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Molecular Mechanisms of Microglial Deactivation by TGF-β-inducible Protein βig-h3

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Abstract: βig-h3 is a secretory protein that is induced by TGF-β and implicated in various disease conditions including fibrosis. We have previously reported that βig-h3 expression is implicated in astrocyte response to brain injury. In this study, we further investigated potential roles of βig-h3 protein in the injured central nervous system (CNS). We specifically assessed whether the treatment of microglial cells with βig-h3 can regulate microglial activity. Microglial cells are the prime effector cells in CNS immune and inflammatory responses. When activated, they produce a number of inflammatory mediators, which can promote neuronal injury. We prepared conditioned medium from the stable CHO cell line transfected with human βig-h3 cDNA. We then examined the effects of the conditioned medium on the LPS- or IFN-γ-mediated induction of proinflammatory molecules in microglial cells. Preincubation with the conditioned medium significantly attenuated LPS-mediated upregulation of TNF-α, IL-1β, iNOS and COX-2 mRNA expression in BV2 murine microglial cells. It also reduced IFN-γ-mediated upregulation of TNF-α and COX-2 mRNA expression but not iNOS mRNA expression. Assays of nitric oxide release correlated with the mRNA data, which showed selective inhibition of LPS-mediated nitric oxide production. Although the regulatory mechanisms need to be further investigated, these results suggest that astrocyte-derived βig-h3 may contribute to protection of the CNS from immune-mediated damage via controlling microglial inflammatory responses.

Key words: TGF-β, βig-h3, microglia, brain inflammation, cytokine

Microglial cells are the prime effector cells involved in immune and inflammatory responses in the central nervous system (CNS) (Aloisi, 2001). They are activated during neuropathologic conditions to restore CNS homeostasis. Microglial activation involves proliferation and migration to the sites of injury, increased expression of immunomodulators, and the transformation into phagocytes capable of clearing damaged cells and debris (Bruce-Keller, 1999; Aloisi, 2001). However, as with peripheral tissue inflammation, activation of counterregulatory mechanisms is also essential to avoid escalation of CNS inflammatory processes (Aloisi, 2001). Activated microglia can promote neuronal injury through the release of proinflammatory and cytotoxic factors, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and nitric oxide (NO) (Bruce-Keller, 1999). In this regard, chronic microglial activation has been implicated in neuronal destruction associated with various neurodegenerative diseases (Stoll and Jander, 1999). To better understand mechanisms of CNS immune modulation, it is important to identify positive- and negative-regulators of microglial activation and the underlying molecular mechanisms.

Transforming growth factor (TGF)-β-inducible gene-h3 (βig-h3) is inducible by TGF-β in several cell types (Skonier et al., 1992; Skonier et al., 1994; Lee et al., 2000). It is a 68-kDa secretory protein that contains four internal repeat domains and an RGD (Arg-Gly-Asp) motif, a recognition sequence for some integrins (Skonier et al., 1992). The repeat domains are homologous to osteoblast specific factor 2 and insect fasciclin I, a neuronal adhesion protein (Skonier et al., 1992). βig-h3 is expressed preferentially on the periphery of cell surface and often associated with extracellular matrix molecules (Escribano et al., 1994; Skonier et al., 1994; Rawe et al., 1997). The importance of βig-h3 in cell growth, differentiation, and wound healing has been demonstrated (Skonier et al., 1994; Rawe et al., 1997; Kim et al., 2000; Bae et al., 2002). Notably, the aberrant expression of βig-h3 has been implicated in diverse tissue pathologies, such as corneal

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Table 1. PCR primer sequences

Gene	Sense	Antisense
GAPDH	5'-agc cca tca cca tct tcc ag-3'	5'-cct gct tca cc acct tct tg-3'
TNF-α	5'-atg agc aca gaa agc atg atc-3'	5'-tac agg ctt gtc act cga att-3'
IL-1β	5'-gca act gtt cct gaa ctc-3'	5'-ctc gga gcc tgt agt gca-3'
iNOS	5'-ccc ttc cga agt ttc tgg cag cag c-3'	5'-ggc tgt cag agc ctc gtg gct ttg g-3'
COX-2	5'-cca gat gct atc ttt ggg gag ac-3'	5'-gct tgc att gat ggt ggc tg-3'

dystrophies, bone abnormalities and fibrotic lens opacification (Munier et al., 1997; Kim et al., 2000; Lee et al., 2000).

