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iNSC suppress macrophage-induced inflammation by repressing COX-2

Jin Hee Kim · Woong Sun · Dong Wook Han · Hong-Joo Moon · Jangbo Lee

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Abstract Brain inflammation causes cell damage and death in diseases such as Alzheimer's and Parkinson's. In this study, we investigated whether early induced neural stem cells (iNSCs) could protect against cell death after treatment with THP1-derived macrophages. We developed an inflammatory model system with THP1-derived macrophages and cortical neuronal cells and investigated the therapeutic efficacy of iNSC against macrophage-induced inflammation in this model. Apoptosis was confirmed by double immunocytochemistry with NeuN and 4′,6-diamidino-2-phenylindole using terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP-biotin nick-end labeling. Cortical neuronal cells cultured with iNSCs exhibited fewer apoptotic cells than did cultures without iNSCs. The levels of inflammatory cytokines and vascular endothelial growth factor (VEGF) were analyzed by enzyme-linked immunosorbent assay. Cells cultured with iNSCs had lower levels of inflammatory cytokines and higher VEGF levels than those cultured without iNSCs. Western blot analysis for cyclooxygenase-2 (COX-2) showed a significantly lower level of COX-2 in cells cultured with iNSCs than in those cultured without iNSCs. Thus, early iNSCs administration reduced inflammation associated with neurological recovery, and this effect is mediated by COX-2 regulation. Our results suggest that iNSCs have potential therapeutic relevance, because they display strong anti-inflammatory functions that promote neuroprotection thorough the inflammatory response.

Keywords Induced neural stem cells (iNSCs) · Inflammatory · Cyclooxygenase-2 (COX-2)

J. H. Kim · J. Lee (✉)
Department of Neurosurgery, College of Medicine, Korea University, Seoul, Korea
e-mail: jblee42@gmail.com

W. Sun
Department of Anatomy, College of Medicine, Korea University, Seoul, Korea

D. W. Han
Department of Stem Cell Biology, School of Medicine, Konkuk University, Seoul, Korea

H.-J. Moon
Department of Neurosurgery, Guro Hospital Korea University, Seoul, Korea

Introduction

Brain inflammation and redox imbalance is a major cause of cell damage and death. Excess production of reactive oxygen species and NADPH oxidase causes tissue injury associated with brain injury, inflammation, and degenerative diseases (Contestabile and Contestabile 2001; Anderton 2002; Halliwell 2006; Uttara *et al.* 2009). Many inflammatory target proteins, including matrix metalloproteinase-9, cyclooxygenase-2 (COX-2), and inducible nitric oxide (NO) synthases are induced by proinflammatory factors (Chiurchiù and Maccarrone 2011; Lee and Yang 2012; von Bernhardi and Eugenín 2012).

Macrophages exhibit marked phenotypic heterogeneity (Mosser and Edwards 2008) and exogenous stimuli such as microorganisms influence phenotype selection. Although they are differentiated, there is considerable plasticity in the tissue macrophage phenotype; any given phenotype is dependent on the prevailing pattern of stimulation. The major functions of macrophages include maintaining tissue homeostasis and responding to microorganisms (Mosser and Edwards 2008). M1 macrophage polarization is associated with inflammation and tissue destruction (Gordon and Martinez 2010; Sica and Mantovani 2012), whereas M2 macrophages have an anti-inflammatory phenotype associated with wound repair and angiogenesis (Mantovani and Locati 2009; Xu *et al.* 2012).

M1 macrophages have been studied with differentiation protocols in which THP1 cells are stimulated with phorbol-12-myristate-13-acetate (PMA) (Murao *et al.* 1983; Olsson *et al.* 1983; Fleit and Kobasiuk 1991). The macrophage phenotypes in these protocols vary with the differentiation treatment and duration (Kohro *et al.* 2004; Park *et al.* 2007). THP1 cells differentiate toward a macrophage-like cell in response to PMA (Rovera *et al.* 1979). PMA activates protein kinase C (PKC) and induces a greater degree of differentiation in THP1 cells, which exhibit increased adherence and expression of surface markers associated with macrophage differentiation (Schwende *et al.* 1996). Activated macrophage (M1), induced by proinflammatory stimuli, increase production of chemokines and inflammatory cytokines, including tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1) and activate inflammatory signaling pathways (Shoelson *et al.* 2006).

