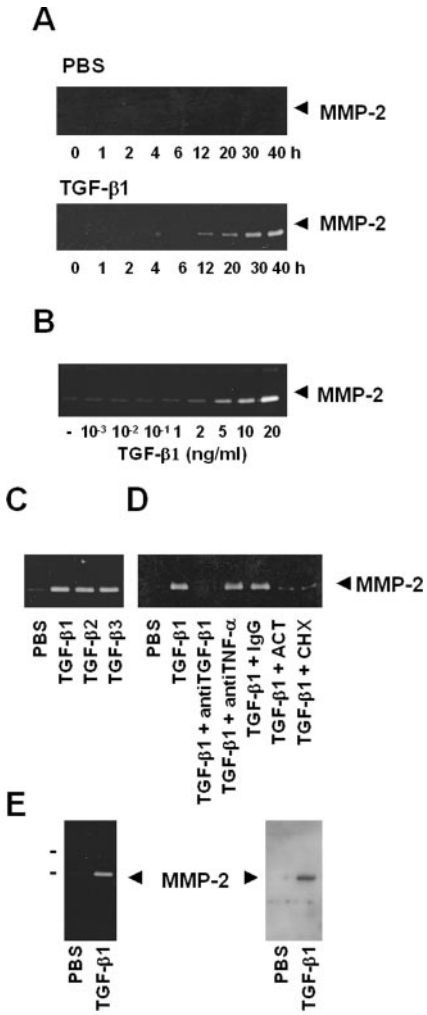

FIG. 1. **Effect of TGF-βs on the expression of MMP-2 in human monocytic THP-1 cells.** Zymographic analyses show that TGF-β1-induced MMP-2 activities in conditioned media in a time- and dose-dependent manner. THP-1 cells were incubated with either the same concentration of TGF-β1 (10 ng/ml) for various periods of time (*A*) or with increasing amounts of TGF-β1 for 30 h (*B*) in serum-free RPMI 1640 medium supplemented with glucose (0.5%). MMP-2 activities present in the conditioned media from THP-1 cells treated with TGF-β1, TGF-β2, or TGF-β3 (10 ng/ml each) were measured (*C*). Cells were pretreated with actinomycin (*ACT*, 50 nM) or cycloheximide (*CHX*, 1 μM) for 30 min followed by incubation in the absence or presence of TGF-β1 (10 ng/ml) for 30 h. TGF-β1 (10 ng/ml) was preincubated for 30 min at room temperature with anti-TGF-β1 antibody, anti-TNF-α antibody, or preimmune IgG (each 5 μg/ml) before addition to the cell cultures, at which time the samples were incubated for an additional 30 h (*D*). *E*, the immunoblot (*lane 2*) of the 68-kDa gelatinase A for verification of MMP-2 induced by TGF-β1. *Bars* on the *left side* indicate the locations of 88- and 68-kDa markers, and *arrowheads* indicate location of MMP-2, respectively. Results are representative of five independent experiments.

