TNF-α down-regulates S100B expression in primary human astrocytes

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Directed by Professor Se Jong Kim

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This certifies that the Master's Thesis of

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Abstract

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S100B is a calcium-binding protein predominantly produced and secreted by astrocytes in the central nervous system (CNS). S100B is suggested to function as a cytokine with both neurotrophic and neurotoxic effects depending on its concentration. S100B would take part not only in the pathophysiology of neurodegenerative disorders but also in brain inflammatory diseases. Therefore, the evaluation of the expression and regulation of S100B is important for understanding its role in CNS diseases. In this study, the effect of TNF-α on the expression of S100B was studied. As results, S100B mRNA expression in primary human astrocytes was down-regulated in a dose- and time-dependent manner by TNF-α. S100B protein expression in primary human astrocytes was also decreased by TNF-α. Pyrrolidine dithiocarbamate, an inhibitor of NF-κB, and cycloheximide inhibited TNF-α-induced decrease of S100B expression. These results suggest that activation of NF-κB and newly synthesized proteins are necessary to mediate TNF-α-induced down-regulation of S100B expression.

As a conclusion, TNF-α could modulate the neurodegenerative disorders in the CNS through regulation of S100B expression.

Key words : astrocyte, S100B, TNF-α, NF-κB

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I. Introduction

The central nervous system (CNS) has been considered as an immunologically privileged site, since the CNS shows a poor alloreactive response to tissue grafts[1] and is protected from blood by the blood-brain barrier (BBB) that restricts the passage of cells and many soluble molecules (immunoglobulins, cytokines, growth factors) from the circulation.[2] But, recent studies have demonstrated that the brain is not separated from the immune system even under normal conditions. A limited number of activated T cells can transmigrate across the BBB for purposes of immune surveillance[3],

and resident cells of the CNS, in particular astrocytes and microglia, can be activated to participate in CNS immune response.[4]

Astrocytes are the most prominent glial cells of the CNS and have important roles in the development and support of neurons, repair of injured neurons, formation and maintenance of the BBB, and maintenance of ion and metabolite homeostasis, and uptake of neurotransmitters such as glutamate.[5] Astrocytes can both produce and respond to many immunoregulatory cytokines including interleukin (IL)-1, IL-4, IL-6, IL-8, IL-10, and IL-12; colony stimulating factors (CSFs) GM-CSF, M-CSF, and G-CSF; interferon (IFN)-γ, transforming growth factor (TGF)-β), tumor necrosis factor (TNF)-α, and chemokines including RANTES, MCP-1, and IP-10.[6] In addition, astrocytes are capable of expressing a variety of immunologically relevant proteins such as class I and II MHC molecules, complement components, costimulatory molecules (B7-1, B7-2), and adhesion molecules (ICAM-1, VCAM-1, E-selectin) upon cytokine stimulation.[4] These functions suggest that astrocytes are involved in immunological and inflammatory events occurring within the CNS.

S100 is a multigenic family of low molecular weight (M_r between 9 and 14 kDa) Ca^{2+}-modulated proteins of the EF-hand (i.e. helix-loop-helix) type comprising 19 members. The most of S100 proteins are composed of two closely related molecules, S100α and S100β, and they exist as dimers

(S100αα, S100αβ, and S100ββ) within cells. S100 proteins have been implicated in the regulation of protein phosphorylation, the dynamics of cytoskeleton components, transcription factors, Ca^{2+} homeostasis, and cell proliferation and differenciation.[7,8] S100ββ (S100B) is abundant in the brain where it is mainly produced and secreted by astrocytes. Extracellular S100B exerts neurotrophic or neurotoxic effects depending on its concentration. S100B stimulates neurite outgrowth and enhances survival of neurons during development and after injury through activation of the Cdc42/Rac and the Ras/MAP kinase/NF-κB pathway at nanomolar concentrations.[9,10] In addition, it stimulates the proliferation of astrocytes by activating the extracellular signal-regulated kinases (ERK).[11] However, the micromolar concentrations of S100B cause the neuronal death by elevation of cytochrome C release following activation of the caspase cascade.[12] Additionally, high concentrations of S100B cause the death of astocytes by inducing the expression of inducible nitric oxide synthase and secretion of nitric oxide.[13] These high levels of S100B have been detected in brains from patients with Down's syndrome because the human gene coding for S100B is located on chromosome 21q22.3 which is trisomic in this syndrome.[14] The activated astrocytes associated with the neuritic plaques of Alzheimer's disease also contain elevated levels of S100B.[15] These observations suggest that S100B might have a role in the pathogenesis of neurodegenerative disorders as well

as in the development of brain. These extracellular effects of S100B are mediated by a cell surface receptor and receptor for advanced glycation end products (RAGE) has been identified as a signal-transducing receptor for S100B.[16]

TNF-α is a proinflammatory cytokine that functions immunologically through transcriptional up-regulation of genes encoding inflammatory cytokines.[17] In the CNS, TNF-α is produced by astrocytes, microglia and neurons[18, 19] and involved in brain inflammatory diseases such as multiple sclerosis, AIDS dementia complex, experimental allergic encephalomyelitis and Alzheimer's disease.[6] TNF-α modulates immune reactivity by affecting the expression of class I and II MHC molecules, adhesion molecules and costimulatory molecules on a variety cell types, including astrocytes and microglia. In addition, TNF-α stimulates many cell types to produce cytokines, including IL-1, IL-6, IFN-β, CSFs and TNF-α itself.[6] In general, TNF-α activates two signaling pathways, one leading to the activation of gene transcription and the other leading to cell death. Cytotoxic effects of TNF-α involve receptor-dependent apoptosis mediated through the action of death-domain-containing proteins.[20-22] TNF-α induced gene transcription events are primarily dependent on the activation of NF-κB.[23,24] The transcription factor, NF-κB plays a critical role in immune and inflammatory responses. NF-κB, a protypic heterodimer of p50 and p65 (Rel-A), is sequestered in the cytoplasm

through association with cellular inhibitors, member of the IκB family of proteins (IκBs). Upon exposure to a wide variety of conditions and stimuli, IκBs are phosphorylated by IκB kinases and then undergo ubiquitination and degradation in proteosomes that lead to nuclear translocation of NF-κB.[25,26]

Extracellular S100B might participate in brain inflammation by activating astrocytes, microglia and neurons,[27-29] however the regulation of S100B is not clear. In this study, we present evidences that S100B is down-regulated by TNF-α in mRNA and protein levels, and its regulation is mediated by NF-κB pathway.

