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Effects of the β-Amyloid and Carboxyl-terminal Fragment of Alzheimer's Amyloid Precursor Protein on the Production of the Tumor Necrosis Factor-α and Matrix Metalloproteinase-9 by Human Monocytic THP-1*

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Young Hae Chong‡§¶, Ji Hye Sung‡, Soon Ah Shin‡, Jang-Hyun Chung‖, and Yoo-Hun Suh‡‡**

*From the Departments of ‡Microbiology and ‖Ophthalmology, College of Medicine, Division of Molecular Biology and §Neuroscience, Medical Research Center, Ewha Womans University, 911-1, Mok-6-dong, Yangcheonku, Seoul, 158-056, Korea and the **Department of Pharmacology, College of Medicine, National Creative Research Initiative Center for Alzheimer's Dementia and Neuroscience Research Institute, MRC, Seoul National University, Seoul 110-799, South Korea*

To explore the direct role of β-amyloid (Aβ) and carboxyl-terminal fragments of amyloid precursor protein in the inflammatory processes possibly linked to neurodegeneration associated with Alzheimer's disease, the effects of the 105-amino acid carboxyl-terminal fragment (CT_{105}) of amyloid precursor protein on the production of tumor necrosis factor-α (TNF-α) and matrix metalloproteinase-9 (MMP-9) were examined in a human monocytic THP-1 cell line and compared with that of Aβ. CT_{105} elicited a marked increase in TNF-α and MMP-9 production in the presence of interferon-γ in a dose- and time-dependent manner. Similar patterns were obtained with Aβ despite its low magnitude of induction. Autocrine TNF-α is likely to be a main mediator of the induction of MMP-9 because the neutralizing antibody to TNF-α inhibits MMP-9 production. Genistein, a specific inhibitor of tyrosine kinase, dramatically diminished both TNF-α secretion and subsequent MMP-9 release in response to CT_{105} or Aβ. Furthermore, PD98059 and SB202190, specific inhibitors of ERK or p38 MAPK respectively, efficiently suppressed CT_{105}-induced effects whereas only PD98059 was effective at reducing Aβ-induced effects. Our results suggest that CT_{105} in combination with interferon-γ might serve as a more potent activator than Aβ in triggering inflammatory processes and that both tyrosine kinase and MAPK signaling pathways may represent potential therapeutic targets for the control of Alzheimer's disease progression.

The Alzheimer's disease (AD)[1] brain is characterized by selective neuronal loss, neurofibrillary tangles, and abundant extracellular deposits of insoluble β-amyloid protein (Aβ), which is a primary constituent of senile plaque (1). Several mutations of amyloid precursor protein (APP) around the Aβ domain in certain types of early onset familial AD support its causal role in the pathogenesis of AD, and extensive studies have implicated Aβ in neurodegeneration (2, 3). An earlier report suggests that Aβ, a 39–43 amino acid product derived from APP, may not be the sole active component involved in the pathogenesis of AD (4). Therefore, the potential effects of other cleavage products of APP need to be explored.

A number of studies have reported that Aβ-bearing carboxyl-terminal fragments (CTs) have been found not only in senile plaques, paired helical filaments, and brain microvessels of AD patients but also in the white matter of Down's syndrome (DS) brains (5–7). Evidence for correlating CTs with neurodegeneration has come from cell transplantation models, transgenic mice, and the investigation of postmortem brains (8–11). Furthermore, a recombinant carboxyl-terminal fragment bearing the full-length Aβ (CT_{105}) elicited greater neurotoxic potency than Aβ on cultured neuronal cells and on memory deficiencies and neuropathological changes in mice (12, 13). Meanwhile, a recent study (14) reported that CT had a proapototic effect on N2a cells and that its cytotoxic properties might be entirely caused by the generation and release of CT_{31}, which appears to amplify the cell death paradigm. These findings together implicate that CT itself may be an alternative contributing factor to the neurodegeneration processes *in vivo*.

Early association of activated microglial cells and reactive astrocytes in senile plaques and the appearance of inflammatory markers indicate a state of chronic inflammation in AD. The neuropathological significance of inflammatory response is strongly supported by multiple epidemiological studies demonstrating that patients taking anti-inflammatory drugs have a decreased risk of developing AD (15). Indeed, patients with AD exhibited increased levels of proinflammatory cytokines that have increased expression on activated microglia in the vicinity of senile plaque (16). In particular, tumor necrosis factor-α (TNF-α) has been implicated as a potent neurotoxic agent that was elevated in brain tissue with plaques and/or the cerebro-

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¶ To whom correspondence may be addressed: Dept. of Microbiology, College of Medicine, Ewha Womans University, 911-1, Mok-6-dong, Yangcheonku, Seoul, Korea, 158-056. Tel.: 822-02-650-5739; Fax: 822-653-8891; E-mail: younghae@mm.ewha.ac.kr.

‡‡ To whom correspondence may be addressed: Dept. of Pharmacology, College of Medicine, National Creative Research Initiative Center for Alzheimer's Dementia, Seoul National University, Seoul, 110-799, South Korea. Tel.: 822-740-8285; Fax: 822-740-8285; E-mail: yhsuh@plaza.snu.ac.