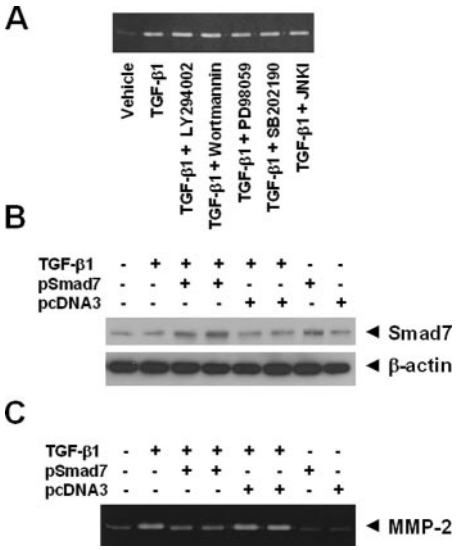

FIG. 2. **Effects of various PK inhibitors and Smad7 expression on TGF-β1-mediated MMP-2 release in THP-1 monocytic cells.** In *A*, pharmacological inhibition of different signal transduction pathways did not affect MMP-2 induction by TGF-β1. In *A*, THP-1 cells were pretreated with either various chemical inhibitors or vehicle (Me_2SO) for 30 min followed by incubation in either the absence or presence of TGF-β1 (10 ng/ml) for 30 h. Specific inhibitors for phosphatidylinositol 3-kinase (5 μM LY294002 and 10 nM wortmannin), MEK1 (10 μM PD98059), p38 MAPK (5 μM SB202190), and JNK (1 μM JNKI) were used, respectively. Zymographic analysis was performed as described in Fig. 1. Results are representative of four independent experiments. In *B*, THP-1 cells were transfected with inhibitory Smad7 expression vector or empty pcDNA3 vector for 24 h. Cells were then treated with TGF-β1 (10 ng/ml) for 4 h. Cytoplasmic fractions were blotted with antibodies against Smad7 or β-actin, respectively. In parallel, transfected cells, as described in *B*, were incubated for 30 h for zymographic analysis (*C*). Smad7 expression increased in Smad7-transfected THP-1 cells, concomitant with decreased MMP-2 activity. The data are representative of five independent analyses. Smad7 level in *B* was 1.0 (control), 1.0 (TGF-β1), 2.6 (TGF-β1/pSmad7), 3.3 (TGF-β1/pSmad7), 1.2 (TGF-β1/pcDNA3), 1.3 (TGF-β1/pcDNA3), 2.5 (pSmad7), and 1.2 (pcDNA3). In *C*, enzymatic activity of MMP-2 in zymogram was 1.0 (control), 3.0 (TGF-β1), 1.8 (TGF-β1/pSmad7), 1.8 (TGF-β1/pSmad7), 3.1 (TGF-β1/pcDNA3) and 3.1 (TGF-β1/pcDNA3), 0.8 (pSmad7), and 0.8 (pcDNA3).

vious papers (33, 37, 38, 44) where these inhibitors were used, the concentrations employed in the experiment should have been enough to inhibit their respective kinases without significantly affecting cell viability. To further delineate the potential involvement of Smad signaling in MMP-2 induction, we inhibited Smad signaling by overexpressing inhibitory Smad7. Because Smad7 competes with Smad-2 and Smad-3 to bind to the activated TGF-β type I receptor, an overexpression of Smad7 should result in the attenuation of Smad2/3 signaling (14–16). Interestingly, Smad7 overexpression in THP-1 cells significantly inhibited TGF-β1-induced MMP-2 expression, in contrast to its effects in vector-transfected cells (Fig. 2, *B* and *C*). These results are consistent with the notion that, in THP-1 cells, MMP-2 induction by TGF-β1 is primarily mediated through Smad signaling.

Aβ1–42 Inhibits TGF-β1-induced MMP-2 Production in THP-1 Cells—To date, the study on TGF-β1 has centered on its involvement in amyloid plaque metabolism. Because dysregulation of TGF-β1 function is also implicated in chronic inflammation, leading to neurodegeneration, it is important to uncover any synergistic or antagonistic modulation of TGF-β1-mediated effects by Aβ1–42 to generate effective therapeutic strategies involving TGF-β1 and Aβ. To this end, we examined the effect of Aβ1–42 on TGF-β1-induced MMP-2 production. Interestingly, Aβ1–42 treatment of THP-1 cells resulted in a decrease of TGF-β1-induced MMP-2 activity over the level seen

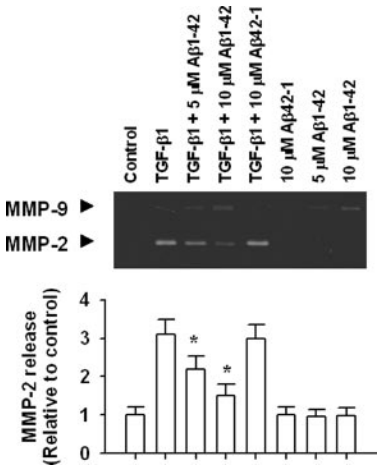

FIG. 3. **Aβ1–42 inhibits the TGF-β1-induced MMP-2 production in THP-1 cells.** THP-1 cells were incubated with TGF-β1 (10 ng/ml) in the presence or absence of Aβ1–42 or Aβ42–1 for 30 h at the indicated concentrations, and zymographic analysis was performed as described in Fig. 1. The densities of the bands on the zymograms were quantified by densitometry for MMP-2 enzymatic activity. The data are expressed as the means ± S.E. ($n = 5$). *, $p < 0.05$ *versus* TGF-β1 alone.

in TGF-β1-treated cells, whereas the reverse peptide, Aβ42–1, elicited only minimal effects under the same experimental conditions (Fig. 3). In contrast, Aβ1–42 at a concentration of 10 μM slightly induced MMP-9 expression in THP-1 cells, as was previously observed in our study (33). However, this Aβ1–42-mediated MMP-9 production was not significantly modulated by TGF-β1. These observations suggest that Aβ1–42 could act as a negative modulator of TGF-β1-induced production of MMP-2.