II. Materials and methods

1. Culture of astrocytes and reagents

Human fetal astrocytes were kindly provided by Dr. Joo Young Park (Yonsei University, Wonju, Korea). The samples were obtained from therapeutic abortion. Astrocytes were cultured at 37 ℃ under 5% CO_2 in 10% fetal bovine serum (GIBCO BRL, Grand Island, NY, USA)-DMEM (GIBCO BRL) containing 1% nonessential amino acids (Sigma, St. Louis, Mo, USA). Culture medium was changed every week. The indirect immunofluorescence staining for glial fibrillary acidic protein (GFAP) revealed that most of the cultured cells (> 99%) were astrocytes.

To address the effect of cytokines on S100B expression, primary astrocytes were treated with 1,000 U/ml of recombinant human IFN-γ (PharMingen, San Diego, CA, USA), 1,000 U/ml of recombinant human TNF-α (R&D systems, Minneapolis, MN, USA), 100 ng/ml of LPS (*E. coli* 055:B5, Difco, Detroit, MI, USA), 10 ng/ml of TGF-β (R&D systems), and 10 ng/ml of IL-18 (R&D systems) for 24 h. Astrocytes were pretreated with 1,000 U/ml of IFN-γ for 12

h before adding LPS. Cycloheximide (CHX; Sigma, 2 μg/ml) was used as protein synthesis inhibitor. Pyrrolidine dithiocarbamate (PDTC; Sigma, 20 μM) was used as NF-κB inhibitor.

2. Northern blot analysis

Astrocytes were placed in a 60 mm dish. Total RNA was isolated by a RNeasy Kit (Qiagen, Santa Claris, CA, USA). Ten μg of total RNA was separated on a 1% agarose gel containing 6% formaldehyde/1× MOPS, and then transferred to a Hybond TM-N nitrocellulose membrane (Amersham Life Science, Buckinghamshire, England). A *Eco*RV-BamHI fragment (350bp) of S100B cDNA was used to make probe. The DNA fragment was denatured by heating at 95 ℃ for 5 min, followed by quick chilling on ice. The DNA fragment was labeled with [α-^{32}P] dCTP using a random primer labeling kit (Promega, Madison, WI, USA). The DNA fragment was incubated with 2 ㎕ of BSA, 2 ㎕ of dNTPs (without dCTP), 10 ㎕ of 5× Klenow buffer, 5㎕ of [α-^{32}P] dCTP and 1 unit of Klenow for overnight at room temperature (RT). The labeled fragment was purified by Sephadex G-50 and used as a probe when specific activity was measured greater than 2×10^7 cpm/μg DNA. Hybridization was carried out in a hybridization buffer containing 50%

formaldehyde, 0.25 M sodium phosphate buffer, 1 mM EDTA, 0.25 M NaCl, 7% SDS and 100 /ml salmon sperm DNA at 42 ℃ for overnight. After hybridization, the membrane was washed with 1× SSC containing 0.1% SDS two times for 10 min at RT. Wash with 0.2× SSC containing 0.1% SDS for 5 min at 55 ℃ was washed for several times. Autoradiography was performed with an intensifying screen at -70 ℃ for several days. To determine whether the equal amount of RNA was used, the expression of β-actin was examined.

3. RNA isolation, cDNA synthesis and reverse transcriptase-polymerase chain reaction (RT-PCR)

Astrocytes were seeded in a 60 mm dish. Total RNA was isolated by a RNeasy Kit (Qiagen). To synthesize cDNA, 2 μg of total RNA was mixed with 100 ng random hexamer (Pharmacia, Uppsala, Sweden), 5 ㎕ of 5× first strand buffer, 2.5 ㎕ of 0.1 M DTT, 5 ㎕ of 10 mM dNTPs and 200 units of Murine molony Leukemia Virus-Reverse Transcriptase (MMLV-RT) (GIBCO BRL) and incubated at 42 ℃ for 2 hr. The reaction mixture was boiled at 95 ℃ for 5 min, quickly chilled on ice. PCR reaction was performed using specific primers for S100B. PCR reaction mixture was prepared with 1 ㎕ of cDNA, 2 ㎕ of 10 mM dNTPs, 1 ㎕ of 10 pmol up-stream primer, 1 ㎕ of

down-stream primer, 2.5 ㎕ of 10× PCR buffer, 17.3 ㎕ of distilled water and 1 unit of Taq polymerase (TaKaRa, Shiga, Japan). PCR reaction was performed by the following cycling condition : 94 ℃ for 30 sec: 62 ℃ for 30 sec: 72 ℃ for 1 min for 27 cycles. To determine whether the equal amount of RNA was used, the expression level of β-actin mRNA was examined. RT-PCR for S100B was performed using the following primer set: forward: 5′-ACT ACT GCC TGC CAC GAG TT-3′, reverse: 5′-GGC CAG TCA GCT TAC ACA CA-3′.

4. Flow cytometry

Astrocytes were plated at 5×10^5 cells in 60 mm dish. Cells were trypsinized and washed with PBS containing 0.1% BSA, then fixed in 75% ethanol for 30 min at -20 ℃ and permeabilized with 0.5% Triton X-100 for 10 min at RT. After washing, cells were stained with anti-S100B monoclonal antibody (mAb) or anti-GFAP mAb (PharMingen) for 30 min at 4 ℃. Then, cells were washed twice and incubated with FITC-conjugated goat anti-mouse IgG (Becton Dickinson, San Jose, CA, USA) for 30 min at 4 ℃. The Cells were washed twice and analyzed by FACstar (Becton Dickinson).