[1] The abbreviations used are: AD, Alzheimer's disease; Aβ, β-amyloid; APP, amyloid precursor protein; CTs, carboxyl-terminal fragments; CT_{105}, the 105 amino acid carboxyl-terminal fragment; TNF-α, tumor necrosis factor-α; MMP-9, matrix metalloproteinase 9; IFN-γ, interferon-γ; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcription-polymerase chain reaction; TK, tyrosine kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAP kinase-ERK kinase; bp, base pair(s); PBS, phosphate-buffered saline.

spinal fluid of AD patients (17, 18). This immune-mediated pathophysiology is further evidenced by recent data reporting the elevated level of MMP-9 activity capable of degrading the extracellular matrix in AD hippocampus (19). Several *in vitro* studies using either rodent microglia or human macrophage/monocytes have demonstrated the capacity of Aβ to activate these cells in order to generate inflammatory mediators (20, 21). However, less information is available on the ability of CTs to induce the inflammatory components, and the exact molecular mechanisms involved in CT- or Aβ-specific inflammatory responses have not been fully identified, though these are currently receiving attention as key therapeutic targets.

The present study therefore aims to investigate the critical role of Aβ-bearing CTs and the underlying molecular mechanisms in inducing proinflammatory responses possibly linked to the chronic AD neuropathology. The capacity of CT_{105} to induce TNF-α and MMP-9 in a human monocytic THP-1 cell line as a model for microglia (23, 24) was specifically studied and compared with the values obtained using Aβ peptides. The effects of the combined application of CT_{105} and interferon-γ (IFN-γ) were measured because of recent reports showing the synergistic effect of the combination on microglia activation in response to Aβ (20, 25) and the increased production of IFN-γ from the immune cells of AD patients (26). A possible association between TNF-α production and subsequent MMP-9 release was also analyzed. In addition, specific inhibitors of various signal cascades were tested to identify the molecular mechanisms mediating CT specific proinflammatory responses compared with Aβ.

EXPERIMENTAL PROCEDURES

Preparation of CT_{105} and Aβ Peptides—Recombinant CT_{105} peptide was synthesized and purified as previously detailed (27). CT_{105} peptide was purified by a combination of urea solubilization and ion exchange chromatography and then subjected to dialysis against 10 mM Tris-HCl (pH 7.4) followed by lyophilization. Previous protein conformational studies using circular dichroism and immunoblot analysis confirmed that the CT_{105} peptide has the β-sheet structure, which can induce self-aggregates similar to Aβ derived from AD brains (28). Protein concentration was determined with bicinchoninic acid using bovine serum albumin as a standard. Aβ-(1–42), Aβ-(1–40), and Aβ-(40–1) were purchased from US peptide (Fullerton, CA) and Aβ-(12–28) and Aβ-(25–35) from Sigma, respectively. As previously described (12, 29), peptides were dissolved in sterile dH_2O at 2 mg/ml and aged by incubation at 37 °C for 6 days, which caused the aggregation states of CT_{105} or Aβ mimic to monomeric, dimeric, and trimeric components from neuritic and vascular amyloids of AD brain (30). TNF-α and anti-TNF-α antibodies were purchased from Upstate Biotechnology (Lake Placid, NY) and R & D (Minneapolis, MN), respectively. IFN-γ and other chemicals including genistein were purchased from Sigma. PD98059 and SB202190 were obtained from Calbiochem (La Jolla, CA).

Cell Culture and Treatment for Preparation of Conditioned Medium—The human monocytic cell line THP-1 used was obtained from ATCC (Manassas, VA) and maintained in RPMI 1640 containing 10% heat-inactivated fetal calf serum as described previously (31). THP-1, a mononuclear cell line of human origin, 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 activate signal transduction pathways (23, 24), but also because of functional differences in the metabolism of rodent and human microglial cells (32). Confluent THP-1 cells suspended in serum-free RPMI 1640 medium supplemented with glucose (0.5%) were seeded into 24-well culture plates (~5×10^5/well) and incubated for 2 h at 37 °C before stimulation. The cells were stimulated by the addition of CT_{105} or Aβ peptides for the indicated times in the presence or absence of INF-γ. To determine the effects of specific enzyme inhibition of CT_{105}- or Aβ-induced responses, cells were pretreated with various concentrations of the protein kinase inhibitors, genistein, PD98059, or SB21090 for 20 min before stimulation. After incubation with the inhibitors for the indicated periods, conditioned medium was collected for subsequent analysis. In the control experiment, a recombinant TNF-α was preincubated with anti-TNF-α neutralizing antibodies for 2 h at 37 °C with the indicated concentration before addition to the cell cultures.

Measurement of TNF-α Levels by an Enzyme-linked Immunosorbent Assay (ELISA)—The concentration of TNF-α in the cell culture medium was measured by ELISA using monoclonal antibodies and the procedure recommended by the supplier (PharMingen, San Diego, CA). A standard curve using recombinant human TNF-α was set up for the ELISA according to the manufacturer's instructions, and the levels of secreted TNF-α were expressed as pg/ml/10^6 cells.

Measurement of TNF-α mRNA Levels by RT-PCR—Total RNA was isolated with the RNAzol B reagent (Tel-Test) as recommended by the manufacturer. cDNA synthesis and RT-PCR were conducted to detect expression of human TNF-α and β-actin mRNAs as previously described (29). 2 μg of total RNA from unstimulated and CT_{105}-stimulated and THP-1 cells in the presence of IFN-γ was reverse transcribed using oligo dTs (Life Technologies, Inc.) and 4 μl of cDNA was amplified with specific primers. PCR primers were designed based on the published sequences for human TNF-α (33) and human β-actin (34) as follows: (*a*) human TNF-α: sense, 5′-CAGAGGGAAGAGTCCCCCAG-3′; antisense, 5′-CCTTGGTCTGGTAGGAGACG-3′; probe, 5′-GGCGTGGAGCTGAGAGATAA-3′; (*b*) human β-actin: sense, 5′-GACAGGATGCAGAAGGAGAT-3′; antisense, 5′-CTAGAAGCATTTGCGGTGGA-3′; probe, 5′-TACTCCGTGTGATCGGCGG-3′. Semiquantitative PCR amplification was carried out at 94, 60, and 72 °C for 1, 0.5, and 1 min, respectively, for 30 cycles (for which there was a linear amplification of each product) using a Perkin-Elmer 9600 Thermal Cycler (Cetus). The expected sizes for the amplified fragments are 323 bp for TNF-α and 200 bp for β-actin. Nonradioactive Southern blot hybridization with an internal fluorescein-labeled probe was performed as a specificity control according to the instruction of the manufacturer (ECL, Amersham Pharmacia Biotech). To normalize the values of human TNF-α mRNA levels, human β-actin was amplified in parallel tubes. A portion (20 μl) of the PCR mixture was size separated in 2% agarose gels. The gel was stained with ethidium bromide and photographed. Signals on the negative (Polaroid 665 film) were quantified by densitometric scanning using UltroScan XL laser densitometer (LKB, Model 2222–020) to determine the ratio of intensity of TNF-α *versus* β-actin amplification products.