Time-dependent Aβ1–42 Effect on TGF-β1-inducible MMP-2: Effect of Pretreatment and Posttreatment with Aβ1–42 Relative to TGF-β1—Although the inhibitory effect of Aβ1–42 on TGF-β1-mediated MMP-2 production could be seen when Aβ1–42 was given concomitantly with TGF-β1 (as described above), we wondered whether Aβ1–42 was also effective if applied to cells before or after TGF-β1. As shown in Fig. 4, TGF-β1-induced MMP-2 release was maximally reduced when Aβ1–42 was applied 1 h before TGF-β1. However, posttreatment of Aβ1–42 after TGF-β1 still reduced TGF-β1 effect on MMP-2. Additionally, the inhibitory effect of Aβ1–42 was seen in human astroglial T98G cells. These observations together suggest that the functional antagonism between Aβ1–42 and TGF-β1 involves a direct activation of signaling cascade downstream of Aβ receptors rather than simple sequestration of TGF-β1 by Aβ1–42.

Aβ1–42 Inhibits TGF-β1-induced PAI-1 Production—An earlier study (27) demonstrated that PAI-1 containing SBEs and its expression is induced by TGF-β1. To further evidence the antagonistic modulation of TGF-β1-mediated effects by Aβ1–42, we examined the effect of Aβ1–42 on TGF-β1-induced PAI-1 production. Under the same experimental conditions described for Fig. 4, Aβ1–42 could suppress TGF-β1-induced PAI-1 production in THP-1 cells with maximal inhibitory effect by Aβ pretreatment (Fig. 5, *A*). This inhibitory effect of Aβ1–42 was also seen in T98G cells (Fig. 5, *B*). These observations clearly confirmed that Aβ1–42 could act as a negative modulator of TGF-β1-induced effects on MMP-2 and PAI-1 in both human monocytic and astroglial cells.

Aβ1–42 Induced Smad7 in THP-1 Cells—Given the critical role of Smad7 in negatively modulating TGF-β1 effects on MMP-2 (as described above), we reasoned that Aβ1–42 might be inducing Smad7 expression, thereby exerting its effects. Therefore, Aβ1–42 would have a detectable effect on the cellular levels of Smad7, acting as a negative regulator of the TGF-

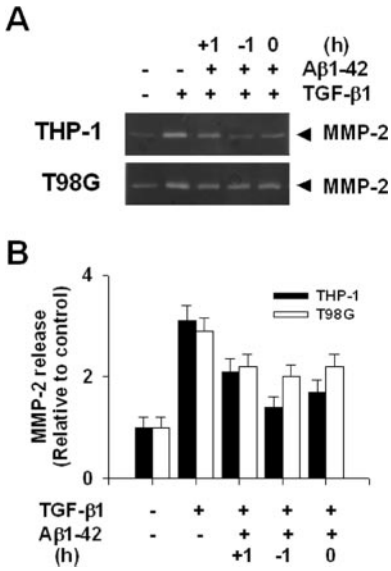

FIG. 4. **Effect of pretreatment and posttreatment of Aβ1–42 relative to TGF-β1 on MMP-2 production.** *A*, either human monocytic THP-1 (*upper panel*) cells or human astroglial T98G cells (*lower panel*) were incubated with TGF-β1 (10 ng/ml) for 30 h, and Aβ1–42 (10 μM) was given 1 h (*+1*) after TGF-β1, concomitantly with TGF-β1 (*0*), or cells were pretreated with Aβ1–42 for 1 h (*−1*) as indicated. Zymographic analysis of supernatants was performed as described in Fig. 1. *B*, the means ± S.E. ($n = 5$) of values obtained from densitometric analysis of all of the experiments are shown.

β/Smad signaling cascade. As shown in Fig. 6, Aβ1–42 consistently induced the cytoplasmic expression of Smad7 in both TGF-β1-treated and TGF-β1-untreated cells, whereas the reverse peptide had no significant effect (Fig. 6, *A*). Moreover, this Aβ1–42-mediated Smad7 induction was dose-dependent and inhibited when *de novo* mRNA expression and protein synthesis were inhibited, indicating that Aβ1–42-mediated Smad7 production is dependent on both transcriptional and translational activities (Fig. 6, *B*). Together, these results indicate that the molecular mechanism underlying Aβ1–42 suppression of TGF-β1-mediated effect on MMP-2 production in THP-1 cells appears to involve Smad7 expression.

Aβ1–42 Inhibits Transcriptional Activation of TGF-β1-inducible SBE-containing Promoters in THP-1 Cells—Given that SBEs are present in the promoter regions of MMP-2 (26), we further examined the effects of Aβ1–42 on the well characterized TGF-β1-inducible SBE-containing promoter-reporter construct, p3TP-Lux (34, 35), which was utilized in THP-1 cells to examine the induction of transcriptional activity by TGF-β1. As shown in Fig. 7, TGF-β1 effectively stimulated the transcriptional activation of luciferase expression. TGF-β1 treatment of transfected THP-1 cells elicited statistically significant inductions of SBE-directed luciferase activity, which were ~4-fold greater than the activity detected in the control cell cultures. Furthermore, Aβ1–42 inhibited the transcriptional activation of TGF-β1-inducible luciferase reporter activity by 2.2-fold, whereas the reverse peptide, Aβ42–1, exhibited little, if any, influence. Importantly, Smad7 transfection mimicked the inhibitory effect of Aβ1–42 on TGF-β1-inducible luciferase reporter activity. These results confirm a direct correlation between Aβ1–42-mediated Smad7 induction and the reduction of TGF-β1-inducible transcriptional activation.