5. Transient transfection

Astrocytes were transfected by using the FuGENE^{TM} 6 (Roche), at a DNA/Fugene6 ratio of 1:2. Cells were seeded at 3×10^5 cells per well in 6 well plates. DNA transfecting complexes (1 μg) contained the reporter gene (NF-κB response element with a luciferase gene, 0.5 μg), β-gal expression vector (0.2 μg), pUSE-IκB dominant negative (S32A and S36A double mutant, 0.25 μg) and empty control vector (Upstate biotech, Lake Placid, NY, USA). FuGENE 6 transfection reagent was diluted in serum-free medium, to a total volume of 100 ㎕. DNA mixture was added into the prediluted FuGENE 6 reagent and was incubated for 30 min at RT. DNA solution was added into a well drop-wise and the plates were incubated at 5% CO_2 incubator for 3 hr, then complete medium were added into the well and the plates were incubated for overnight. At the end of incubation, medium was changed and treated with TNF-α (1,000 U/ml) for 24 hr. Cells were lysed with 200 ㎕ of 1× reporter lysis buffer and luciferase activity was measured by adding 50 ㎕ luciferin (Promega) into 10 ㎕ of cell lysate using an analytical luminescence luminometer. To measure β-gal activity, 50 ㎕ of cell lysate was incubated with 142.5 ㎕ of 0.1 M sodium phosphate buffer (pH 7.4), 55 ㎕ of o-nitrophenyl β-D-galactopyranoside solution (ONPG) (Sigma) and 2.5 ㎕ of

$MgCl_2$ mixture at 37 ℃ in microwell plate. ONPG solution was prepared with 0.4 g ONPG in 100 ml of 0.1 M $MgCl_2$ and 5 M β-mercaptoethanol. Microwell plate was read in 405 nm using ELISA reader. Luciferase activity was normalized for transfection efficiency by the corresponding β-gal activity.

III. Results

1. Effects of cytokines on S100B gene expression in primary human astrocytes.

To address the effect of cytokines on the expression of S100B, primary astrocytes were treated with IFN-γ (1,000 U/m), TNF-α (1,000 U/ml), LPS (100 ng/ml), IL-18 (10 ng/ml), TGF-β (10 ng/ml) alone or together. To determine the expression of S100B mRNA, Northern blot analysis was performed. Untreated primary astrocytes expressed S100B mRNA constitutively. LPS and IL-18 increased S100B mRNA level by 40-50% and IFN-γ showed additive effect on LPS-mediated increase of S100B mRNA by 10-20%. TGF-β did not alter basal S100B mRNA level. In contrast, TNF-α treatment significantly reduced S100B mRNA level by about 80% (Fig. 1). Thus, this study is focused on the effect of TNF-α upon the S100B expression.

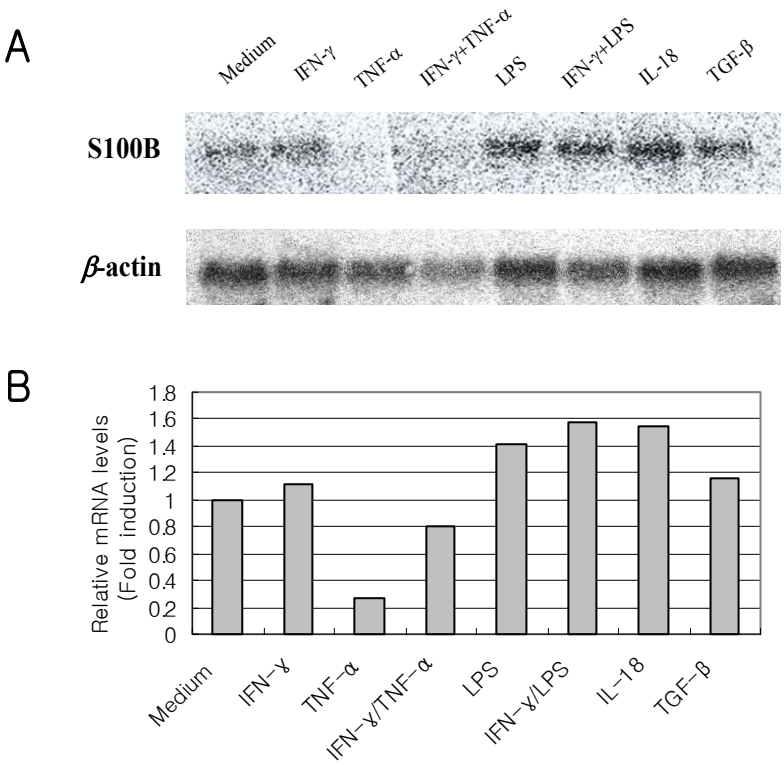


Figure 1. Northern blot analysis of S100B mRNA in primary human astrocytes after treatment of cytokines. *A*, Primary human astrocytes were treated with IFN-γ (1,000 U/ml), TNF-α (1,000 U/ml), IFN-γ (1,000 U/ml) and TNF-α (1,000 U/ml), LPS (100 ng/ml) for 24 h, IFN-γ (1,000 U/ml) pretreatment for 12 h and then LPS (100 ng/ml) for 24 h, IL-18 (10 ng/ml) and TGF-*β* (10 ng/ml) for 24 h. The expression of *β*-actin was monitored as a control. *B*, Relative mRNA levels are presented as fold induction calculated as (ratio of S100B/*β*-actin intensity in study group)/(ratio of S100B/*β*-actin intensity in control group).

2. TNF-α down-regulates S100B mRNA expression in primary human astrocytes.

To confirm the TNF-α-induced down-regulation of S100B, we examined dose-response experiment by using Northern blot analysis. S100B mRNA expression was decreased by 30%, 50%, 75%, 88% when primary astrocytes were treated with 1,10,100 and 1,000 U/ml of TNF-α for 24 hours, respectively (Fig. 2). In addition, primary astrocytes were incubated with 1,000 U/ml TNF-α for 24 hours in the presence or absence of anti-TNF-α antibody (2 μg/ml or 10 μg/ml). Anti-TNF-α antibody blocked TNF-α-induced decrease of S100B mRNA expression (Fig. 3).

