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Leukemia inhibitory factor inhibits the proliferation of primary rat astrocytes induced by oxygen-glucose deprivation

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Leukemia inhibitory factor (LIF) is a neuroprotective cytokine that is necessary for the normal development of astrocytes. Oxygen-glucose deprivation (OGD) can induce astrocyte proliferation by increasing hypoxia-inducible factor alpha (HIF-1α) and vascular endothelial growth factor (VEGF). Here, we studied whether LIF affects the proliferation of cultured primary rat astrocytes under OGD conditions by measuring EdU incorporation into astrocyte DNA and the expression of proliferating cell nuclear antigen (PCNA) mRNA and protein. Our findings show that low concentrations of LIF (5 and 10 ng/mL) significantly decreased EdU incorporation and downregulated the expression of PCNA mRNA and PCNA protein in astrocytes subjected to OGD. A low concentration of LIF (10 ng/mL) clearly inhibited astrocyte proliferation induced by OGD, while a higher concentration (50 ng/mL) had no effect. To investigate the mechanism of this inhibition by LIF (10 ng/mL), the expression of 3 related genes (LIF receptor, HIF-1α, and VEGF) was assessed using real-time PCR; VEGF protein expression was measured by Western blot. Our results indicate that LIFR mRNA was downregulated in astrocytes subjected to OGD. Interestingly, treatment with LIF further reduced LIFR mRNA expression in these cells. LIF treatment also decreased the expression of HIF-1α mRNA, VEGF mRNA, and VEGF protein induced by OGD. Low concentrations of LIF were observed to inhibit astrocyte proliferation induced by OGD.

Key words: leukemia inhibitory factor, astrocyte, oxygen-glucose deprivation, proliferation, VEGF

INTRODUCTION

Leukemia inhibitory factor (LIF) has a variety of important functions in the developing and mature nervous system (Hilton 1992, Heinrich et al. 2003). Studies on the central nervous system (CNS) have shown that this member of the IL-6 family of cytokines has neuroprotective properties. A range of beneficial effects of LIF on the oligodendrocyte lineage and myelination have been extensively studied and reported (Azari et al. 2006, Ishibashi et al. 2006, Slaets et al. 2008, Ishibashi et al. 2009). A number of studies have also shown that LIF is an important neuronal survival factor in the CNS and can promote neurogenesis and regeneration (Suzuki et al. 2005, Covey and Levison 2007, Ueki et al. 2008, Leibinger et al. 2009, Liu and Zang 2009, Simamura et al. 2010). LIF is thus a promising candidate for therapeutic use in diseases involving neurodegenerative and inflammatory components. Indeed, therapeutic effects of LIF have been observed in animal models of multiple sclerosis (MS), including models of experimental autoimmune encephalomyelitis (Slaets et al. 2010a,b, Gresle et al. 2012).

LIF plays a role in the normal development of astrocytes (Weible and Chan-Ling 2007, Cohen and Fields 2008, Asano et al. 2009, Fukushima et al. 2009). Apart from normal development, astrocytes proliferate in response to hypoxic ischemic injury of the CNS. This process, known as astrogliosis or glial scarring, interferes with subsequent neural repair and axonal regeneration and can cause myelination deficiencies. Hypertrophic reactive astroglio-

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sis is a well-known feature of human periventricular white matter injury (PWMI). The primary mechanism of myelination failure after PWMI is related to the burden of astrogliosis, whereby preoligodendrocytes fail to differentiate in diffuse astrogliotic lesions (Segovia et al. 2008, Franklin and Ffrench-Constant 2008, Huang et al. 2009, Buser et al. 2012). Diminishing astrocyte proliferation is thus an attractive goal in the prevention and treatment of such injuries.