Zymographic Analysis—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 (35). The clear bands on the zymograms were photographed as negatives (Polaroid 665 film), and the signals were quantified by densitometric scanning using UltroScan XL laser densitometer to determine the intensity of the MMP activity. The arbitrary densitometric units were expressed or converted to a fold of the response of the PBS-treated controls for each individual experiment.

Western Blot Analysis—The proteins in conditioned medium were separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. The blots were blocked by incubation with 5% nonfat dry milk in Tris-buffered saline containing 0.15% Tween 20 for 2 h. The blots were then probed for 2 h with mouse monoclonal IgG antibodies specific for MMP-9 (Oncogene Science, Cambridge, MA; diluted 1:1000) followed by incubation for 1 h with a goat anti-mouse IgG conjugated with horseradish peroxidase (diluted 1:3000; Amersham Pharmacia Biotech). The proteins were visualized using an enhanced ECL Western blotting detection system.

Data Analysis—Data are expressed as the mean ± S.D. and were analyzed by two-tailed Student's *t* test for unpaired observations and analysis of variance in order to study the relationship between the different variables. Values of $p < 0.05$ were considered to be significant.

RESULTS

Stimulation of TNF-α Production by CT_{105} or Aβ—To test whether the interaction of CT_{105} with human monocytes could induce the production of proinflammatory and potentially cytotoxic mediators, we measured the levels of secreted TNF-α from THP-1 stimulated with CT_{105}, Aβ-(1–40), Aβ-(1–42) or its subfragments for comparison. As shown in Fig. 1*A*, CT_{105} at a concentration of 100 nM produced a small effect on the levels of secreted TNF-α after a 20-h incubation (an increase from about 2 to 11 pg/ml). However, IFN-γ markedly potentiated the accumulation of TNF-α induced by CT_{105} (an increase from about 11 to 83 pg/ml). The effect of IFN-γ was somewhat lower when the cells were exposed to Aβ-(1–40) at 10 μM concentration, but the levels of TNF-α were considerably higher than the amounts observed by peptide alone (an increase from about 3.4 to 7.2 pg/ml). The effect of Aβ-(1–42) closely mimicked that of Aβ-(1–

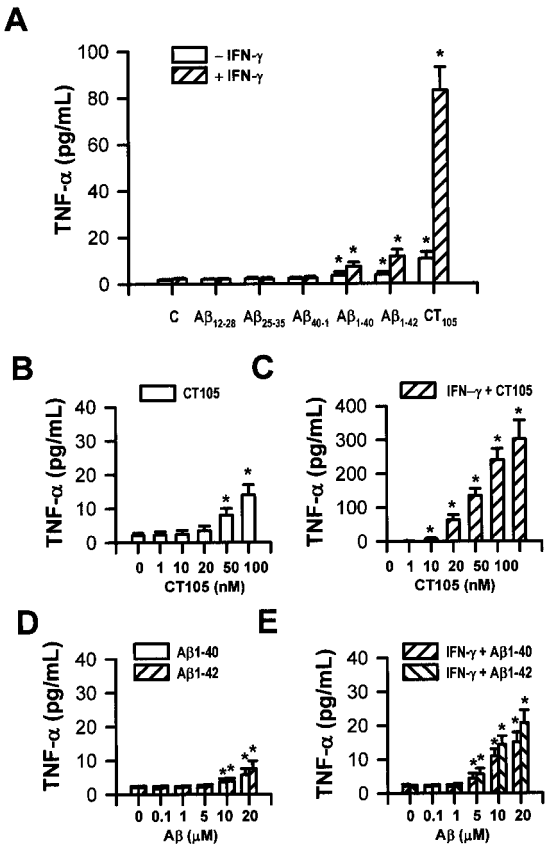

FIG. 1. **Effects of CT_{105} or Aβ peptides on TNF-α production.** In *A*, human monocytic THP-1 cells were incubated in the presence or absence of peptides, CT_{105} (100 nM), Aβ-(1–42) (10 μM), Aβ-(1–40) (10 μM), Aβ-(40–1) (10 μM), Aβ-(12–28) (10 μM), Aβ-(25–35) (10 μM) and/or IFN-γ (50 ng/ml). After a 20-h incubation in serum-free RPMI 1640 medium supplemented with glucose (0.5%), the concentration of TNF-α in the culture supernatants was measured using a sandwich ELISA kit and expressed in pg/ml/10^6 cells. Dose response of TNF-α production by CT_{105}, Aβ-(1–40), or Aβ-(1–42) was also examined by incubation with increasing concentrations of CT_{105} (*B*), Aβ-(1–40) or Aβ-(1–42) (*D*) alone and CT_{105} (*C*), Aβ-(1–40) or Aβ-(1–42) (*E*) in combination with IFN-γ (50 ng/ml) as indicated for 44 h. Data are mean ± S.D. ($n = 5$). *, $p < 0.05$, significantly different from the appropriate untreated cells.