For the kinetic analysis of S100B mRNA expression, primary astrocytes were treated with 1,000 U/ml of TNF-α and S100B mRNA was measured by RT-PCR. S100B mRNA was constitutively expressed and little changed for initial 8 hours after treatment of TNF-α and then, slowly declined between 8 and 24 hours and the low level was sustained over 48 hours (Fig. 4). The cells remained viable for 72 hours (data not shown).

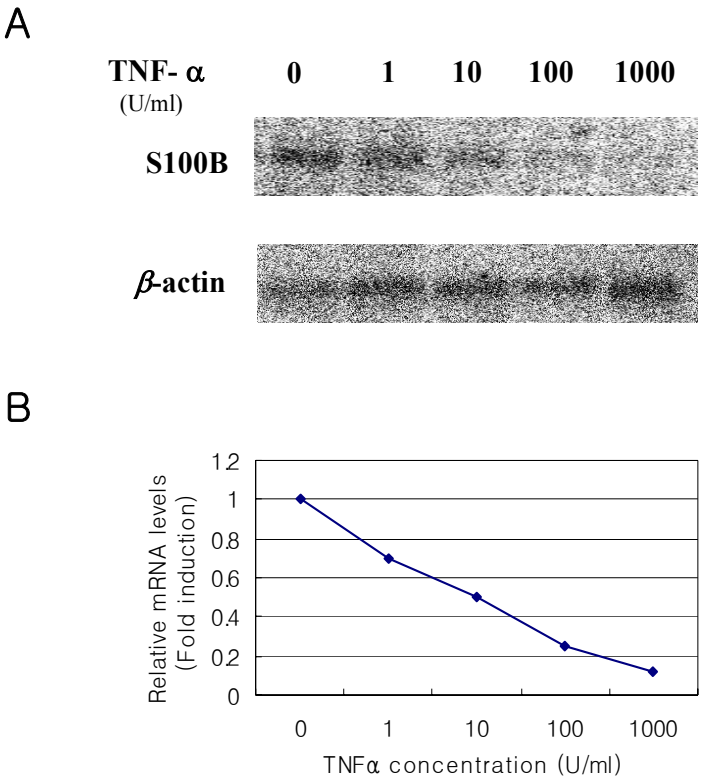


Figure 2. Dose-dependent inhibition of S100B mRNA expression by TNF-α in primary human astrocytes. *A*, Primary human astrocytes were incubated with indicated concentration of TNF-α (1-1,000 U/ml) for 24 h. S100B mRNA was analyzed by Northern blotting. The expression of *β*-actin was monitored as a control. *B*, Relative mRNA levels are presented as fold induction calculated as (ratio of S100B/*β*-actin intensity in study group)/(ratio of S100B/*β*-actin intensity in control group). Data shown are representative of three experiments.

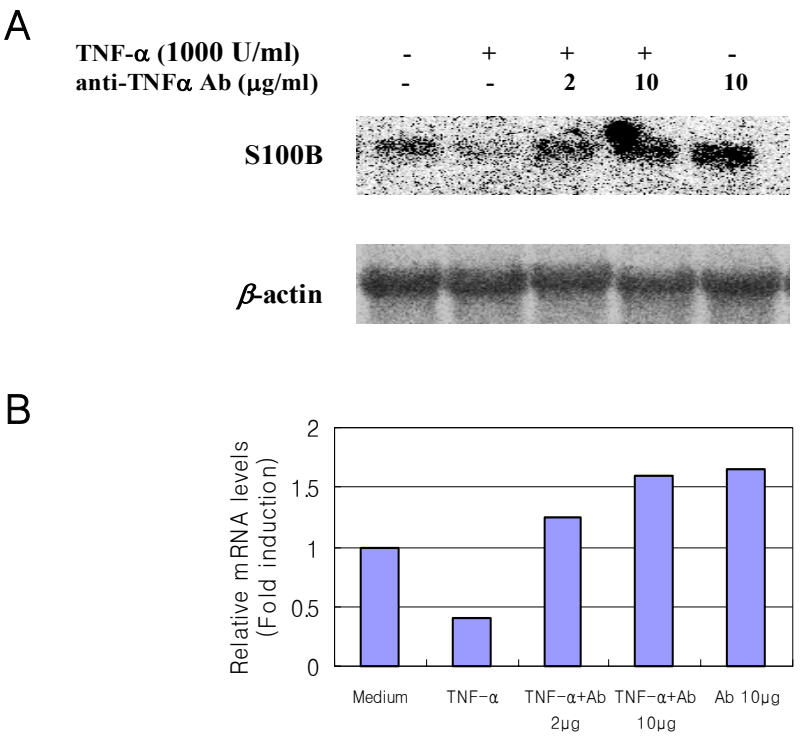


Figure 3. Anti-TNF-α antibody blocks TNF-α-induced decrease of S100B mRNA expression. *A*, Primary human astrocytes were incubated with the indicated concentration of anti-TNF-α-antibody in the presence or absence of TNF-α (1,000 U/ml) for 24 h. S100B mRNA was analyzed by Northern blotting. The expression of *β*-actin was monitored as a control. *B*, Relative mRNA levels are presented as fold induction calculated as (ratio of S100B/*β*-actin intensity in study group)/(ratio of S100B/*β*-actin intensity in control group). Data shown are representative of two experiments.