Whether LIF affects the proliferation of astrocytes has been a matter of debate, as the few studies related to this subject have reported conflicting results. One study demonstrated that LIF did not enhance astrocyte proliferation but did counteract the growth stimulatory effects of epidermal growth factor (EGF) (Gadient et al. 1998). In another study it was found that LIF over-expression activates astrocytes but does not stimulate astrocyte proliferation (Kerr and Patterson 2004). Levison and cowerkers (2000) reported that IL-6-type cytokines enhanced EGF-induced astrocyte proliferation. One *in vivo* study reported that LIF administration during the neonatal period in rats induced cortical astrogliosis (Watanabe et al. 2004). The therapeutic value of LIF would be limited if this cytokine is proven to promote astrogliosis and the formation of glial scarring. Thus, understanding the effects of LIF on astrocyte proliferation and its mechanism of action is important to expanding treatment options for CNS diseases.

The mechanism of LIF action in astrocytes is not well understood. LIF signals through the heterodimeric complex of the LIF receptor (LIFR) and gp130 (Heinrich et al. 2003, Schmid-Brunclik et al. 2008) have shown that astrocytes proliferate in cultures deprived of oxygen and glucose (OGD), an *in vitro* model mimicking *in vivo* astrogliosis after hypoxic ischemic brain injury. Increases in the expression of hypoxia-inducible factor alpha (HIF-1α) and vascular endothelial growth factor (VEGF) are responsible for astrocyte proliferation after OGD. The VEGF gene is a major target of the transcription factor HIF-1. The expression of the α-subunit of this transcription factor is upregulated by oxygen. However, in chronic injury from hypoxia/ischemia HIF-1 may only be partially responsible for astrocyte responses mediated by VEGF (Schmid-Brunclik et al. 2008).

In this study, we aimed to determine the effect of LIF on the proliferation of primary rat astrocytes induced by oxygen-glucose deprivation. To further elucidate the mechanism whereby LIF affects astrocyte proliferation under OGD, we examined the expression of LIFR, HIF-1α, and VEGF in response to LIF treatment.