Neural stem cells (NSCs) are a well-characterized somatic stem cell type with extensive proliferation and self-renewal capacity; they also differentiate into daughter cell types (Reynolds and Weiss 1992). Reprogramming of fibroblasts into induced NSCs (iNSCs) is a potentially unlimited source of neurons; indeed iNSCs exhibit characteristics similar to those of brain tissue-derived NSCs (Han *et al.* 2012). iNSCs are nearly identical to control NSCs in morphology, gene expression profile, and epigenetic features. They can be engrafted in the stem cell niches of the mouse adult brain, where they continue to proliferate and differentiate into neurons, astrocytes, and oligondendrocytes, suggesting potential therapeutic applications (Han *et al.* 2012).

We sought to determine whether earlier iNSC administration could protect cell death after THP1-derived macrophage treatment and to identify potential underlying mechanism. We used a characterized neuronal cell culture model of inflammatory reaction and investigated the therapeutic effect of iNSC. We found that the inflammatory cytokines level decreased in the cells cultured with iNSCs, which inhibit activation of proinflammatory gene products such as IL-1 and IL-6, which are important in the pathogenesis. We also found that iNSCs downregulates COX-2 expression in macrophages. These observations suggest iNSC mediate anti-inflammation by regulating COX-2 expression. In this study, iNSCs displayed anti-inflammatory functions that promote neuroprotection; we also explored the regulatory mechanisms that underlie these effects.

Materials and Methods

Generation of iNSC. iNSCs were obtained from Dr. Han Dong Wook. iNSCs were cultured as described previously (Han *et al.* 2012). Briefly, fibroblasts (5×10^4 cells) were infected with pMX retrovirus expressing the reprogramming factors in different combinations for 48 h. Cells were cultured in standard NSC medium: DMEM/F-12 supplemented with N2 or B27 (GIBCO-BRL, Gaithersburg, MD), 10 ng/ml EGF, 10 ng/ml basic fiboblast growth factor (bFGF; both from Invitrogen, San Diego, CA), 50 μg/ml bovine serum albumin (BSA; Fraction V; GIBCO-BRL), and 1× penicillin/streptomycin/glutamine (GIBCO-BRL). After the first mature iNSC clusters were observed, we either manually picked a mature iNSC clump or passaged and seeded whole dishes of cells onto either gelatin- or laminin-coated dishes and changed the medium every 24 h. Animal handling was in accordance with the Ministry for Primary Industries's animal protection guidelines and the German animal protection laws.

Differentiation of THP1 cells. The THP1 cell line was obtained from ATCC and maintained at 2×10^5 cells/ml in RPMI 1640 medium supplemented with 10% FBS and 2 mmol/L L-glutamine. THP1 cells (2×10^5/ml) were differentiated using 162 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO) for 3 d. Differentiation of PMA treated cells was enhanced after the initial 3 d stimulus by removing the PMA containing media then incubating the cells in fresh RPMI 1640 (10% FBS, 1% L-glutamine) for a further 5 d.

Coculturing with a cortical primary culture. Cortical neurons obtained from cerebral cortices of 17-d-old rat embryos were cultured as described previously (Beaudoin *et al.* 2012). Briefly, the brains of embryonic rats (E16–E18) were removed from the skull, and both cortices were dissected from the brain stem and transferred into Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; both, GIBCO-BRL). Cortical tissue was mechanically minced and centrifuged (320×*g* for 2 min). Tissue pellets were resuspended in Hanks—buffer free of Ca2+ and Mg2+ and centrifuged again. After resuspending, 1 ml of ethylenediaminetetraacetic acid (EDTA)/phosphate-buffered saline (PBS) solution (GIBCO-BRL) was added before additional centrifugation Trypsination was performed by adding 2 ml Trypsin/EDTA solution (0.25%/0.02%, GIBCO-BRL) to the 6 ml culture suspension and 2 min incubation at room temperature under gentle agitation. After resuspending in DMEM with 10% FBS, samples were squirted twice through a syringe equipped with a 23-gauge needle. After resuspending the cells in the same medium and counting with a Neubauer hemocytometer, 1,000,000 cells were seeded per well into a 12-well plate (1 ml/well) coated with glass coverslips. Cells were grown under standard conditions at 37°C and 5% CO_2 in Neurobasal medium with B27 (Invitrogen) and glutamine (Invitrogen). Immediately afterwards, iNSC were added. The coculture was maintained for 48 h before treatment with macrophage [Toku *et al.* 1998], and then, macrophages were added to cultures of cortical neuronal cells for 48 h.