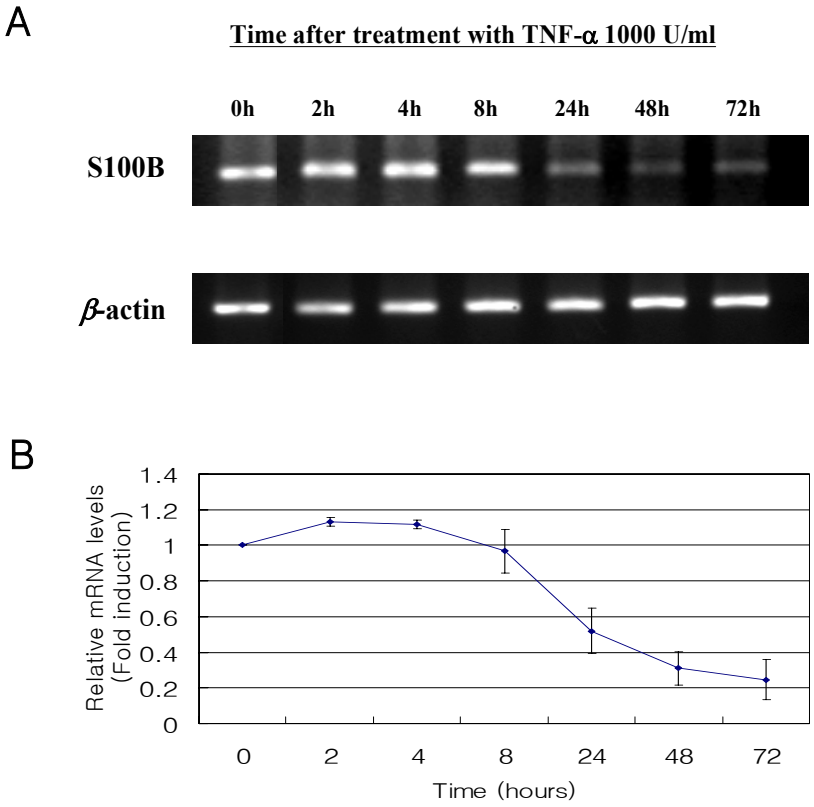


Figure 4. Time-dependent effect of TNF-α on S100B mRNA expression : Kinetic analysis. *A*, Primary human astrocytes were incubated with medium alone or with TNF-α (1,000 U/ml) for the time indicated. S100B mRNA was analyzed by RT-PCR. *β*-actin was used as an internal control. *B*, Relative mRNA levels are presented as fold induction calculated as (ratio of S100B/*β*-actin intensity in study group)/(ratio of S100B/*β*-actin intensity in control group). Data shown are representative of three experiments and are expressed as mean ± S.D.

3. S100B protein expression in primary human astrocytes is reduced by TNF-α.

To know whether TNF-α-mediated down-regulation of S100B mRNA lead to a decrease of S100B protein, intracellular S100B protein expression was evaluated by flow cytometric analysis and mean fluorescence intensity (MFI) was measured. The MFI of untreated primary astrocytes was 2.25±0.1 and the levels were reduced to 1.47±0.15, 1.31±0.08 after the treatment of 100, 1,000 U/ml TNF-α for 24 hours respectively (Fig 5). Thus, the results demonstrates that the reduction of S100B mRNA expression results in decreased S100B protein expression.

4. Regulation of S100B gene expression

To determine the requirement for protein synthesis in S100B mRNA reduction, primary astrocytes were pretreated with protein synthesis inhibitor, cycloheximide (CHX) (2 μg/ml) and then, treated with TNF-α (1,000 U/ml) for 24 hours. TNF-α-induced decrease of S100B mRNA was inhibited by CHX pretreatment (Fig. 6).

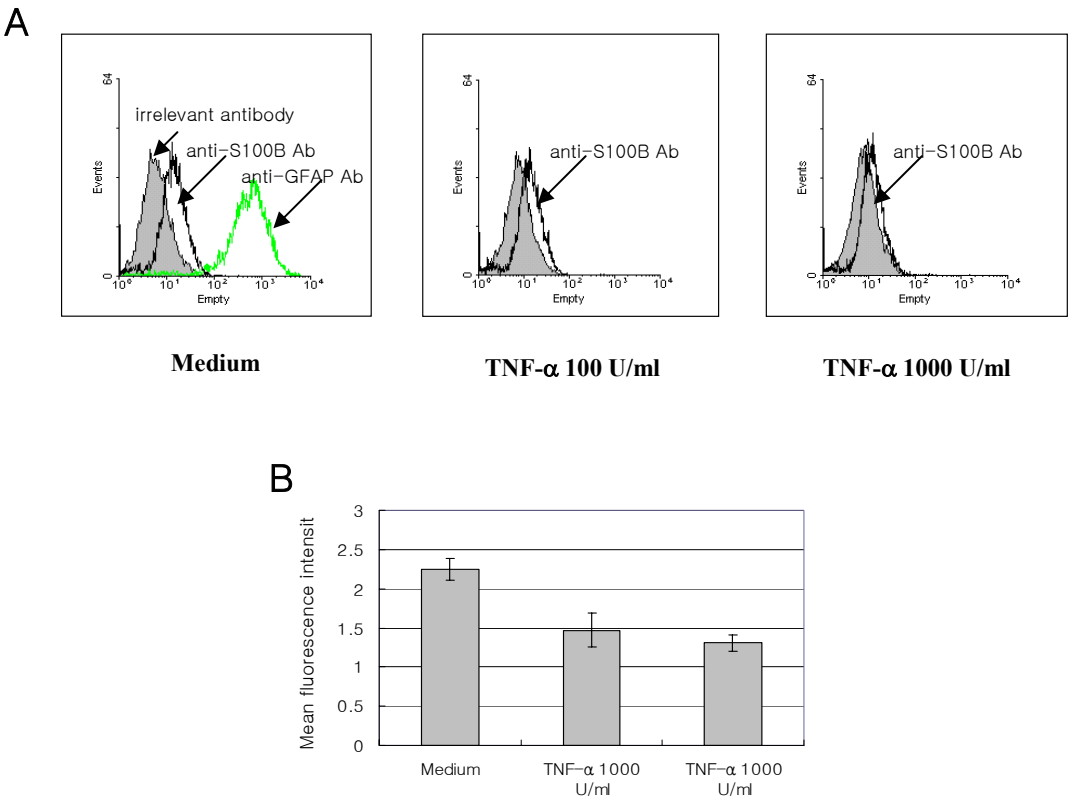


Figure 5. Effect of TNF-α on S100B protein expression in primary human astrocytes. *A*, The expression of S100B protein was determined by flow cytometry. Primary human astrocytes were incubated medium alone or with TNF-α for 24 h. 1×10^5 primary astrocytes were stained with anti-S100B monoclonal antibody and analyzed by FACstar. Filled histogram, stained with irrelevant antibody; open histogram, stained with anti-S100B antibody. GFAP was used as a positive control. *B*, The expression levels of S100B were displayed by MFI values. Data shown are representative of three experiments and are expressed as mean ± S.D.