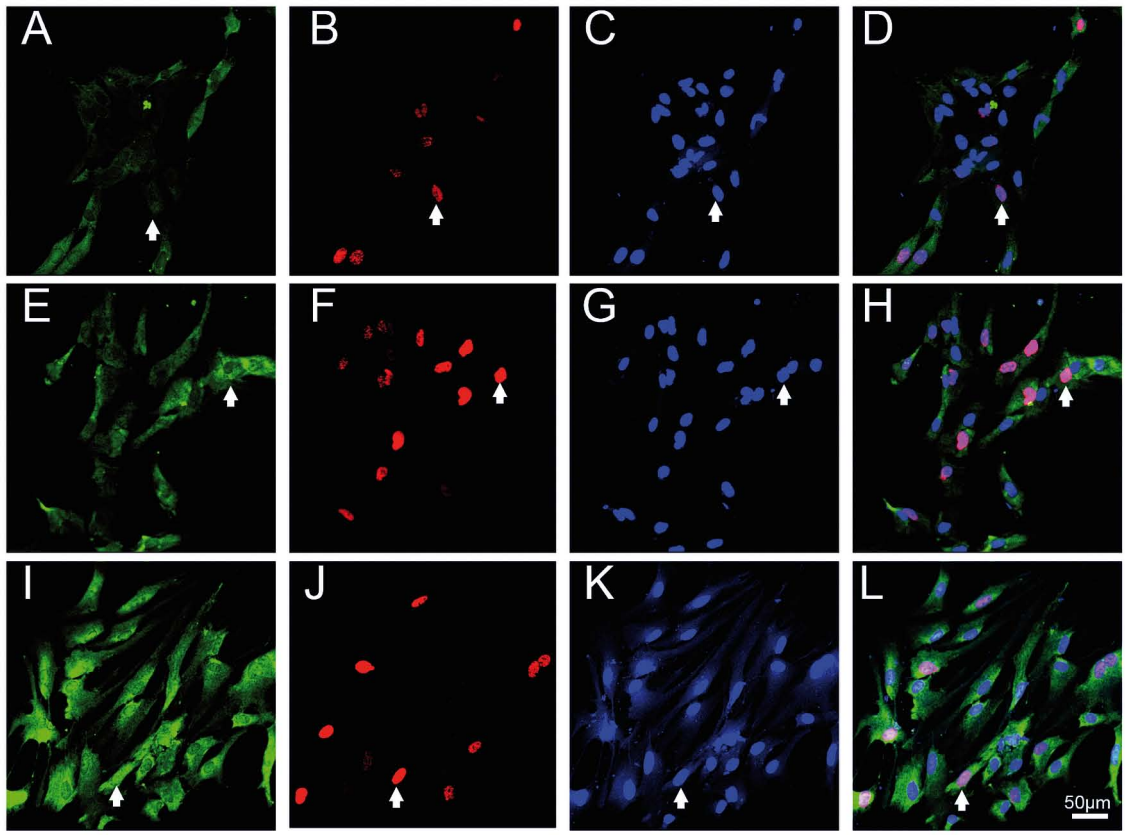
METHODS

Primary culture of astrocytes

All animal procedures were carried out in accordance with the NIH guidelines for care and use of animals in research, and the protocols were approved by the Local Animal Ethics Committee of China Medical University. Primary astrocytes were prepared from newborn Wistar rat pups as previously described (Chow et al. 2001) with modifications according to the method of Hertz and colleagues (1998). Briefly, neonatal rats were anesthetized by hypothermia, decapitated, and the cerebral cortices removed. The meninges were removed, and the cortices were minced and placed in culture medium. Homogenized tissue was vortexed, filtered through nylon mesh with pore sizes of 80 μm and 10 μm, and diluted in culture medium. The culture medium was Dulbecco's medium with 7.5 mM glucose, initially containing 20% horse serum. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. The culture medium was exchanged with fresh medium of similar composition on day 3 and subsequently every 3–4 days. On day 3, the serum concentration was reduced to 10%. Contaminating cells were removed by shaking the flasks on a rotary shaker overnight at 260 rpm. Astrocytes were passaged a maximum of 3 times and used at 80–90% confluence. The cultures displayed uniform (>95%) immunoreactivity to glial fibrillary acidic protein.

Hypoxia and glucose deprivation experiments

Hypoxia and glucose deprivation experiments were carried out as in a previous study (Schmid-Brunclik et al. 2008). All experiments were performed at least in triplicate. Briefly, primary astrocytes in glucose-free culture medium were subjected to 6 h of hypoxia (1% oxygen) balanced with N_2 in a 3-gas incubator (Thermo Scientific, Waltham, MA, USA) with a humidified atmosphere.