In the CNS, βig-h3 gene is not normally expressed (Escribano et al., 1994; Skonier et al., 1994). Interestingly, however, our recent finding demonstrates that the expression for βig-h3 gene is induced at the site of stab wound in the cerebral cortex of adult rats (Yun et al., 2002). βig-h3 protein enhanced astrocyte adhesion via α6β4 integrin-mediated signaling (Kim et al., 2003b). To further understand the scope of βig-h3 action in the CNS, we investigated potential roles of this protein in the regulation of microglial activation. As TGF-β is a well-known anti-inflammatory cytokine (Bottner et al., 2000), we specifically assessed whether βig-h3 could limit lipopolysaccharide (LPS)- and interferon (IFN)-γ-induced inflammatory responses in BV2 mouse microglial cells.

MATERIALS AND METHODS

Cell culture and treatment

BV2 mouse microglial cells were kindly provided by Dr. EJ Choi (Korea University, Korea) and maintained in DMEM containing 2 mM glutamine and 5% fetal bovine serum (FBS; Invitrogen). BV2 cells were plated in 6-well plates at a density of 3×10^5 cells/well, and were incubated for overnight. Then the cells were stimulated with *E. coli* LPS (Sigma) or IFN-γ (Peprotech) in fresh DMEM containing 0.5% FBS. CHO cells stably transfected with a βig-h3-containing expression vector (Kim et al., 2003a) were kindly provided by Dr. IS Kim (Kyungpook National University, Korea) and cultured in RPMI 1640 supplemented with 10% FBS. For preparation of conditioned medium, CHO cells were seeded at a density of 6×10^5 cells/25 cm^2-flask in 5-ml RPMI1640 supplemented with 10% FBS and cultured for 48 h. The culture medium was centrifuged for 10 min at 12,000×g to remove cell debris. An aliquot of the conditioned medium was then pretreated for 30 min before the addition of LPS or IFN-γ, as indicated in the figure legends. The conditioned medium was directly added to BV2 culture without further manipulation. At the end of stimulation, BV2 culture supernatant was collected for nitrite assay and the cells were subjected to RNA isolation.

Reverse transcription (RT)-PCR

Total cellular RNA was isolated from microglial cells by using Trizol reagent (Invitrogen). A 1-μg amount of RNA was reverse-transcribed in a 20-μl reaction mixture by using Moloney murine leukemic virus reverse transcriptase (Invitrogen). The cDNA (0.2 μl) was amplified using gene specific primers. The amounts of amplified products were determined using an image documentation system (ImageMaster VDS; Pharmacia) with image analysis software (ImageMaster TotalLab; Pharmacia). DNA size markers (MBI) were run in parallel to validate the predicted sizes of the amplified bands. The primer sequences specific for the genes examined are shown in Table 1.

Nitrite assay

Nitrite in culture supernatants was measured as an indicator of NO production. An aliquot of the culture supernatant was mixed with a volume of Griess reagent (Molecular Probes). The absorbance was then determined at 570 nm on a microplate reader. Sodium nitrite at concentrations of 0-100 μM, was used as the standard to calculate nitrite concentrations.