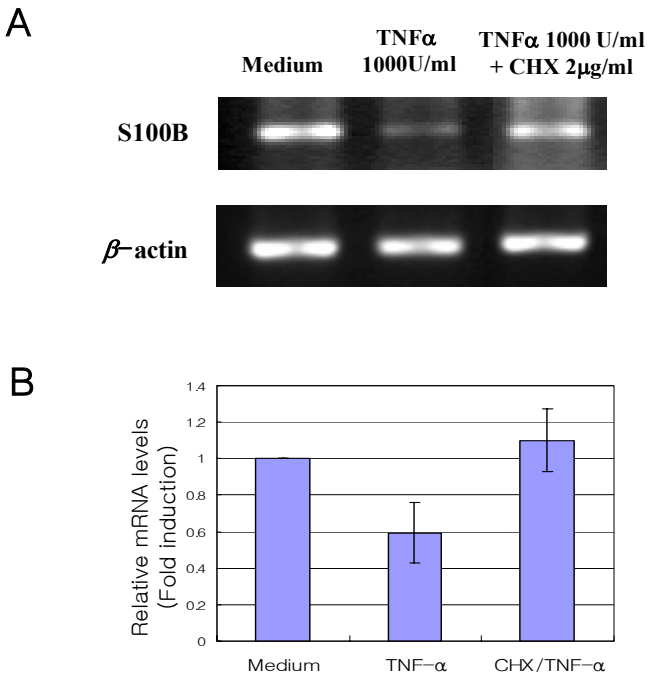
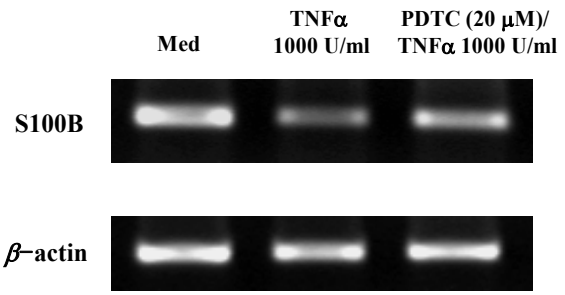


Figure 6. Effect of CHX on TNF-α-mediated down-regulation of S100B mRNA expression in primary human astrocytes. *A*, Primary human astrocytes were treated with TNF-α (1,000 U/ml) for 24 h in the presence or the absence of CHX (2 μg/ml). S100B mRNA was analyzed by RT-PCR. *β*-actin was used as an internal control. *B*, Relative mRNA levels are presented as fold induction calculated as (ratio of S100B/*β*-actin intensity in study group)/(ratio of S100B/*β*-actin intensity in control group). Data shown are representative of three experiments and are expressed as mean ± S.D.

A



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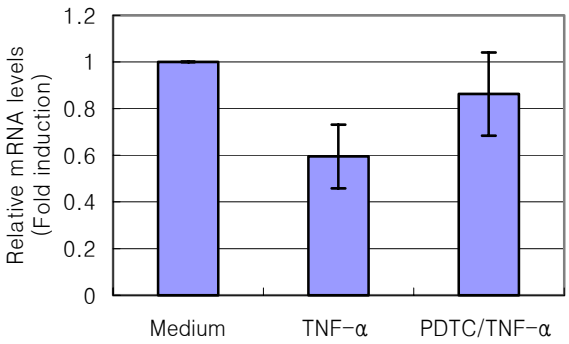

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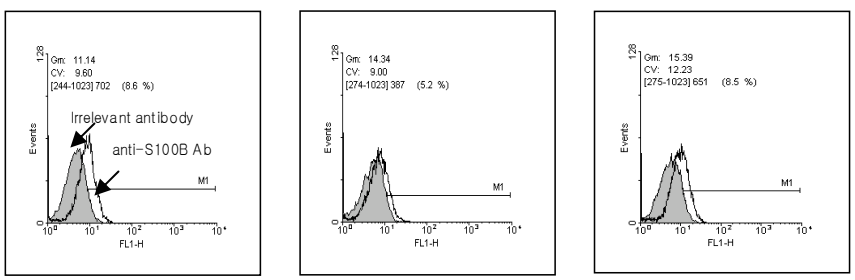

Medium TNF-α PDTC & TNF-α

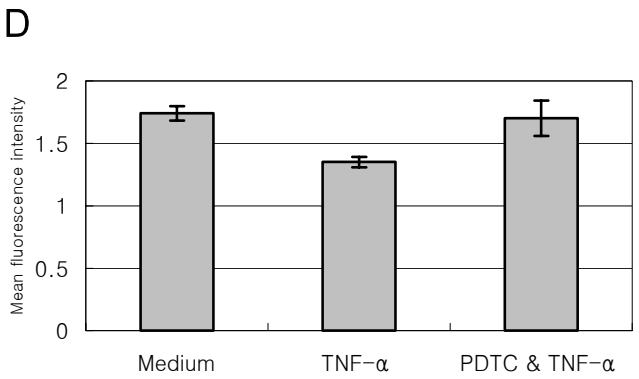

Figure 7. Effect of PDTC on TNF-α-mediated down-regulation of S100B expression in primary human astrocytes. *A*, Primary human astrocytes were pretreated with 20μM PDTC for 30 min, a specific inhibitor of NF-κB, and then treated with TNF-α (1,000 U/ml) for 24 h. S100B mRNA was analyzed by RT-PCR. *β*-actin was used as an internal control. *B*, Relative mRNA levels are presented as fold induction calculated as (ratio of S100B/*β*-actin intensity in study group)/(ratio of S100B/*β*-actin intensity in control group). Data shown are representative of four experiments and are expressed as mean ± S.D. *C*, The expression of S100B protein was determined by flow cytometry. Primary human astrocytes were pretreated with 20 μM PDTC for 30 min and then treated with TNF-α (1,000 U/ml) for 24 h. A total of 1×10^5 primary astrocytes was stained with anti-S100B mAb and analyzed by FACstar. Filled histogram, stained with irrelevant antibody; open histogram, stained with anti-S100B mAb. *D*, The expression levels of S100B were displayed by MFI values. Data shown are representative of two experiments and are expressed as mean ± S.D.