Depletion of Smad7 with siRNA Reversed the Inhibitory Effects of Aβ1–42 on TGF-β1-induced MMP-2 Production—To further verify the inhibitory effect of Aβ1–42 on TGF-β1-induced MMP-2 activity via Smad7 induction, Smad7 depletion was performed using the siRNA method. Inhibition of Smad7 expression with Smad7-siRNA (Fig. 8, *A*) significantly reversed Aβ1–42-mediated suppression of the TGF-β1-induced MMP-2 production in a dose-dependent manner. The maximal reversal effect occurred at a final RNA concentration of 100 nM (Fig. 8, *B*). On the other hand, little effect was observed in the nonspecific control siRNA-transfected THP-1 cells. Furthermore, consistent with the rescue of the TGF-β1-mediated MMP-2 activity, the Aβ1–42 suppression of TGF-β1-inducible transcriptional reporter activity was restored in the Smad7-siRNA-treated cells (Fig. 8, *C*). These results clearly confirm that the inhibitory effect of Aβ1–42 on TGF-β1-induced MMP-2 production is mediated primarily via Smad7 expression.

DISCUSSION

The results presented in this paper verify a critical role of Aβ1–42 in suppressing TGF-β1-mediated MMP-2 induction via Smad7 expression in human monocytic THP-1 cells. Several lines of observation support this conclusion. First, overexpression of Smad7 significantly decreased TGF-β1-induced monocytic MMP-2 production in a time- and dose-dependent manner, whereas pharmacological inhibition of different signal transduction pathways resulted in only minimal effects. Second, Aβ1–42 treatment inhibited TGF-β1-induced MMP-2 production, concomitant with increased Smad7 expression. Third, Aβ1–42 markedly suppressed the TGF-β-inducible transactivation of transfected reporter constructs containing SBEs in TGF-β1-treated cells. Fourth, Smad7 overexpression mimicked the inhibitory effect of Aβ1–42 on TGF-β1-inducible luciferase reporter activity. Finally, the reduction of cellular levels of Smad7 via siRNA treatment reversed the Aβ1–42-mediated suppression of TGF-β-inducible transactivation reporter activity and TGF-β1-mediated MMP-2 production. Overall, these data point to the presence of negative modulation of functional TGF-β1-induced MMP-2 production by Aβ1–42 via Smad7 expression in human monocytic THP-1 cells. This antagonistic regulation of TGF-β1 by Aβ1–42 is further supported by our additional findings that Aβ1–42 could also reduce TGF-β1-mediated increase of PAI-1, another endogenous gene containing SBEs, and that these inhibitory effects of Aβ1–42 were also observed in human astroglial T98G cells.

Earlier examination of the promoter sequences of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) (26, 45) revealed the presence of a putative SBE, which corresponds to the CAGAC consensus site reported by Dennler's group (17). Furthermore, a previous study (46) also reported that both MMP-2 and MMP-9 were transcriptionally regulated by TGF-β through direct promoter activation. Results from the present study clearly demonstrate that transcription of the MMP-2 gene containing the three putative SBEs can be functionally activated by all three TGF-β isoforms, thus confirming both the functionality of the Smad-mediated TGF-β signaling pathway and the presence of the three TGF-β receptors in human monocytic THP-1 cells. This Smad-mediated MMP-2 production, in response to TGF-β1, is in good agreement with recent studies (47). However, the minimal effect on monocytic MMP-9 activity exhibited by TGF-βs in this study is not in good agreement with published reports (47–50) in which contrasting results are reported. Treatment with TGF-βs has been demonstrated to result in either down-regulation or up-regulation of MMP-9 production, possibly due to differences in cell types and/or experimental conditions.