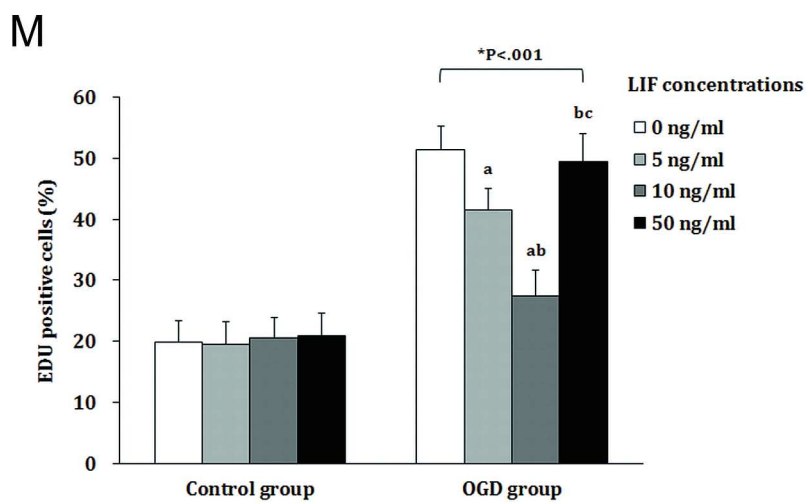


Fig. 1. Cells under all culture conditions were pulsed with EdU. The cultures were stained for GFAP (A, E, I) and EdU (B, F, J) and counterstained with DAPI (C, G, K). Merged color overlays are presented in panels D, H and L. Panels A–D illustrate astrocytes without LIF treatment in the normal culture group. Panels E–H illustrate astrocytes without LIF treatment in OGD culture group. Panels I–L illustrate astrocytes with 10 ng/mL LIF treatment in the OGD culture group. Arrows indicate astrocytes with immunofluorescent staining. Scale bar is 50 μm. Fig. 1M: Effect of LIF on the incorporation of EdU into astrocyte DNA. Data are presented as the mean ± SD by LIF concentration for the control and OGD groups (n=8 for each condition). (*) significant difference P<0.05 among LIF concentrations within the OGD group using the Kruskall-Wallis test with a Mann-Whitney U test for pair-wise comparisons; furthermore the actual calculation to reach statistical difference P<0.001 had been marked within figure. When compared to LIF concentrations with corresponding groups, including 0 ng/mL(^{a}P), 5 ng/mL (^{b}P), and 10 ng/mL (^{c}P), respectively, which P<0.01 (0.05/4) indicates a significant difference.

LIF administration

Rat recombinant LIF (Chemicon LIF 3010, USA) was dissolved in culture medium and used at low concentrations (5 and 10 ng/mL) and a relatively high concentration (50 ng/mL). Control cultures were stimulated with an equal volume of culture medium for the same time period. Primary astrocytes were treated with LIF for 6 h.

Incorporation of ethynyl deoxyuridine (EdU)

Briefly, astrocytes were passaged onto poly-D-lysine (20 mg/mL)-coated 24-well glass slides at a density of 5×10^3 cells/cm^2. Cells were cultured for 18 h in Dulbecco's medium with 7.5 mM glucose containing 10% horse serum and 1/1 000 EdU (volume ratio). The astrocytes were then randomly divided into normal and OGD culture groups, and the medium was replaced with fresh medium containing EdU. After culturing for 6 h, EdU detection was performed according to the protocol of the Cell-Light EdU DNA Cell Proliferation Kit (Guangzhou RiboBio, China). EdU-positive cells were counted in 15 randomly chosen fields of total 1 000 cells under 400× magnifications with 3.8 mm^2 microscopic observatory areas. All experiments were performed at least in triplicate.

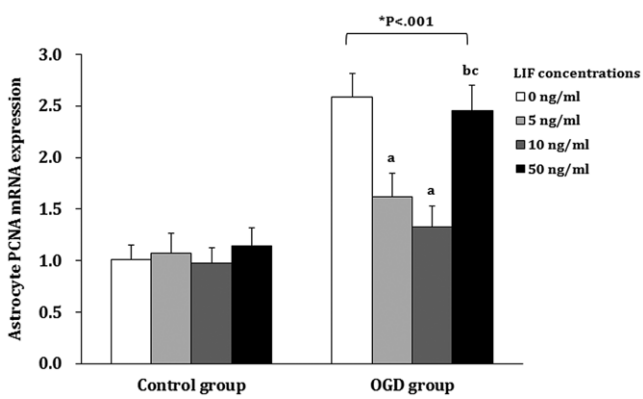


Fig. 2. PCNA mRNA expression in astrocytes under different culture conditions. Data are presented as mean ± SD by group for each LIF concentration. An asterisk (*) means a significant difference $P<0.05$ among LIF concentrations within the OGD group using the Kruskall-Wallis test with a Mann-Whitney U test for pair-wise comparisons; furthermore the actual calculation to reach statistical difference $P<0.001$ had been marked within figure. When compared to LIF concentrations with corresponding groups, including 0 ng/mL (^{a}P), 5 ng/mL (^{b}P), and 10 ng/mL (^{c}P), respectively, which $P<0.01$ (0.05/4) indicates a significant difference.

Combined EdU and GFAP immunolabeling

After EdU immunolabeling as described above, cells were incubated for 1 h at room temperature with mouse primary antibody to GFAP (1:200, Ab-6 Neomarkers, Fremont, CA, USA). Cells were rinsed 4 times in PBS and then incubated for 1 h at room temperature with a fluorescein-conjugated secondary antibody and DAPI. Positive cells were counted in 8 randomly chosen fields of total 1 000 cells under 400× magnifications with 3.8 mm^2 microscopic observatory areas. All experiments were performed at least in triplicate.