RESULTS

Suppression of LPS-induced expression of TNF-α, IL-1β, iNOS and COX-2 by βig-h3

To evaluate the effects of βig-h3 on microglial activation, BV2 cells were activated with LPS in the absence or presence of various doses of βig-h3-conditioned CHO cell medium, then the mRNA expression was measured for different proinflammatory mediators such as TNF-α, IL-1β, inducible NO synthase (iNOS), and cyclooxygenase (COX)-2. LPS stimulation of BV2 cultures resulted in a significant increase in the expression of these factors (Fig. 1). Optimal time periods of stimulation were set based on preliminary results, in which peak mRNA levels were observed around 6 h in the case of TNF-α, IL-1β and iNOS, and 24 h in the case of COX-2 (data not shown). βig-h3-containing CHO cell medium suppressed, generally in dose-dependent manners, the expression of all factors examined (Fig. 1). Secretion of βig-h3 protein in the conditioned medium was confirmed by the presence of a

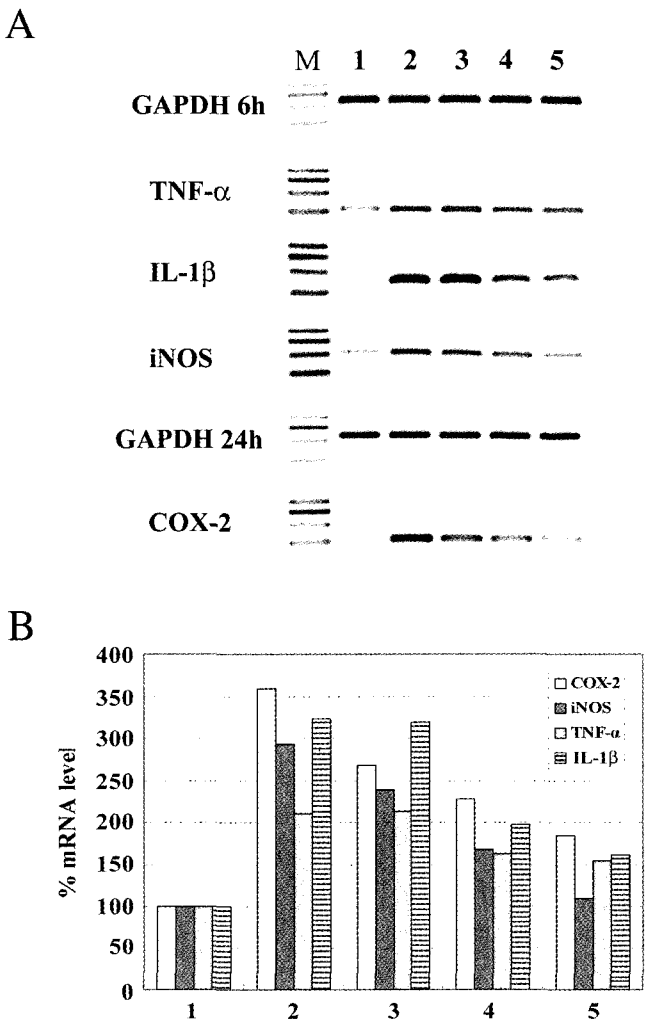

Fig. 1. Effects of βig-h3 on mRNA expression of proinflammatory mediators in LPS-stimulated microglial cells. A, BV2 cells were incubated in the absence (lane 1) or presence (lanes 2-5) of LPS (100 ng/ml). Conditioned medium derived from CHO cells overexpressing βig-h3 was pretreated with graded amounts (10, 20, and 30 μl in 2-ml culture; lanes 3-5) for 30 min before the addition of LPS. After the indicated time periods (6 h for TNF-α, IL-1β, and iNOS; 24 h for COX-2), total RNA was extracted and then subjected to RT-PCR. M, molecular size standards. The data presented are from one of three independent experiments that produced similar results. B, The intensities of amplified bands were determined from the gel shown in (A). Then, the amounts of target cDNA were normalized against GAPDH cDNA in the corresponding sample and shown in the graph. Values for control were set to 100%.