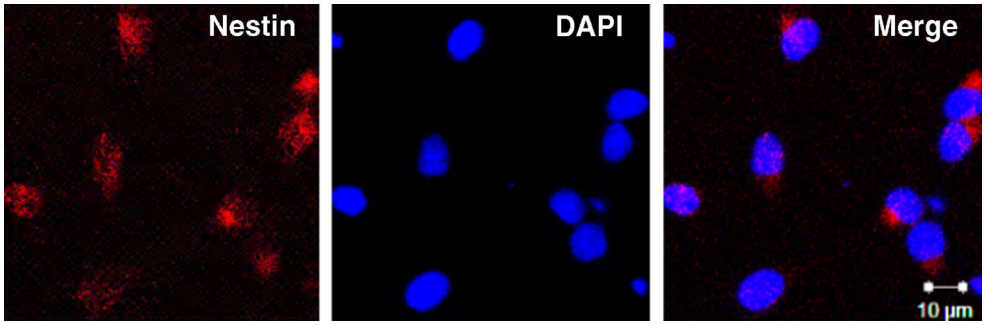

Figure 1. The differentiation potential of induced neural stem cells (iNSCs) in vitro. The differentiation potential of iNSCs was determined by immunocytochemistry with the antibodies neural stem cell marker (Nestin) and 4′,6-diamidino-2-phenylindole (DAPI), iNSC cultures contained Nestin-positive cells, indicating that fibroblasts were reprogrammed into an NSC-like state.

Coculture systems. To determine the interaction of cortical neuronal cells, iNSCs and macrophages, an indirect coculture system was assembled using transwell culture plates (0.4-μm pore size, 24-mm diameter; Corning/Costar, Corning, NY). This system allowed cells to maintain contact through shared culture medium without mixing the cell lines; 1×10^6 cortical neuronal cells were plated on the fibronectin-coated lower chamber, 2×10^5 iNSC were plated in the upper inserts, and 2×10^5 macrophages were plated in the upper inserts. The cocultures were maintained in media.

Immunocytochemistry and terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP-biotin nick-end labeling assay. The terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP-biotin nick-end labeling (TUNEL) assay using the In Situ Cell Death Detection Kit (Roche, Penzberg, Germany) was used to confirm apoptosis by demonstrating apoptotic bodies in primary cortical neurons. Briefly, primary cortical neurons were fixed with 4% formaldehyde and incubated at room temperature for 40 min. This was followed by several rinses in PBS and permeabilization in 0.2% Triton X-100 solution on ice for 5 min. Then, 50 μl of TUNEL reaction mixture was added on coverslips before being incubated for 60 min at 37°C in a dark humidified chamber. Finally, the coverslips were incubated with 4′,6-diamidino-2-phenylindole (DAPI; Sigma) for 20 min at room temperature and examined with a model LSM 510 confocal microscope (Carl Zeiss, Jen, Germany). Data were expressed as the ratio of apoptotic neurons to total nuclei.

For immunostaining of cortical neuronal cells, cells were plated onto fibronectin-coated glass coverslips in 10% FBS, for 4 h. Cells were then fixed with 4% paraformaldehyde. Fixed cells were washed in PBS containing 0.1% BSA and permeabilized with PBS-blocking buffer (PBS with 0.1% BSA and 0.3% Triton X-100) for 40 min at room temperature. Fixed cells were incubated with anti-NeuN primary antibody (Abcam, Newcastle, UK) or anti-Nestin primary antibody