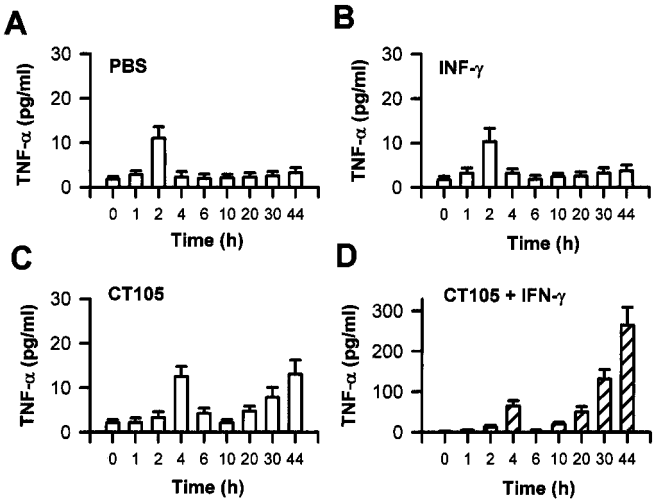

FIG. 2. **Time course of TNF-α production in response to CT_{105}.** THP-1 cells received PBS (*A*), IFN-γ at 50 ng/ml (*B*) 50 nM CT_{105} alone (*C*), or 50 nM CT_{105} plus 50 ng/ml of IFN-γ (*D*), and were incubated for indicated times in serum-free RPMI 1640 medium supplemented with glucose (0.5%). The levels of secreted TNF-α were measured and expressed as described in the legend to Fig. 1. Data are mean ± S.D. ($n = 5$).

40) despite a slightly higher magnitude of induction (an increase from about 3.8 to 11.7 pg/ml). There was no detectable TNF-α in the medium from untreated THP-1 cells, and IFN-γ on its own did not significantly increase TNF-α secretion. The specificity of the effects of CT_{105}, Aβ-(1–40) and Aβ-(1–42) was demonstrated by the fact that Aβ subfragments such as Aβ-(12–28) or Aβ-(25–35), and the reverse peptide Aβ-(40–1) also failed to induce TNF-α secretion under the same experimental conditions.

Dose- and Time-dependent Effects on TNF-α Secretion by CT_{105} or Aβ—The dose response was evident despite the small increases with peptide alone after a 44-h incubation (Fig. 1, *B* and *D*). A significant increase of TNF-α release was obtained as low as 10 nM CT_{105} or at 5 μM Aβ-(1–40) or Aβ-(1–42) in the presence of IFN-γ at 50 ng/ml, and further increases were observed at higher concentrations (Fig. 1, *C* and *E*). Moreover, IFN-γ resulted in a dose-dependent stimulatory effect (active at concentrations as low as 10 ng/ml) and the degree of stimulation was greater at higher IFN-γ concentrations (data not shown). In time course experiments over a 44-h incubation, TNF-α rapidly accumulated at low levels in the cell culture supernatant by 2 h and returned to basal levels in both untreated and only IFN-γ-treated THP-1 cells (Fig. 2, *A* and *B*). However, stimulation with CT_{105} led to a rapid TNF-α secretion as seen in the control at 4 h, and the second phase of TNF-α secretion was induced at 20 h and increased steadily thereafter (Fig. 2*C*). It is noteworthy that IFN-γ markedly enhanced this biphasic pattern, starting the second phase of CT_{105}-induced TNF-α secretion at 10 h (Fig. 2*D*). Similar patterns were observed with Aβ-(1–40) or Aβ-(1–42) despite the low magnitude of induction as seen in Fig. 1 (data not shown).

Transcriptional Control of TNF-α Production by CT_{105}—TNF-α synthesis is controlled at several levels. Whereas transcriptional, translational, and post-translational mechanisms play important roles, TNF-α transcription appears to be the primary regulatory site. To study the mechanism of action of CT_{105} on THP-1 monocytes, mRNA levels of TNF-α were examined by RT-PCR. As shown in Fig. 3 (*B* and *D*), CT_{105} in combination with IFN-γ increased TNF-α mRNA levels in a near parallel fashion compared with their stimulation of TNF-α secretion (Fig. 2*D*). Furthermore, this biphasic transcription pattern preceded the peak in the secretion of TNF-α. In contrast, TNF-α mRNA accumulation was only faintly detected in PBS-treated control cells at 2 h and decreased to almost below the detection limit at 4 h (Fig. 3, *A* and *C*), which was consistent with the protein data obtained by sensitive ELISA (Fig. 2*A*). These observations indicate that CT_{105}-induced TNF-α production basically occurs at the transcriptional level.