To understand the signaling pathway mediating S100B down-regulation in primary astrocytes by TNF-α, the cells were incubated with the NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC). After pre-incubation with 20 μM PDTC for 30 min, cells were treated with 1,000 U/ml TNF-α for 24 hours in the presence of PDTC. PDTC was able to prevent down-regulation of S100B mRNA and protein by TNF-α (Fig. 7). The inhibitor had no significant effect on the level of S100B in the absence of TNF-α (data not shown).

The involvement of NF-κB in TNF-α-mediated down-regulation of S100B expression was also determined using the luciferase reporter analysis. Experiments were carried out using an IκB dominant negative [IκB(DN)] cDNA expression kit. IκB(DN) plasmid has mutations at residues 32 and 36 by substitutions of alanine for serine, which prevents the phosphorylation and subsequent degradation of the IκB. Thus, IκB(DN) competes with IκB for binding to NF-κB and thereby maintains NF-κB in an inactive state. Primary astrocytes were transiently transfected with IκB(DN) and NF-κB response element constructs and then treated with 1,000 U/ml TNF-α for 24 hours. Luciferase activity in IκB(DN)-transfected cells was significantly lower than in untransfected cells. In addition, cells transfected with the IκB(DN) showed

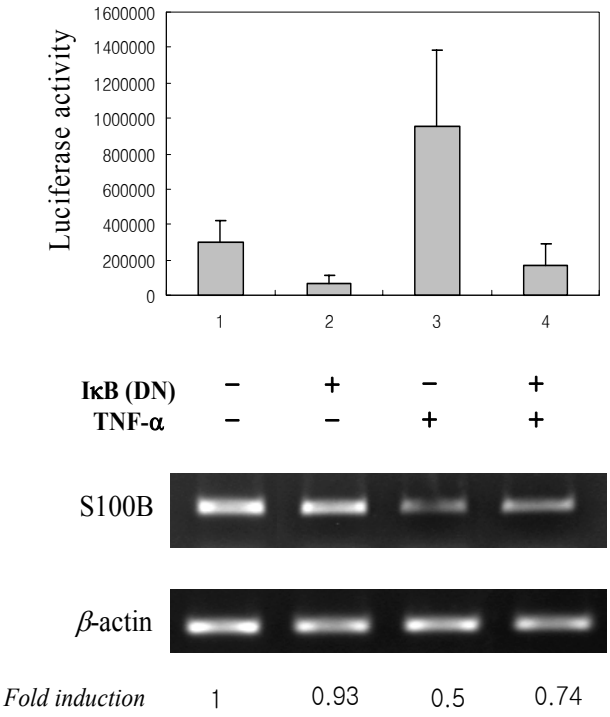

Figure 8. NF-κB mediates TNF-α-induced decrease of S100B protein expression in primary astrocytes. IκB dominant negative [IκB(DN)] and NF-κB response element-Luc reporter plasmids with β-gal expression vector were transiently transfected into astrocytes. Transfected cells were treated with or without 1,000U/ml TNF-α for 24 hr. After incubation, luciferase activity was measured and normalized by β-gal activity. Effect of NF-κB on the expression of S100B transcripts was examined by RT-PCR. Relative mRNA levels are presented as fold induction calculated as (ratio of S100B/β-actin intensity in study group)/(ratio of S100B/β-actin intensity in control group).

that TNF-α-induced reduction of S100 mRNA was inhibited (Fig. 8). These results support that NF-κB is involved in the down-regulation of S100B expression by TNF-α.

IV. Discussion

As immunocompetent cells within the CNS, astrocytes can secrete or respond to a broad array of cytokines and chemokines implicated in the development of inflammation within the CNS and in progression into diseases, as well as resolution of diseases.[6] S100B is a glial-derived protein and is abundant in the brain.[7] A secreted disulfide-linked S100B is known to have a beneficial role in the development of brain, stimulating neurite outgrowth at low nM concentrations.[30,31] However, high μM concentrations of S100B induce neuronal death with the consequence of brain dysfunction.[32] Extracellular S100B might also participate in brain inflammation by activating astrocytes, microglia and neurons.[27-29] S100B induces the expression and secretion of proinflammatory cytokine IL-6 in neurons at concentration more than 100 nM and the induced IL-6 progresses the neurodegenerative cascades in Alzheimer's disease.[27] Therefore, the regulation of S100B expression is important in the development and maintenance of the CNS, and in the pathophysiology of CNS diseases.

In this study, we showed that S100B mRNA expression in primary human astrocytes was down-regulated by TNF-α in a concentration-dependent manner, and TNF-α-mediated decrease of S100B mRNA resulted in decreased S100B protein expression (Fig. 2 and 5). It was reported that IL-1β also down-

regulates S100B gene expression in astrocytes.[33] This report, together with our findings suggest that the proinflammatory cytokines, TNF-α and IL-1β can regulate the expression of S100B in astrocytes. Kinetic analysis showed that S100B mRNA down-regulation by TNF-α required at least 24 hours to reach significant reduction (Fig. 4). This slow, sustained decrease of S100B by TNF-α suggests an indirect process that may be controlled by some inducible components. This possibility was confirmed by protein synthesis inhibition experiments in which CHX inhibited TNF-α-induced decrease of S100B mRNA expression (Fig. 6). These results suggest that down-regulation of S100B mRNA by TNF-α requires synthesis of new protein such as a negative regulatory factor.