On the other hand, PAI-1 is a serpin that suppresses fibrinolysis by inhibiting the activity of plasminogen activator (PA). Together with PA, PAI-1 is expressed in the central nervous system and may play a role in the regulation of PA activity. Given that PAI-1 has physiological functions for the survival of neurons other than its role as PA inhibitor (51–53), decreased PAI-1 production from astrocytes due to the negative regula-

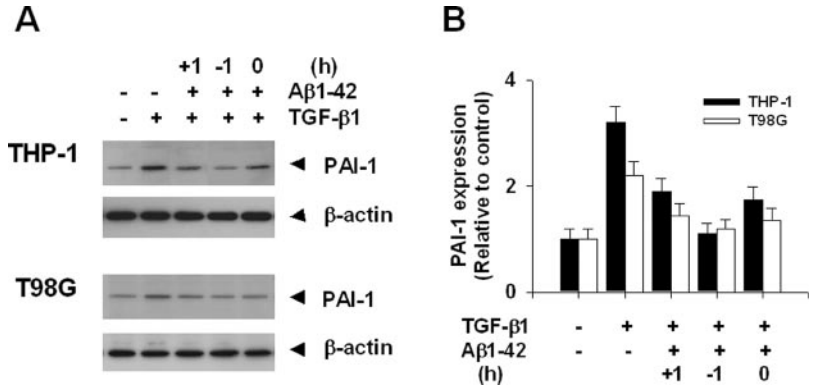

FIG. 5. **Aβ1–42 reduces the TGF-β1-induced PAI-1 production.** *A*, either human monocytic THP-1 (*upper panel*) cells or human astroglial T98G cells (*lower panel*) were treated as described in Fig. 4. Total lysates were analyzed for PAI-1 expression by immunoblotting with anti-PAI-1 antibody. Probing for β-actin was performed as for loading control. *B*, the means ± S.E. ($n = 5$) of values obtained from densitometric analysis of all of the experiments are shown.

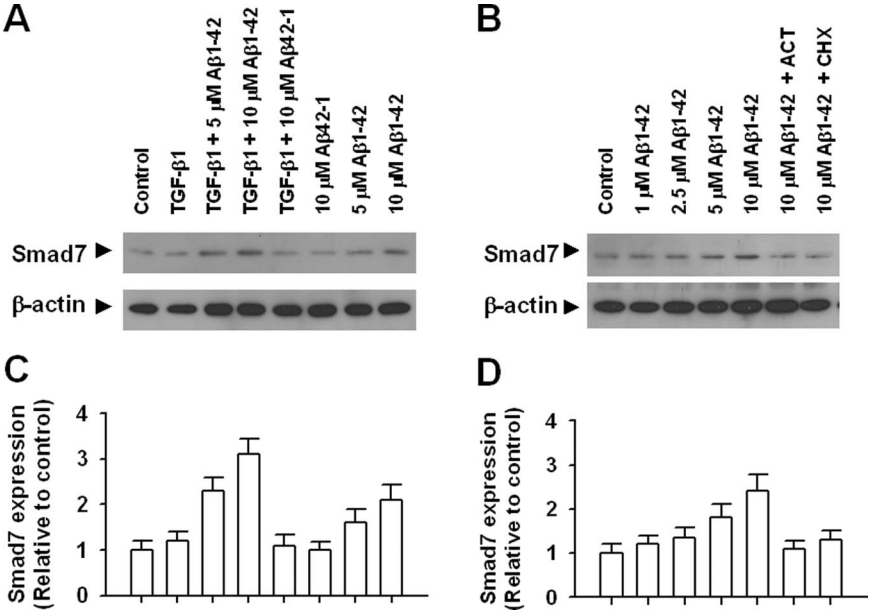

FIG. 6. **Aβ1–42 induces Smad7 in THP-1 cells.** *A*, THP-1 cells were incubated with TGF-β1 (10 ng/ml) in the presence or absence of Aβ1–42 or Aβ42–1 for 4 h at the indicated concentrations. *B*, cells were incubated with increasing amounts of Aβ1–42 for 4 h after pretreatment with actinomycin (*ACT*, 50 nM) or cycloheximide (*CHX*, 1 μM) for 30 min. Cytoplasmic fractions were analyzed by immunoblotting with anti-Smad7 antibody. Probing for β-actin was performed as for loading control. Data are representatives of five independent experiments. *C* and *D* represent the densitometric analysis of *A* and *B*, respectively ($n = 5$).

tion of TGF-β1-mediated effects by Aβ1–42, as seen in our study, might be detrimental to neurons and accelerate Aβ-induced neurodegeneration. It is also interesting to note that PA was shown to inhibit Aβ-induced neurotoxicity through rapid clearance of Aβ (54).