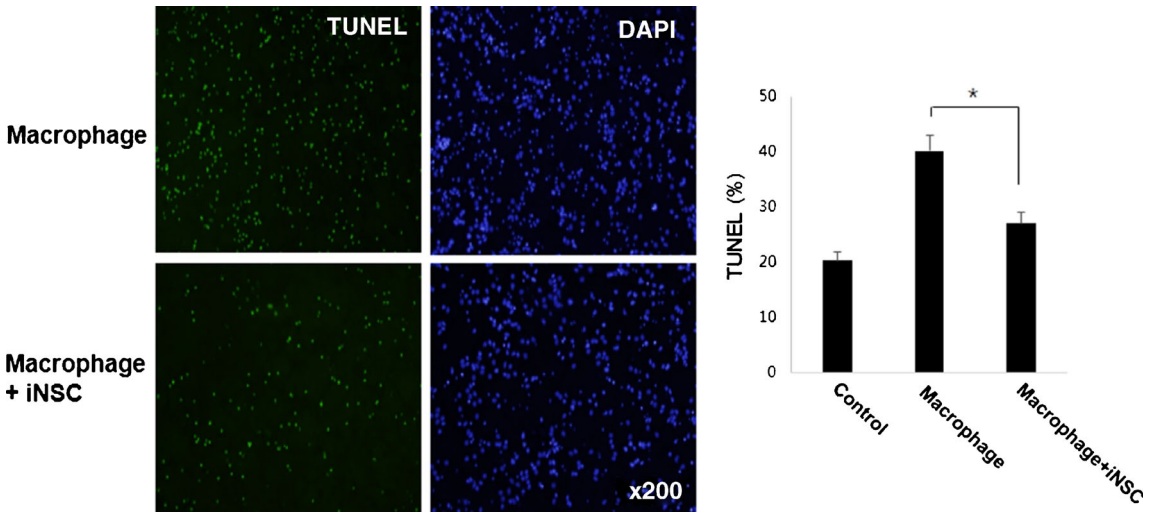

Figure 2. Detection of apoptosis in cortical neuronal cells by terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP-biotin nick-end labeling (TUNEL). To examine the apoptotic cells in the cortical neuronal cells, we performed TUNEL staining. The cultured group with induced neuronal stem cells (iNSC) had fewer apoptotic cells compared with the cultured group without iNSCs. Histograms show the average number of TUNEL- positive cells as percentage for each group.

(Millipore, Roma, Italy). The primary antibodies were detected with Alexa 596-conjugated goat anti-rabbit immunoglobulin (IgG; Molecular Probes, Eugene, OR) and cyanine-conjugated goat anti-mouse IgG. Data were expressed as the ratio of apoptotic neurons to total neurons.

Western blot analysis. Cells were resuspended in ice-cold cell lysis buffer (Cell Signaling Technology, Beverly, MA) with Protease Inhibitor Cocktail (complete mini tablet, Roche). Samples were incubated on ice for 30 min, and supernatants were recovered by centrifuging at 14,000 rpm at 4°C for 30 min. Protein concentrations were determined with a Bradford assay kit (Bio-Rad protein assay, Bio-Rad, Segrate, Italy) according to the manufacturer's instructions. Samples of 50 μg of protein per lane were separated in SDS-PAGE 10% polyacrylamide NuPAGE gels (Invitrogen) and transferred to PVDF membranes (Millipore, Rome, Italy). The membranes were blocked in Tris-buffered saline with 0.1% Tween-20 and 3% milk, incubated with anti-TNF-α (Cell signaling systems), anti-COX-2 (Cell signaling systems), and Actin (Sigma) antibodies. Next, the membranes were then incubated with horseradish peroxidase-conjugated anti-secondary IgG (Invitrogen) antibody and visualized using Super Signal West Pico Chemiluminescent Substrate (Pierce, IL). Densitometric quantification of the bands was performed using ImageJ software (version 1.29x, NIH, Bethesda, MD). Bars represent the mean (±SD) of three replicates.

Enzyme-linked immunosorbent assay. Quantification of cytokines in conditioned medium was performed by enzyme-linked immunosorbent assay (ELISAs; R&D systems, Minneapolis, MN) (Neuhoff *et al.* 2007) according to the manufacturer's instructions. Interleukin (IL)-1β, IL-6, TNF-α, tumor growth factor (TGF)-β, and VEGF expressions were analyzed. The optimal density of the color reaction was