Stimulation of MMP-9 Production by CT_{105} or Aβ—We subsequently investigated whether CT_{105} or Aβ-(1–40) were able to induce the production of MMP-9 by THP-1 cells because expression of MMPs can be influenced by proinflammatory cytokines, which have increased expression on microglia in the vicinity of senile plaques. Using a sensitive zymography method, MMP-9 release was studied in THP-1 cells over a 44-h incubation with the same time course used in the TNF-α production assay (Fig. 3). We found that the unstimulated THP-1 cells did faintly display both 92- and 66-kDa gelatinolytic bands at 20 h (Fig. 4*G*, *lane 1*). A 92-kDa gelatinolytic band in supernatants was markedly induced by TNF-α (consistent with a previous report, Ref. 36) and was confirmed as MMP-9 (Fig. 4*G*, *lanes 2* and *3*). Relative to the untreated control, significantly increased MMP-9 activity (about 2–3-fold increase) was detected in the supernatants of CT_{105}-stimulated cells at 44 h (Fig. 4; *A*, *C*, and *H*). Costimulation with CT_{105} and IFN-γ resulted in enhanced MMP-9 release to about 5–6-fold at 44 h

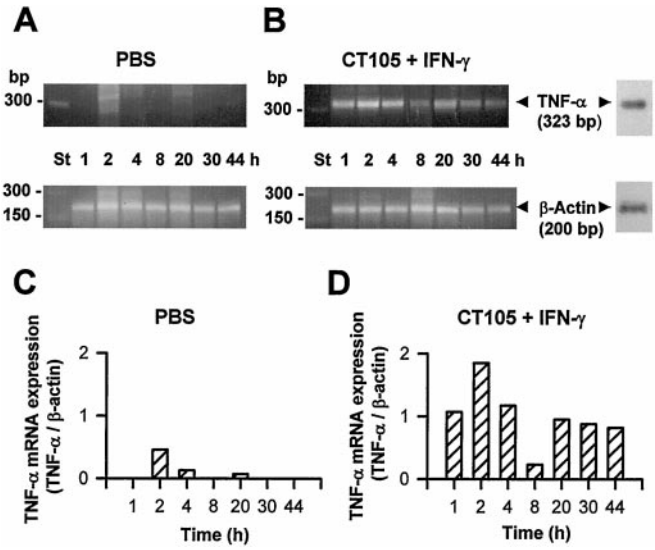

FIG. 3. **Kinetics of TNF-α mRNA expression by CT_{105}.** THP-1 cells were stimulated with CT_{105} (50 nM) in combination with INF-γ (50 ng/ml) (*B*) or treated with PBS only (*A*). Gene expression of TNF-α and β-actin was analyzed by RT-PCR as described in "Experimental Procedures" and the specificity of each amplification product was demonstrated by Southern blot hybridization on the *right.* PCR molecular markers (Promega) indicate 500, 300, or 150 bp on the *left side* of each gel. Probing for β-actin was performed for a loading control. *C* and *D* represent the densitometric analysis of *A* and *B*, respectively. Shown is a gel representative of triplicate experiments.

(Fig. 4, *D* and *H*). MMP-9 production was not significantly affected by IFN-γ incubation (Fig. 4*B*). A similar result was also obtained with Aβ-(1–40) in IFN-γ-treated cells although the degree of potentiation was lower than that achieved by CT_{105} (Fig. 4, *E* and *I*). Thus, these time course experiments indicate the production of TNF-α preceded MMP-9 release. When exogenous TNF-α was used as a control, the maximal effect on MMP-9 induction was obtained at a concentration of 10 ng/ml with appreciable increases observed at concentrations of 0.01 to 1.0 ng/ml, and higher concentrations of this cytokine did not increase MMP-9 release further (Fig. 4*F*).

Evidence for the Role of Endogenous TNF-α in MMP-9 Production—To determine whether the endogenous TNF-α produced in response to CT_{105} could contribute to the observed MMP-9 induction, we performed further studies in which neutralizing antibodies to TNF-α were added to THP-1 cells stimulated with CT_{105} in the presence of IFN-γ. Fig. 5 shows the dose-dependent inhibition of MMP-9 release by neutralizing antibodies. Anti-TNF-α antibodies at 1 μg/ml concentration over a 44-h time course reduced MMP-9 induced by CT_{105} by more than 50%, whereas a 10-fold lower concentration of anti-TNF-α antibodies (0.1 μg/ml) resulted in the same level of reduction in the case of Aβ-(1–40) (Fig. 5; *A*, *B*, and *D*). As a control, anti-TNF-α antibodies at 1 μg/ml concentration completely suppressed the MMP-9 induction by exogenous TNF-α, whereas an isotype matched antibody had little effect (Fig. 5, *C* and *D*).

Effects of Protein Kinase Inhibitors on CT_{105} or Aβ-induced Inflammatory Responses—To determine whether TK is involved in the CT_{105}-induced inflammatory response and to further investigate a possible association between TNF-α production and subsequent MMP-9 induction, THP-1 was stimulated with CT_{105} or Aβ-(1–40) plus IFN-γ in the presence of the TK inhibitor genistein. Genistein led to a dose-related inhibition of TNF-α production at the indicated concentrations shown in Fig. 6. 10 μM genistein reduced CT_{105} or Aβ-(1–40)-induced TNF-α secretion by about 60–80% of the level obtained without this agent (Fig. 6*A*). Additional experiments were undertaken to determine whether MAPK downstream of TK is involved in CT_{105}-induced TNF-α production and subsequent MMP-9 release. Both a highly specific MEK inhibitor (PD98059) and a specific inhibitor of stress-activated p38 MAPK (SB202190) led to the reduction of TNF-α secretion (Fig. 6*B*). Similarly, PD98059 also dose dependently suppressed induction of TNF-α production in response to Aβ-(1–40) (Fig. 6*C*). In contrast to the above finding, SB202190 had little effect on this Aβ-(1–40)-induced inflammatory response (Fig. 6*C*). Furthermore, down-regulation of TNF-α secretion by these agents significantly coincided with the reduction of MMP-9, further confirming the TNF-α-mediated MMP-9 production (data not shown).