Several S100 members have been proposed to have a role in inflammation. Particularly, S100A8 and S100A9 are potent chemoattractants and influence leukocyte margination and transmigration into tissues by increasing leukocyte deformability.[34,35] In contrast with our findings, S100A8 gene expression is up-regulated by IFN-γ and TNF-α in murine macrophage,[36] which suggest that TNF-α regulates expression of S100 proteins differentially, according to the cell type. TNF-α-induced gene transcriptions are primarily dependent on the activation and translocation of NF-κB.[37] Our data showed that the effect of TNF-α on S100B expression in astrocytes involves NF-κB. PDTC, an NF-κB inhibitor, prevented TNF-α-induced decrease of both S100B mRNA and

protein expression (Fig. 7). The ability of PDTC to antagonize down-regulation by TNF-α can be considered to suggest that NF-κB could act as a negative regulator of S100B expression in astrocytes. Luciferase reporter assay confirmed that NF-κB mediated TNF-α-induced decrease of S100B expression (Fig. 8). A repressive function of NF-κB has been found for the choline acetyltransferase gene in PC-12 cells,[38] as well as for the bcl-2 gene in lymphocytes.[39] Human S100B promoter activity is regulated by both positive and negative regulatory elements located upstream in the 5' flanking region.[40] The promoter region of human S100B gene contains several potential regulatory transcription elements, such as general transcription factor binding sequences (TATA box, GC box and CCAAT box), cAMP regulatory element, AP-2 and SP-1 binding site, S100 protein element.[41] However, the direct binding site for NF-κB was not found in the promoter. This fact indicates that NF-κB activation could lead to the activation of a repressor which eventually reduce transcription level by interacting with inhibitory sequence on the S100B promoter. Further characterization of a repressor which influences on the S100B promoter will be needed to understand the regulatory mechanisms of S100B gene expression in response to specific physiologic conditions.

Cytokines play critical roles in the development and progression of Alzheimer's disease. Cells associated with extracellular plaques within the brains of Alzheimer's disease patients can produce a variety of cytokines and

other related proteins that can ultimately influence plaque and tangle formation. Cytokines typically associated with amyloid plaques (*e.g.*, IL-1, IL-6 and TNF-α) may influence the expression of additional factors that participated in the pathogenesis of Alzheimer's disease. For instance, elevated levels of IL-1 in Alzheimer's disease brain tissue can up-regulate expression of the neuritic extension factor S100B by activated astrocytes.[42] The finding that overexpression of S100B in the neuritic plaques of Alzheimer's disease correlates with the degree of neuritic amyloid-β plaques in this disease suggests that high levels of S100B may be an important pathogenic factor in the genesis and evolution of neuritic plaques in Alzheimer's disease.

In summary, our results demonstrate that TNF-α down-regulates the expression of S100B and this regulation can be a potential mechanism for down-regulation of pro-inflammatory response pathways associated with degenerative conditions of the CNS.

V. Conclusion

1. TNF-α down-regulated the expression of S100B in a dose- and time-dependent manner in primary human astrocytes.

2. Cycloheximide inhibited the effect of TNF-α on S100B mRNA expression, which suggests that S100B down-regulation by TNF-α in primary human astrocytes is an indirect process and requires for new protein synthesis.

3. Pyrrolidine dithiocarbamate inhibited the effect of TNF-α on S100B expression. These results suggest that the down-regulation of S100B expression by TNF-α in primary human astrocytes was mediated by transcription factor NF-κB.

4. Transient transfection with the dominant negative form of IκB experiment demonstrated that NF-κB mediated TNF-α-induced decrease of S100B expression in primary human astrocytes.

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국문 요약

사람 뇌성상세포에서 TNF-α에 의한 S100B의 발현 조절

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뇌성상세포는 중추신경계에서 가장 많은 수를 차지하는 교세포로서 친염증성 사이토카인 및 신경성장인자를 생성하고 분비하여 중추신경계 내에서 염증반응을 유발하고 면역 반응을 조절하는데 중요한 역할을 한다. S100B는 칼슘 결합 단백질로서 중추신경계에서 주로 뇌성상세포에서 생성되어 분비되며 다양한 세포 내, 외 기능을 수행하고 있다. 세포내에서 S100B는 단백질의 인산화, 세포골격 구성물질의 역동성, 세포주기 그리고 효소의 활성도를 조절하여 칼슘 이온의 항상성 유지에 중요한 역할을 한다. 활성화된 뇌성상세포에서 분비된 S100B는 저농도(nM)에서는 신경세포의 분화와 신경 교세포의

증식을 유도하지만 고농도(μM)에서는 신경계 세포의 세포죽음을 유발하여 신경계의 발달 및 유지에 중요한 역할을 한다. 이처럼 사이토카인과 같은 기능을 하는 S100B는 퇴행성 뇌질환 뿐만 아니라 뇌성상세포, 소교세포 및 신경세포를 활성화시켜 뇌 염증성 질환에도 관여한다. 따라서 S100B의 발현 조절은 뇌신경계 질환을 이해하는데 중요할 것으로 생각된다. 이에 본 연구에서는 일차배양한 사람 뇌성상세포에서 사이토카인 처리에 따른 S100B의 발현 변화를 관찰하였다. 그 결과 친염증성 사이토카인인 TNF-α에 의해 S100B mRNA가 시간별, 농도별 의존적으로 감소하는 것을 확인하였다. 또한 S100B의 단백 발현도 TNF-α에 의해 감소하였다. 단백 합성 억제제인 cycloheximide와 NF-κB 억제제인 pyrrolidine dithiocarbamate의 전처리에 의해서는 TNF-α에 의한 S100B 발현 감소가 억제되는 것을 확인할 수 있었다. 이는 TNF-α에 의해 S100B의 발현이 조절되기 위해서는 NF-κB가 활성화되어야 할 뿐만 아니라 조절 억제 인자와 같은 새로운 단백질의 합성이 필요함을 제시한다. 이상의 결과로 일차배양한 사람 뇌성상세포에서 TNF-α가 S100B의 발현을 조절함으로써 여러 퇴행성뇌질환을 조절하는데 중요한 역할을 담당할 것으로 생각된다.

핵심되는 말 : 뇌성상세포, S100B, TNF-α, NF-κB