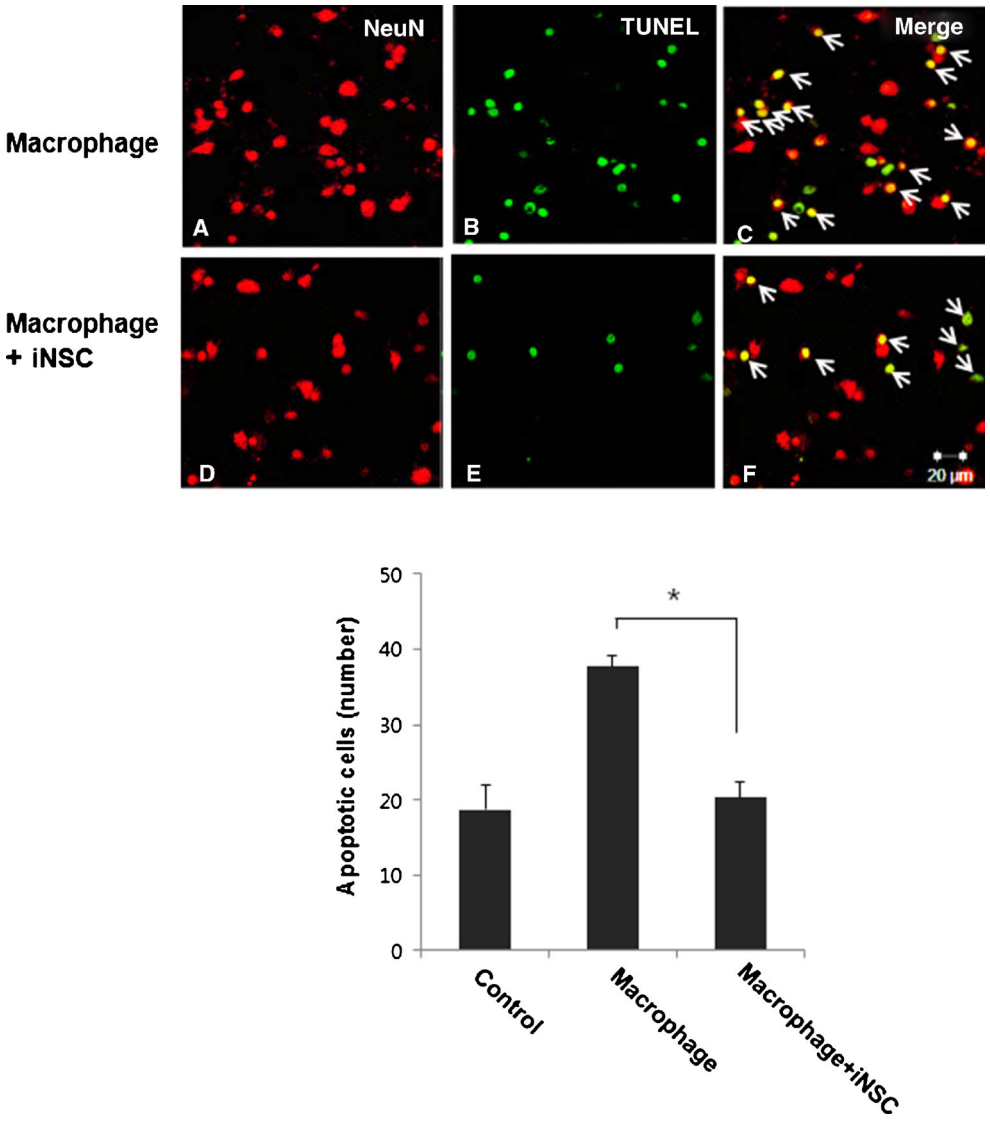

Figure 3. Detection of apoptosis in cortical neuronal cells by TUNEL and NeuN staining. We used immunocytochemistry to confirm the presence of neuronal apoptotic cells in cortical neuronal cell culture. The neuronal specific marker NeuN and TUNEL confirmed the occurrence of apoptosis. Scale bar 20um. Histograms show the average number of apoptotic cells as percentage for each group.

detected at a wavelength of 450 nm using a chemiluminescence reader. The background signal detected at 450 nm was subtracted from the values. Delta values were normalized to the extinction obtained from standard curves, and protein contents were calculated for each sample. The ELISA presented represents three independent experiment with similar results.

Statistical analyses. Data are shown as average and standard deviation. When comparing means between two groups, Student's *t* test was applied. The level of statistical significance was based on the *p* values ($p<0.05$).

Results

Induction of NSC fate on fibroblasts. Stem cell factors with neural specific transcription factors were used for the direct reprogramming of fibroblasts into iNSCs. The result was obtained within 5 wk of infection with this 5-factor (5F) combination: Brn4, Sox2, Klf4, c-Myc, and E47; BSKME. After 5 wk in neural differentiation medium, NSC cultures contained Nestin-positive cells, indicating that fibroblasts were successfully reprogrammed to an NSC-like state with defined factors. Immunofluorescent analysis demonstrated the expression of neural lineage markers (Fig. 1).