DISCUSSION

Previously, we have reported that Aβ containing CT_{105} elicited stronger potency in mediating neurotoxic effect than those of any of the Aβ fragments studied, implicating it as an alternative toxic element important for the generation of memory deficit and the neuropathological changes characteristic of AD (12, 13). The mechanism underlying CT_{105}-induced neurotoxicity appears to involve its nonselective channel-forming activity (37, 38). Our study here clearly demonstrates that CT_{105} at sublytic concentrations has a greater capacity for inducing a major inflammatory cytokine TNF-α production and subsequent MMP-9 release than the Aβ in the presence of IFN-γ from human monocytic THP-1 cells. This CT_{105} evoked inflammatory responses dependent on TK and ERK/p38 MAPK signal cascades and seems to be different from the Aβ-induced effect involving the TK and ERK MAPK signaling pathways. Together, these findings provide the first evidence implicating an important role for CT_{105} in the triggering of the inflammatory process, possibly linked to chronic neurodegeneration in AD.

These Aβ-bearing amyloidogenic CTs could be generated from APP through an endosomal-lysosomal pathway by the action of β-secretase and possibly further cleaved by γ-secretase to release Aβ (6, 39, 40). In contrast, at least two other APP processing pathways produce fragments with generally opposing properties. In nonamyloidogenic secretory pathway, soluble neuroprotective ectodomain APP (sAPPα) is released through the action of α-secretase, thus preventing Aβ formation, and the combined actions of β-and γ-secretases yield Aβ products in the coated pit-mediated endocytosis pathway (40, 41). CTs with molecular masses of between 12 and 22 kDa have been found in both the medium and the cytosol of lymphoblastoid cells obtained from patients with early- or late-onset familial AD (42) and DS (43). In addition, the carboxyl-terminal peptides have been identified in plaques, microvessels, and the neurofibrillary tangles in the brains of AD patients (5–7, 44). Earlier studies showed that Aβ-bearing CT fragments were released from several different cells and/or more easily released from the damaged neurons into the medium or extracellular fluids (45–48). Moreover, a recent study reported that APP mutations found in familial AD increased the intracellular accumulation of potentially amyloidogenic and neurotoxic CTs in neurons (49). Our previous studies demonstrated that Aβ-bearing CT was detected in the medium of cultured PC-12 cells and the intracellular injection of CT_{105} into *Xenopus oocyte* caused the same channel effects but to a greater extent than the extracellular application of CT_{105}, suggesting that the extracellular effect reflects intracellular ones (37). Thus, these observations together strongly imply that highly increased levels of CTs (intracellular or extracellular) may directly cause neuronal cell death. If not directly causal, CTs released upon cell death could act as a stimulator perpetuating inflammatory responses such as the production of TNF-α and MMP-9, which could also indirectly damage neurons. The far greater effect elicited by CT_{105} and at much lower concentration than Aβ on

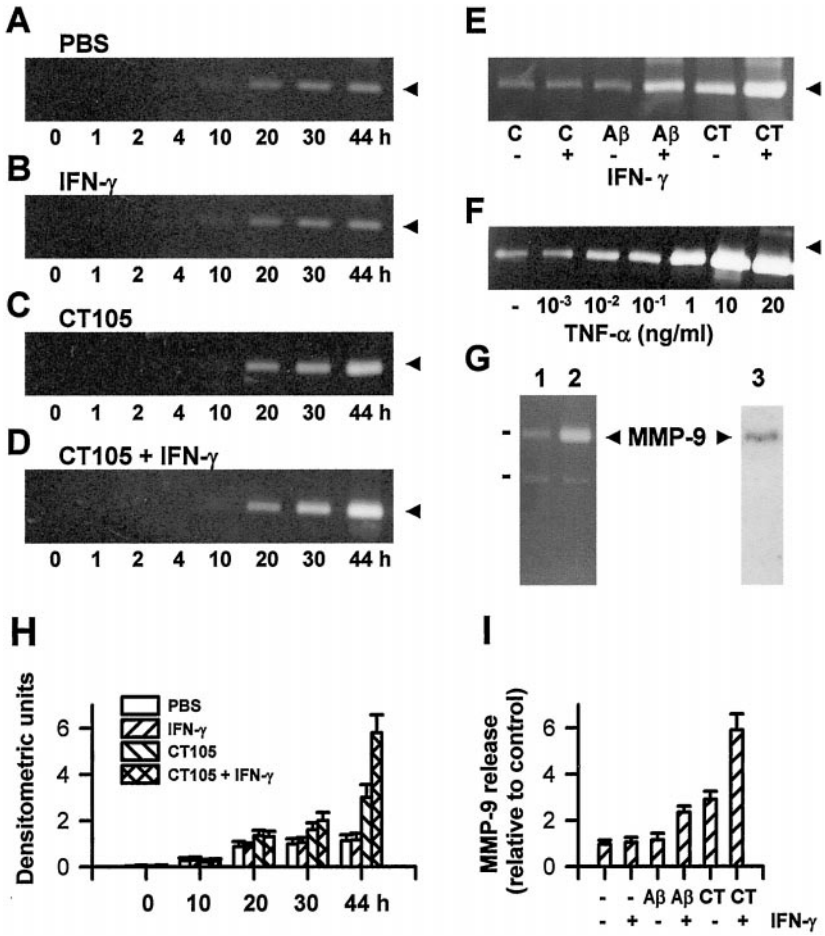

FIG. 4. **Time course of MMP-9 release in response to CT_{105} or Aβ-(1–40).** Zymographic analysis was performed using the same cell supernatants prepared for TNF-α assay in Fig. 3 to determine CT_{105}-induced MMP-9 activities: *A,* PBS; *B,* IFN-γ at 50 ng/ml; *C,* 50 nM CT_{105} alone; and *D,* 50 nM CT_{105} plus 50 ng/ml of IFN-γ. In *E*, MMP-9 activities induced by Aβ-(1–40) were compared with those by CT_{105} in conditioned medium from THP-1 cells treated for 44 h as indicated. *F,* a dose-dependent effect of TNF-α on MMP-9 release from THP-1 cells incubated for 44 h. *G,* the zymographic analysis of gelatinolytic activities in conditioned medium from THP-1 cells incubated for 20 h without (*lane 1*) and with TNF-α (1 ng/ml) and the immunoblot (*lane 3*) of the 92-kDa gelatinase B for verification of MMP-9. *Bars* on the *left* indicate the locations of 88 and 68 kDa markers, and the *arrowheads* locate MMP-9, respectively. Results are representative of five independent experiments. *H* and *I,* densitometric analysis of zymograms for *A, B, C, D,* and *E*, respectively. Data are mean ± S.D. ($n = 5$).