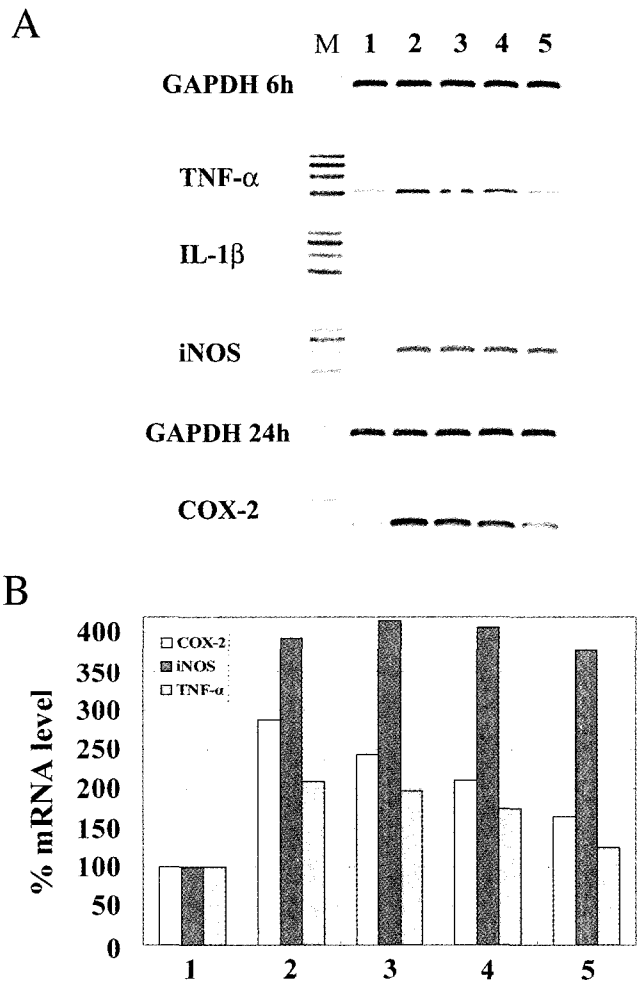

Fig. 2. Effects of βig-h3 on mRNA expression of proinflammatory mediators in IFN-γ-stimulated microglial cells. A, BV2 cells were treated as described in the legend for Fig. 1A except the stimulation with IFN-γ (100 unit/ml; lanes 2-5). B, The mRNA levels were determined as described in the legend for Fig. 1B.

major 64 kDa-immunoreactive band on the Western blot (data not shown). We observed no effect with the culture medium derived from untransfected control CHO cells (data not shown).

Suppression of IFN-γ-induced expression of TNF-α and COX-2 by βig-h3

We next determined whether βig-h3 could suppress inflammatory responses elicited by other stimuli. IFN-γ induced the expression of TNF-α, iNOS and COX-2 in BV2 cells. IFN-γ-mediated induction of TNF-α and COX-2 but not iNOS was suppressed by βig-h3 (Fig. 2), which was distinct from the suppressive effects on LPS-mediated induction. The inhibitory effects were not the result of a decreased number of microglial cells, as βig-h3 did not affect cell viability of stimulated BV2 cells during the periods of assays (data not shown).

Suppression of LPS-induced but not IFN-γ-induced nitrite production by βig-h3

We tested the effects of βig-h3 on NO production by measuring nitrite amount in BV2 culture supernatants. In correlation with RT-PCR data, βig-h3 attenuated LPS-induced but not IFN-γ-induced NO production from BV2 cells (Fig. 3).