Anti-apoptotic effect of iNSCs. To determine whether macrophage activation is associated with apoptosis of cortical neuronal cells, we performed TUNEL assays to visualize DNA damage in single cells. Apoptotic cells were detected after coculture with iNSCs. Confocal microscopy showed fewer apoptotic cells in iNSC coculture (26.97±2.1%) than in cultures without iNSCs (40.06%±2.9%, both $P<0.05$; Fig. 2). The cultured cells were fixed and stained for a neuron-specific marker (NeuN) and for TUNEL assay. Coculture with iNSCs yielded fewer apoptotic neuronal cells (20.33±2.1) than did cultures without iNSCs (37.67±1.5, both $P<0.05$; Fig. 3). A

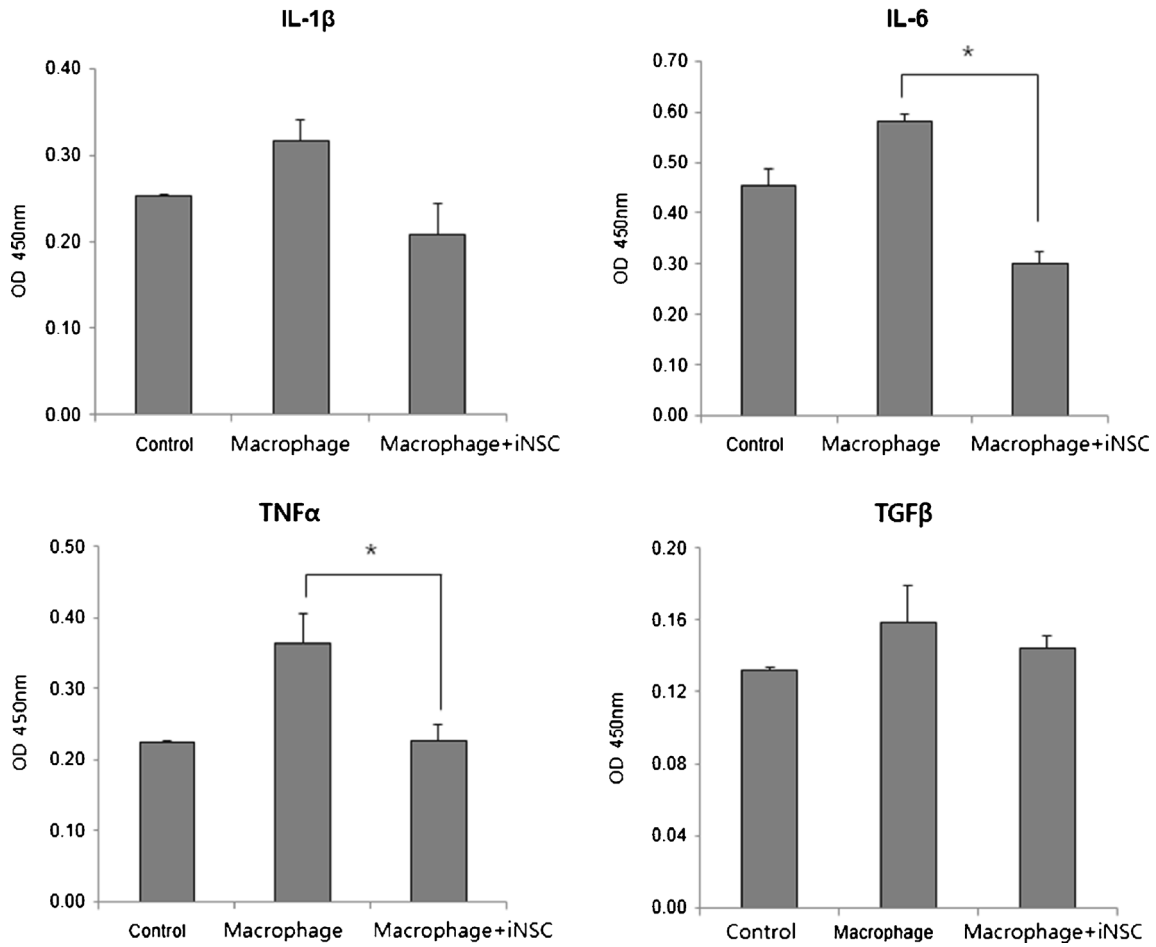

Figure 4. Inflammation markers in coculture supernatants. To examine whether iNSC coculturing results in the secretion of inflammatory cytokines, we performed enzyme-linked immunosorbent assays (ELISA). ELISA showed that cells cultured with iNSCs exhibited reduced secretion of IL-1β, IL-6, TNFα and TGFβ-1 in comparison to cells cultured without iNSCs.