the induction of these major inflammatory components suggests synergistic effects with the CT domains downstream of the Aβ sequence within CT_{105}. Our recent results suggest that CT fragments without Aβ and the transmembrane domain may also participate in the neuronal degeneration in AD (50).

IFN-γ, a predominantly proinflammatory cytokine, has been implicated to be a strong immunological component capable of inducing mononuclear cells in order to release factors that actually lead to neuronal death in response to Aβ (20, 51). Our study clearly demonstrates that CT_{105} enhanced TNF-α transcription and secretion in combination with IFN-γ, which primed THP-1 cells for a rapid and enhanced response to CT_{105} although the precise mechanism is unknown. This enhancing effect of IFN-γ on CT_{105}-induced TNF-α production from human mononuclear cells is significantly comparable with recent studies showing its synergistic effect on the Aβ-mediated increase of TNF-α production from the mouse microglia and the BV-2 mouse microglia cell line (20, 51). Consistent with a recent study, Aβ elicited a modest induction of TNF-α release from THP-1 cells, although there were some discrepancies in the patterns of TNF-α induction possibly because of culture conditions and treatment schemes (21). Thus, CTs as well as Aβ might be in part responsible for the elevated level of brain TNF-α, which has a critical role in the initiation and/or progression of the inflammatory processes leading to neurodegeneration in AD. Earlier studies demonstrating dense immunostaining for TNF-α on microglia associated with amyloid plaques and Aβ-induced neurodegeneration accompanied by enhanced TNF-α release further support this possibility (18, 52). Moreover, these observations together support the current hypothesis that IFN-γ could act as an inflammatory amplifier aggravating the neurodegenerative process through priming microglia or monocytes/macrophages for secretion of proinflammatory cytokines (53, 54). In fact, there have been reports of hyperproduction of IFN-γ in the DS thymus and increased IFN-γ in the sera of DS patients (55, 56). In addition, a recent study has demonstrated an increased generation and the release of TNF-α and IFN-γ from the immune cells of AD patients (26) further supporting *in vivo* relevance of this study and a potential immunological mechanism associated with AD neuropathology. On the other hand, recent data reporting that the combination of TNF-α and IFN-γ increases Aβ production inhibiting sAPP secretion further support the mechanism by which inflammatory components can exacerbate the fundamental pathology in AD (57).

The present study demonstrates that CT_{105} or Aβ to a lesser extent could elicit a significant induction of MMP-9 activity by human monocytic cells. The main mediator of this response appeared to be endogenous TNF-α production in response to CT_{105} or Aβ based on the observations that MMP-9 production was significantly inhibited by TNF-α neutralizing antibodies and TNF-α production preceded MMP-9 release as well as the observation that the induction pattern of MMP-9 was mimicked by exogenous TNF-α treatment. Furthermore, the fact that protein kinase inhibitors could reduce both TNF-α production and MMP-9 release further supports the idea of autocrine TNF-α as a key mediator for subsequent MMP-9 induction in response to either CT_{105} or Aβ. The demonstration of up-regulation of MMP-9 in AD brain tissue and the induction of MMP-9 by Aβ in microglia and astocytes as well as macrophages/monocytes implicates its potential role in disease progression during the chronic inflammatory state in AD (19, 58, 59). In particular, monocytes differentiating into microglia-like cells in the vicinity of cerebrovascular plaques containing CTs as well

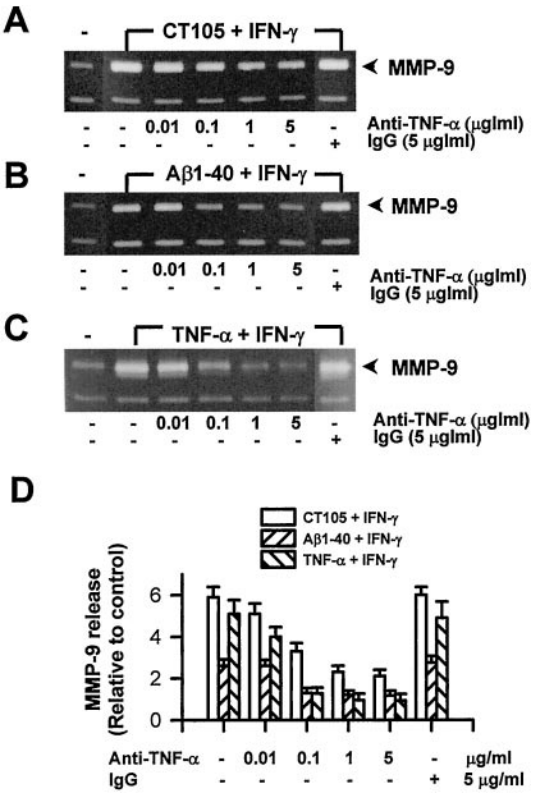

FIG. 5. **Effect of anti-TNF-α antibodies on CT_{105} or Aβ-(1–40)-induced MMP-9 release.** MMP-9 activities present in conditioned media from THP-1 treated with either 50 nM CT_{105} (*A*) or 10 μM of Aβ-(1–40) (*B*) in combination with IFN-γ (50 ng/ml) in the absence or presence of increasing anti-TNF-α antibodies or preimmune IgG (1 μg/ml) for 44 h were analyzed. In *C*, TNF-α (1 ng/ml) plus IFN-γ (50 ng/ml) were preincubated for 2 h at 37 °C with the indicated concentration of anti-TNF-α antibodies before addition to the cell cultures. Results are representative of four independent experiments. *D,* densitometric analysis of zymograms for *A, B,* and *C*. Data are mean ± S.D. (n = 4).