Interestingly, our results showed that overexpression of inhibitory Smad7 significantly decreased TGF-β1-induced MMP-2 production, suggesting that TGF-β-mediated Smad signaling is primarily responsible for MMP-2 production in THP-1 cells. In fact, the Smad pathway is not the sole one that transduces the TGF-β signal and, hence, differential activation of the varied TGF-β signaling pathways has been proposed as a mechanism for selectivity of TGF-β action in different cell types (42, 43). However, pharmacological inhibition of different signal transduction pathways, as shown in our study, did not significantly affect TGF-β1-induced MMP-2 production. This finding is in good contrast to MMP-9 production, which occurs via multiple integrated signaling pathways within the cell, as described previously (33). Furthermore, depletion of Smad7 with siRNA enhanced TGF-β1-inducible luciferase reporter activity; concomitant with increased MMP-2 activity in TGF-β1-treated cells. Thus, our observations strongly support the notion that TGF-β-mediated MMP-2 production primarily occurs through the activation of the Smad pathway, which is in turn inhibited by the overexpression of Smad7, a negative regulator of the TGF-β/Smad signaling cascade.

To our knowledge, this study constitutes the first report that Aβ1–42 and TGF-β1 have opposite effects on MMP-2 production. Smad7-siRNA data from the present study support the

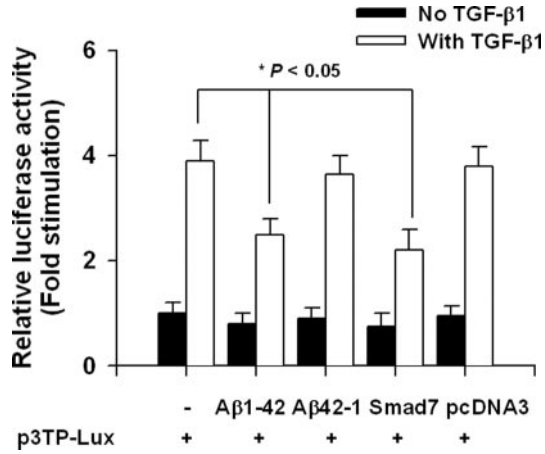

FIG. 7. **Aβ1-42 suppresses transcriptional activation of a TGF-β1-inducible luciferase reporter plasmid in THP-1 cells.** THP-1 cells were co-transfected with the TGF-β-responsive p3TP-Lux and with pRL-TK-*Renilla* for 4 h and then exposed to TGF-β1 (10 ng/ml) in the presence or absence of Aβ1–42 (5 μM) or Aβ42–1 (5 μM) as described under "Experimental Procedures." To confirm the effect of Smad7 overexpression, cells were pretreated with either Smad7 expression plasmid or control pcDNA3 for 24 h prior to transfection with p3TP-Lux. Firefly and *Renilla* luciferase activities were measured and normalized using a dual luciferase reporter assay system. Aβ1–42 consistently suppressed TGF-β1-inducible luciferase reporter activity, and Smad7 mimicked the Aβ1–42-mediated effect. Luciferase data are expressed as the means ± S.E. of two measurements from a representative experiment that was repeated five times. *, $p < 0.05$, TGF-β1-treated cells *versus* Aβ1–42 plus TGF-β1-treated cells ($n = 5$).