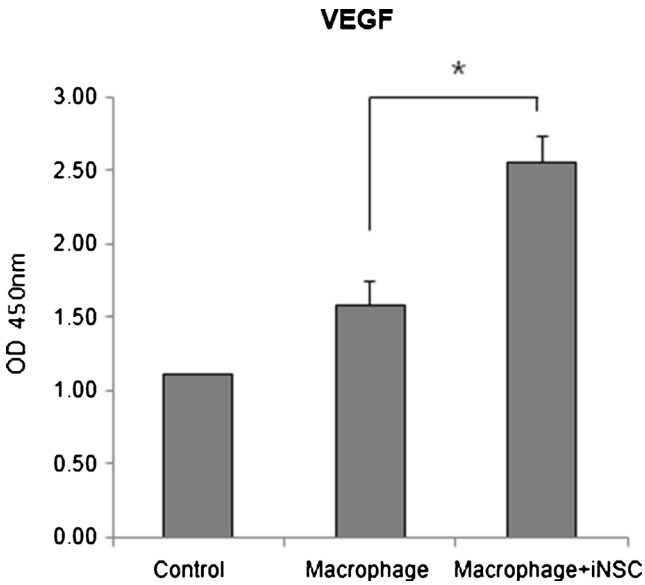

Figure 5. VEGF in coculture supernatants. To examine whether iNSC coculturing results in the secretion of VEGF, we performed enzyme-linked immunosorbent assays (ELISA). ELISA showed that VEGF levels were significantly higher in iNSCs cocultures than in cells cultured without iNSCs.

secondary antibody was used to rule out nonspecific staining (data not shown).

Anti-inflammatory proteins are elevated in the supernatants of cocultured cells. Stem cells release growth factors and cytokines important for repair (Takai *et al.* 1997; Labouyrie *et al.* 1999; Re *et al.* 2000). We performed ELISA to determine whether coculture with iNSCs triggers secretion of inflammatory cytokines. Indeed, iNSCs inhibited expression of IL-1β, IL-6, TNF-α, and TGF-β-1 ($P<0.05$; Fig. 4). Cells cultured with iNSCs showed lower IL-1β (0.209±0.03), IL-6 (0.3±0.02), TNF-α (0.227±0.02), and TGF-β-1 (0.144±0.01) expressions than did cells cultured without iNSCs (0.317±0.02, 0.58±0.01, 0.364±0.04, and 0.158±0.02, respectively; all $P<0.05$). ELISA results also showed a significant increase in VEGF expression in cells cultured with iNSCs (2.559±0.175) compared with those cultured without iNSCs (1.58±0.163, $P<0.05$; Fig. 5).

iNSCs induce an inflammatory response in cortical neuronal cells. COX-2 is an inflammatory regulator induced by proinflammatory cytokines (Kim *et al.* 2002). We performed Western blotting to determine the effect of iNSCs in COX-2 expression. Western blotting showed that cells cultured without iNSCs exhibited higher COX-2 (1.44±0.05) expression than did those cultured with iNSCs (1.15±0.02, $P<0.05$; Fig. 6).

Discussion

Inflammation is an intricate process mediated by the activation of various immune cells. Macrophages play a central role in mediating many immune-pathological phenomena during inflammation, including the overproduction of proinflammatory cytokines and inflammatory mediators, such as interleukin IL-1β, IL-6, TNF-α, and NO synthesized by inducible NO synthase (i-NOS), and prostaglandin PGE-2 synthesized by COX-2 (Uchida *et al.* 2004; Mohamed *et al.* 2011).

We show that cortical neuronal cells undergoing inflammation were protected by prior administration (by coculture) of iNSCs. We successfully generated self-renewing iNSCs that were nearly identical to brain tissue-derived NSCs in terms of their morphology, gene expression profile, and epigenetics. The iNSCs can be engrafted in the stem cell niches of the adult mouse brain, where they can continue to