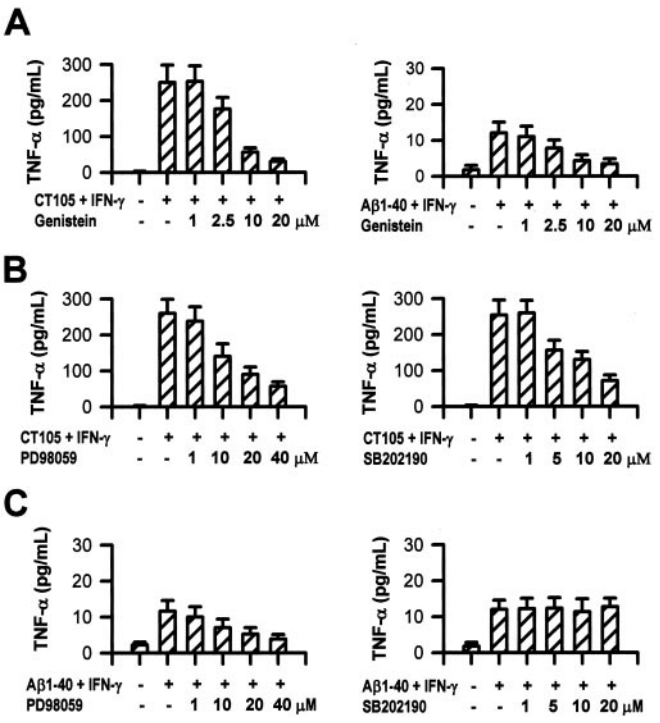

FIG. 6. **Effects of genistein, PD98059, or SB202190 on CT_{105} or Aβ-(1–40)-evoked TNF-α secretion.** TNF-α in supernatants of THP-1 incubated with either 50 nM CT_{105} or 10 μM Aβ-(1–40) plus IFN-γ (50 ng/ml) in the absence or presence of increasing concentrations of genistein (*A*), PD98059 (*B*), or SB202190 (*C*) was quantitated by ELISA as described in Fig. 1. Data are mean ± S.D. (n = 5–7).

as Aβ may produce several cytokines including TNF-α and other chemokines and inflammatory mediators (22, 60). Consequently, highly increased MMP-9 activities may cause destruction of cortical and leptomeningeal vessels, and the penetration of these activated immune cells into the brain leading to severe tissue damage and a disturbed blood brain barrier as seen in AD brains (61). Perturbation of blood brain barrier could in turn allow the passage of INF-γ, a cytokine secreted predominantly by CD4+ lymphocytes, into the brain lesions, subsequently potentiating the inflammatory effect of TNF-α produced in response to CTs or Aβ as observed in this study.

The activation of various components of the signal transduction pathway linked to the inflammatory responses leads to the synthesis of numerous proinflammatory species. Recent studies demonstrate that activation of MAPK pathways in response to Aβ fibrils follow a subsequent downstream TK-dependent inflammatory signaling event (23, 24). Based on dose-related inhibitory patterns by specific PK inhibitors revealed in our study, we report that both the TK cascade and subsequent downstream ERK/p38 MAPK pathways appear to play important roles in the CT_{105}-mediated production of TNF-α and MMP-9 in human monocytic cells in the presence of IFN-γ. In contrast, inflammatory signaling events occurring in response to Aβ may be dependent on both the PTK and ERK MAPK pathways and possibly be independent of the p38 MAPK pathway. Thus, CT_{105}-mediated activation of the p38 MAPK pathway as well as the potential synergistic effect of the CT domain downstream of Aβ sequence within CT_{105} may be correlated with the extensive inflammatory response elicited by CT_{105} compared with Aβ. Recent data reporting that the increased level of active p38 MAPK is associated with senile plaques in AD brains supports the *in vivo* relevance of this study (62).

Taken together, these results suggest that CT_{105} or Aβ to a lesser extent can function as an inflammatory stimulator to activate cells of human mononuclear origin and trigger a marked increase in TNF-α and MMP-9 production in the presence of INF-γ. Additionally, CT_{105}- or Aβ-mediated inflammatory signals were significantly inhibited by the tyrosine kinase inhibitor genistein and specific inhibitors of MAPK. These findings support the hypothesis that CT generated from alternative processing and/or excessive production of APP may be not only an intermediate precursor of neurotoxic Aβ but also an alternative contributing factor stimulating inflammatory processes linked to the delayed neurodegeneration in AD. Accordingly, further detailed characterization of the various signaling pathways implicated may hold promise as potential therapeutic targets for slowing progression of the disease.

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Effects of the β-Amyloid and Carboxyl-terminal Fragment of Alzheimer's Amyloid Precursor Protein on the Production of the Tumor Necrosis Factor- α and Matrix Metalloproteinase-9 by Human Monocytic THP-1

Young Hae Chong, Ji Hye Sung, Soon Ah Shin, Jang-Hyun Chung and Yoo-Hun Suh

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