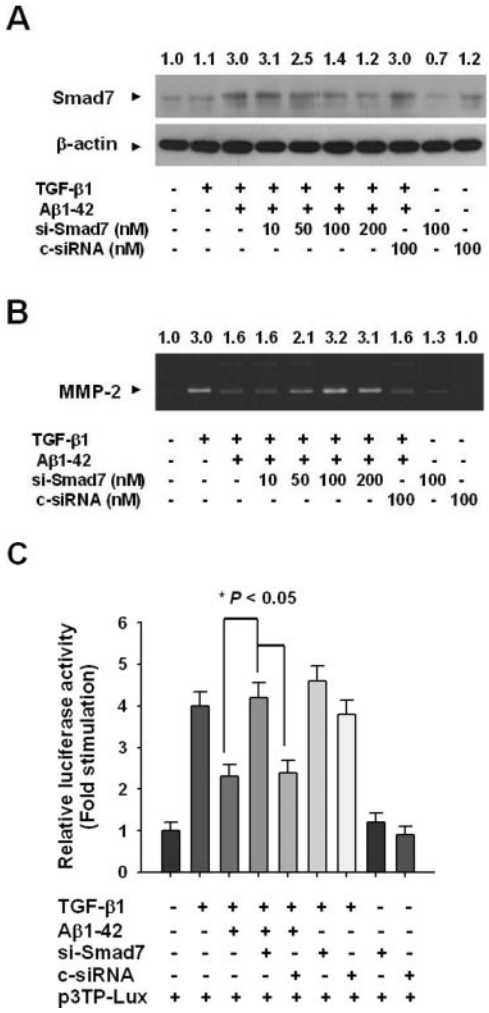

FIG. 8. **Smad7-siRNA reversed the inhibitory effect of Aβ1–42 on TGF-β1-enhanced MMP-2 production and TGF-β1-mediated reporter activity.** THP-1 cells were transfected with increasing concentrations of Smad7-small interference RNA or the control-small interference RNA (*c-siRNA*) pool for 24 h and then were treated with TGF-β1 (10 ng/ml) in the presence or absence of Aβ1–42 (5 μM) for 4 h. Cytoplasmic fractions were then immunoblotted as described in Fig. 2. In *A*, inhibition of Smad7 expression was observed with Smad7-siRNA transfection (Fig. 6, *A*). Relative levels of Smad7 expression, normalized to β-actin, are shown on *top* of the *panel*. In parallel, transfected cells as described in *A* were incubated for 30 h for purposes of zymographic analysis in *B*. Relative levels of MMP-2 release were shown on *top* of the *panel*. Only Smad7-siRNA transfection restored TGF-β1-mediated MMP-2 induction in a dose-dependent manner. Results are representative of five independent experiments. In *C*, Smad7-siRNA or c-siRNA-transfected cells were further transfected with the TGF-β-responsive p3TP-Lux and with pRL-TK-*Renilla* for 4 h and then exposed to TGF-β1 (10 ng/ml) in the presence or absence of Aβ1–42 (5 μM) or Aβ42–1 (5 μM) for 4 h. Firefly and *Renilla* luciferase activities were measured as described in Fig. 5. Only Smad7-siRNA transfection restored TGF-β1-mediated reporter activity of THP-1 cells in the presence of Aβ1–42. The data are expressed as the means ± S.E. *, $p < 0.05$, Smad7-siRNA-transfected cells in response to Aβ1–42 plus TGF-β1 *versus* c-siRNA-transfected cells in response to Aβ1–42 plus TGF-β1 or mock-transfected cells in response to Aβ1–42 plus TGF-β1 ($n = 5$).

notion that the negative modulation by Aβ1–42 of a functional Smad-mediated TGF-β signaling system primarily involves Smad7 induction. This conclusion is further substantiated by our observation that Smad7 overexpression mimicked the inhibitory effect of Aβ1–42 on TGF-β1-induced MMP-2 production. Furthermore, Aβ1–42 consistently suppressed the TGF-β1-mediated activation of p3TP-Lux, a well characterized TGF-β-responsive construct, which contains SBEs capable of conferring TGF-β responsiveness on a luciferase reporter. This construct has been used extensively to characterize transcriptional responsiveness to both TGF-β1 and Smads in a variety of primary cells in culture and established cell lines (17, 34, 47). We observed that Smad7 depletion by the siRNA method significantly ameliorated or reversed the Aβ1–42-mediated suppression of both TGF-β1-inducible luciferase reporter activity and TGF-β1-mediated MMP-2 induction. Conversely, Smad7 overexpression mimicked the inhibitory effect of Aβ1–42 on TGF-β1-inducible luciferase reporter activity. These observations further confirmed the inhibitory effect of Aβ1–42 on TGF-β1-mediated MMP-2 production via Smad7 expression, which results in the inhibition of Smad2/3 phosphorylation and Smad complex nuclear translocation (14–16). Further studies are necessary regarding which receptor(s) and/or signal transduction pathways are involved in Aβ-mediated Smad7 expression. This is because the Smad signaling system through cross-talk with other signal transduction pathways appears to be an important integrator of multiple signaling pathways within the cell (42, 43).

Although the precise role of TGF-β in the neuropathology of AD remains unknown, earlier studies (7, 12) have detected TGF-β1 immunoreactivity in senile plaques and neurofibrillary tangle-bearing neurons in the brains of AD patients. Elevated TGF-β1 levels were found in the cerebrospinal fluid and serum of patients with AD than in control patients without dementia (55), and the expression of TGF-β receptors I and II was increased in the reactive glia of AD brains (56). However, a more recent study has demonstrated that decreased TGF-β1 plasma levels are a potential biomarker for AD (57). Moreover, TGF-β has recently been shown to play a protective role, creating an environment favorable for the survival of the cell in the face of death-inducing insults including Aβ, hypoxia/ischemia, glutamate excitotoxicity, oxidative damage, and the human immunodeficiency virus (58). Consistent with this notion, chronic neuroinflammation could represent the result of a failure in the anti-inflammatory mechanism, in which a significant reduction in the biological function of immunosuppressive cytokines, such as TGF-β1, results in the defective suppression of the inflammatory process and the unrestrained production of proinflammatory cytokines such as TNF-α, thus leading to enhanced neurodegeneration. In this context, the aberrant antagonistic activities of Aβ1–42 against the physiological actions of TGF-β as reported in this study would be detrimental and may even play an important role in the pathogenesis of AD. Nevertheless, recent studies suggest the possibility of an indirect neurotoxic role for TGF-β in terms of its promoting effects with regard to perivascular inflammation and/or amyloid deposition (5, 6, 59). These deleterious effects when bolstered by a genetic polymorphism of the TGF-β1 gene may be associated with higher risks of AD (9). Other possible synergistic risk factors include a direct receptor-independent interaction between TGF-β and Aβ, which enhances Aβ oligomerization, leading to potentiation of the neurotoxic effects of Aβ (60), although an earlier study (61) proposes the TGF-β receptor as one possible cellular Aβ interaction site. Maximal inhibitory effect resulting from Aβ1–42 pretreatment, as shown in our study, suggested that the functional antagonism between Aβ1–42 and TGF-β1 appears to involve a direct activation of signaling cascade downstream of Aβ receptors. However, the possibility of simple sequestration of TGF-β1 due to a direct interaction between TGF-β1 and Aβ1–42 cannot be completely ruled out. Further studies with a dominant active TGF-β receptor will clarify this point.