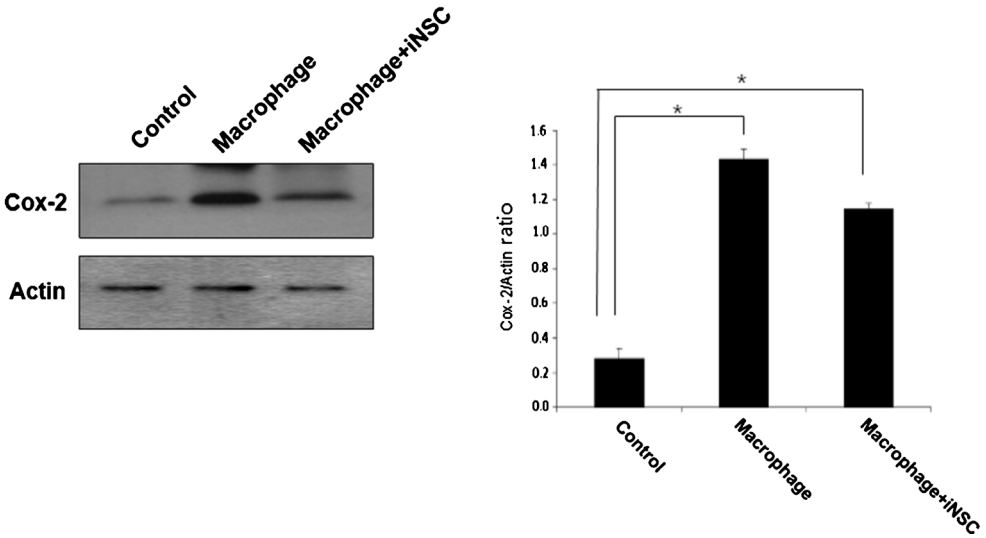

Figure 6. Anti-inflammatory effects are mediated by iNSCs activation. To evaluate the anti-inflammatory effect, we performed western blot analysis of COX-2. Western blot analysis of COX-2 showed reduced expression in cocultures with iNSCs.

proliferate. Therefore, these cells have potential therapeutic applications. In this study, we investigated the effect of iNSC on inflammation in cortical neuronal cells and the regulatory mechanisms that underlie these effects. We hypothesized that iNSC would protect cortical cells against macrophage-induced inflammation. Our findings support our hypothesis and show that prior administration of iNSCs can prevent cortical neuronal death via repression of COX-2.

Stem cells secret a large number of cytokines and growth factors (Takai *et al.* 1997; Labouyrie *et al.* 1999; Re *et al.* 2000). Stem cells show promise in clinical interventions, such as the use of embryonic stem cells (ESCs) and adult stem cells for wound repair and regeneration of injured tissue. ESCs have a capacity for self-renewal and plasticity, but their use is limited by political considerations. We propose that iNSCs are an attractive source of stem cells for use in wound repair and regeneration of injured tissue. Mesenchymal stem cells produce cytokine and chemokines during the inflammation (Singer and Clark 1999). Cytokines such as IL-1β, IL-6, and TNF-α are secreted by cells in different tissues and organs, linking their inflammatory response (Chamorro *et al.* 2007). IL-6 is initially generated by monocytes and neutrophils and initiate the healing response (Finnerty *et al.* 2006); IL-6 expression increases after injury. TNF-α is one of the most well-known cytokines in the inflammatory cytokine. TNF-α expression in the repair response is considered deleterious, and increased expression is detectable after injury. TNF-α is released by local macrophages, where it induces neutrophil recruitment and maturation (Feiken *et al.* 1995; Rumalla and Borah 2001). TGF-β plays an important role in inflammation and connective tissue regeneration. TGF-β levels increase at the site of injury (Kane *et al.* 1991) and upregulate VEGF (Riedel *et al.* 2007). We investigated whether iNSCs produce growth factors and inflammatory cytokines, all of which are regulated during regeneration (Singer and Clark 1999; Fausto 2000; Langer *et al.* 2008). In its final analysis, iNSCs decreased the level of inflammatory cytokines and secreted growth factor.

In this study, we explored the effect of COX-2 expression on macrophage in coculture with cortical neuronal cells and found that exposure of cultured neurons to iNSCs decreased inflammation; this protective effect is mediated by the regulation of COX-2. Cyclooxygenase (COX) catalyzes the conversion of arachidoic acid to prostaglandin, the precursor of a variety of biological effector (Smyth *et al.* 2009; Wang and Dubois 2010). COX-2 is induced by proinflammatory cytokines and growth factors and is involved mainly in the regulation of inflammatory responses (Araki *et al.* 2001; Kim *et al.* 2002). COX-2 expression is induced by various mitogenic and proinflammatory stimuli and is regulated by multiple pathways such as mitogen-activated protein kinases (MAPKs) and NFκB in many different cell types (Singer *et al.* 2003).

In conclusion, we used an inflammatory model of rat cortical neuronal cells. The experiments of the THP1-induced macrophage of cortical neuronal cells showed that iNSCs had potency to inhibit cellular inflammation. Prior treatment with iNSCs reduced expression of inflammatory cytokines associated with neurological recovery; this effect is mediated by the regulation of COX-2. We suggest that iNSCs may be used to treat brain diseases and neuronal damage. Further studies will test this hypothesis and support the development of therapeutics to cure neurodegenerative disease.

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