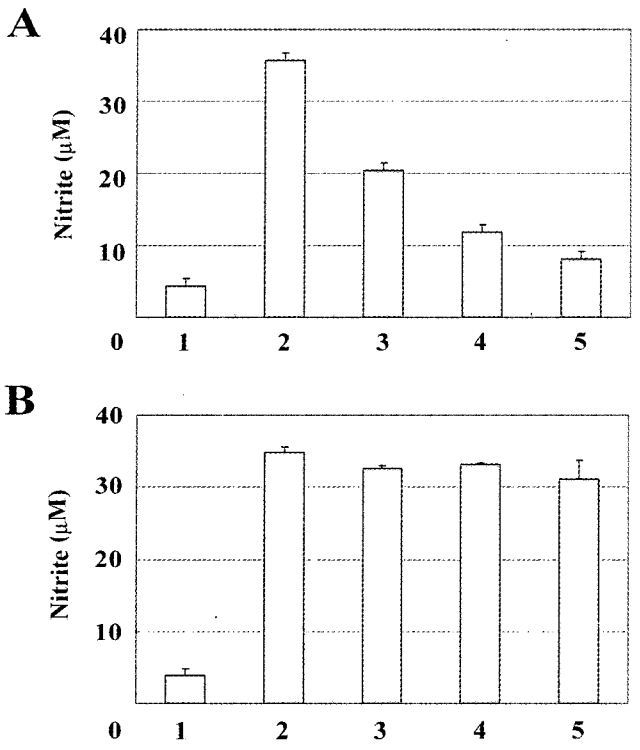

Fig. 3. Effects βig-h3 on NO production in LPS- and IFN-γ-stimulated microglial cells. The culture supernatants of BV2 cells treated as described in the legends for Figs. 1 and 2 were collected at the end of stimulation. After cell debris was discarded by centrifugation, the resultant supernatant was subjected to nitrite assay. A, LPS-stimulated cells. B, IFN-γ-stimulated cells.

DISCUSSION

TGF-β exerts multiple effects on glial cells and neurons both in vitro and in vivo. Particularly, it acts as a suppressor of functions of activated microglia (Bottner et al., 2000). Because βig-h3 is produced by TGF-β, we speculated that the molecule is likely to act as a downstream effector of TGF-β in the regulatory steps by direct or indirect means. As shown in this study, βig-h3 prominently attenuated inflammatory responses of activated microglia. It would be worthwhile to determine the actual amount of βig-h3 protein in the treatments. It has been previously shown that βig-h3 protein stimulates cell attachment, spreading, migration, and proliferation in several cell types (Ohno et al., 1999; Bae et al., 2002; Kim et al., 2003b). Moreover, RGD peptides released from βig-h3 have been shown induce apoptosis (Kim et al., 2003a). The present findings suggest that βig-h3 protein play an important role in brain inflammation, which broadens our knowledge of molecular functions of βig-h3. Inhibition of microglial activation is a promising target in the treatment of neurodegeneration. In this sense, the inhibitory effects on the expression of proinflammatory mediators define βig-h3 as a microglia-deactivating factor with therapeutic potential for neurodegenerative disorders.

In light of astroglial source of βig-h3 expression (Yun et al., 2002), it is expected that βig-h3 protein functions as a mediator to regulate astroglial interactions with microglia and possibly neurons in the injured brain. Experiments performed in our laboratory have demonstrated that βig-h3 interacts with α6β4 integrin on astrocytes and transduce adhesion signals through focal adhesion molecules (Kim et al., 2003b). Further investigation on the molecular mechanisms involved, including the specific microglial receptors and the intracellular signaling pathways that mediate the effect of βig-h3 in microglia is also required. Although underlying mechanisms are not clarified, our data imply that βig-h3 differentially acts on LPS- and IFN-γ-triggered signaling molecules at least in the case of iNOS gene expression. Protein kinase C, p38 mitogen-activated protein kinase (MAPK) and p42/44 MAPK are reported to be involved in the LPS-induced signal transduction, leading to iNOS in microglia (Bhat et al., 1998; Fiebich et al., 1998). Studies of transcriptional regulation of the iNOS gene have revealed that nuclear factor -κB is essential during iNOS induction by LPS (Lowenstein et al., 1993; Xie et al., 1994). IFN-γ-mediated signaling involves the activation of the Janus kinase/signal transducer and activator of transcription pathway, which induces IFN regulatory factor (IRF)-1 (Darnell et al., 1994). IRF-1 subsequently acts as the major transcription factor for IFN-γ-mediated iNOS induction (Kamijo et al., 1994; Martin et al., 1994). Distinct signaling relayed by LPS and IFN-γ to the iNOS gene promoter may provide selective targeting points of βig-h3.

In conclusion, our study invites important future directions, including the possible therapeutic role of βig-h3 in brain disorders, where inflammatory response is uncontrolled. The neuroprotective potential of βig-h3 requires further in vivo investigation in detail.

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