Contrasting results regarding the involvement of MMPs in AD are also present in the literature. Previous studies indicated that MMPs are involved in Aβ degradation (24, 25) and

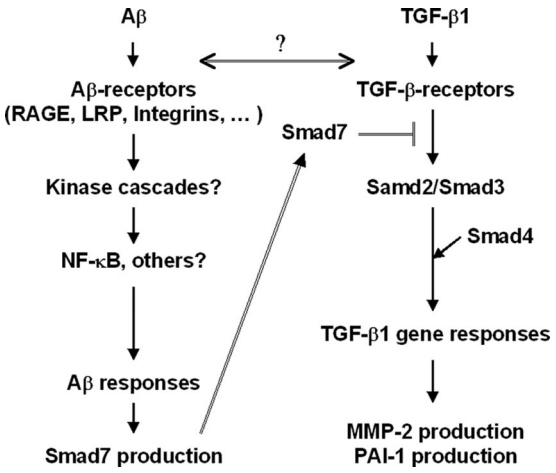

FIG. 9. **Scheme of the proposed role of Aβ1–42 on TGF-β1-mediated response in human monocytic THP-1 cells.** A schematic diagram proposing how Aβ1–42 negatively regulates TGF-β1-mediated MMP-2 production via Smad7 expression in human monocytic THP-1 cells. This scheme presents a molecular mechanism by which Aβ1–42 via Smad7 expression negatively regulates TGF-β1-mediated MMP-2 production. In the absence of Aβ1–42, TGF-β1 induces TGF-β1-mediated transcriptional activity, which in turn induces MMP-2 production. When Aβ1–42 is present, it will activate, through unknown receptors, a series of downstream secondary signaling pathways associated with enhanced expression of the inhibitory Smad7, which inhibits SBE-dependent transcription of target genes such as *MMP-2* or *PAI-1*. As a consequence of this Aβ1–42 action, TGF-β1-mediated production of MMP-2 or PAI-1 will decrease. *RAGE*, receptor for advanced glycosylation end products; *LRP*, low-density lipoprotein-related receptor protein.

that increased MMP expression levels, in particular MMP-9, have been observed in plasma and in proximity to extracellular amyloid plaques in brain tissues from AD patients, whereas plasma levels of MMP-2 were found to be unchanged (21, 22). Recent study has shown a functional polymorphism in the gene for MMP-9 (62), which supports a critical role for MMP-9 in the pathophysiology of AD. Likewise, a recent study has also demonstrated that Dutch variant Aβ, possibly associated with CAA, a major pathological feature of AD and related disorders (8), stimulates the expression and activation of MMP-2. This study also asserts that this process may contribute to loss of vessel wall integrity in CAA, resulting in hemorrhagic stroke (23). Taken together, these findings suggest that MMPs may influence the stability of extracellular matrix or other MMP substrates and thus may play a role in the neurotrophic/neurotoxic events associated with AD.

In conclusion, as both glial cells and monocytes have been observed to express TGF-β and to respond to TGF-β stimulation, our findings support the notion that specific concentrations and combinations of Aβ1–42 and TGF-β1, encountered during the immune response at inflammatory foci, provide a balanced system for regulating MMP-2-mediated activities such as extracellular matrix degradation and PAI-1 activities closely associated with neuroprotection. Therefore, dysfunctions in either TGF-β1 or Aβ1–42 might result in improper MMP-2 and PAI-1 activities, which could result in chronic inflammation followed by secondary neuronal cell death leading to AD. Overall, our results prompted us to propose a molecular mechanism by which Aβ1–42 could negatively modulate TGF-β-mediated MMP-2 production via Smad7 expression as depicted in Fig. 9. However, the *in vivo* relevance of this TGF-β1 inhibitory role of Aβ1–42 in AD pathology remains unknown.

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Eun Ok Lee, Jihee Lee Kang and Young Hae Chong

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