Proliferating cell nuclear antigen (PCNA) immunocytochemistry

Cells were briefly fixed with cold acetone and then incubated with mouse anti-PCNA (1:100 dilution, Boster Biotechnology, Wuhan, China) for 2 h. After rinsing, cells were incubated with goat anti-mouse IgG (1:200, Invitrogen, Beijing, China) for 1 h and then developed using an ABC kit. The immunoreactivity was visualized with 0.05% diaminobenzidine chromogen. All experiments were performed at least in triplicate.

Real-time PCR analysis

The expression of genes involved in LIF-induced astrocyte proliferation was investigated using real-time PCR. PCNA, LIFR, HIF-1, and VEGF mRNA expression was quantitatively analyzed in normal and OGD cells. Total RNA was extracted from primary astrocytes using RNAiso Plus reagent (Takara, Dalian, China) and quantified using a spectrophotometer. Following quantification, 2 μg of RNA was reverse transcribed to cDNA, and real-time quantitative PCR assays were conducted using an ABI PRISM 7500 real-time PCR System (Applied Biosystems, Foster City, CA, USA). PCR amplification was performed using the SYBR PrimeScript RT-PCR kit reagent (Takara, Dalian, China). The PCR conditions for LIFR, HIF-1α, and VEGF consisted of 45 cycles of denaturation at 95°C for 5 s and annealing and extension at 60°C for 30 s. For quantification, a standard curve was generated using various dilutions of the cDNA templates. Target mRNA levels were normalized to those of β-actin. The following oligonucleotide

primers were used: PCNA forward, 5'-TAAGGGCT-GAAGATAATGCTGAT-3'; PCNA reverse, 5'-CCT-GTTCTGGGATTCCAAGTT-3'; LIFR forward, 5'-CCAGATACTTGGCGAGAGTGGAG-3'; PCNA reverse, 5'-CGCTTGATGAACAGGACACATTG-3'; HIF-1α forward, 5'-TGGACCCTGGCTTTACT-GCTG-3'; HIF-1α reverse, 5'-GGCAATAGC TGCGCTGGTAGA-3'; VEGF forward, 5'-CCAGAT-TCAAGATCAG CCAGCA-3'; VEGF reverse, 5'-GCT-GTCCACATCAAAGCAGTACTCA-3'. Gene expression was analyzed using the 2-ΔΔCT method.

Western Blot analysis

Cells were scraped into lysis buffer [0.27 M sucrose, 2 mM EDTA (pH 8.0), 0.1% NP-40, in 0.6 M KCl, 150 mM NaCl, 150 mM HEPES (pH 7.5)] and centrifuged for 10 min at 16 000 g. Proteins were extracted and separated by denaturing SDS-PAGE. After transfer to nitrocellulose, membranes were incubated in 4% milk in PBS for 1 h at room temperature and then incubated overnight at 4°C with antibodies against VEGF (diluted 1:100; Boster Biotechnology, Wuhan, China). Following washes, membranes were incubated with a secondary horseradish peroxidase-conjugated antibody for 1 h at room temperature. Membranes were exposed to X-ray film after luminescent detection (25 µl of 90 mM coumaric acid in DMSO, 50 µl of 250 mM luminol in DMSO in 10 mL of 100 mM Tris, pH 8.5). For loading, control blots were stripped and reprobed for GAPDH. Quantification was carried out by densitometry using Quantity One software (BioRad Laboratories).

Statistical analysis

Data were expressed as mean ± standard deviation (SD). The differences between the 2 groups were analyzed using the Mann-Whitney *U* test. Differences between more than 3 groups were compared using the Kruskall-Wallis test with the Mann-Whitney *U* test for pair-wise comparisons. All statistical assessments were considered 2-tailed and significance was defined as $P<0.05$. An adjusted significance level of 0.01 (0.05/4) was also considered for the *post-hoc* pair-wise comparison approach. Statistical analyses were performed using SPSS 16.0 statistics software (SPSS Inc, Chicago, IL, USA).

RESULTS

Figure 1A–L shows cells under all culture conditions pulsed with EdU. Figure 1M shows the quantitative data for the effect of LIF on the incorporation of EdU into astrocyte DNA. At all LIF concentrations tested, the OGD groups had significantly higher EdU-positive cell rates than the corresponding control groups (all $P<0.05$; not shown in the figure), suggesting that OGD enhanced the EdU-

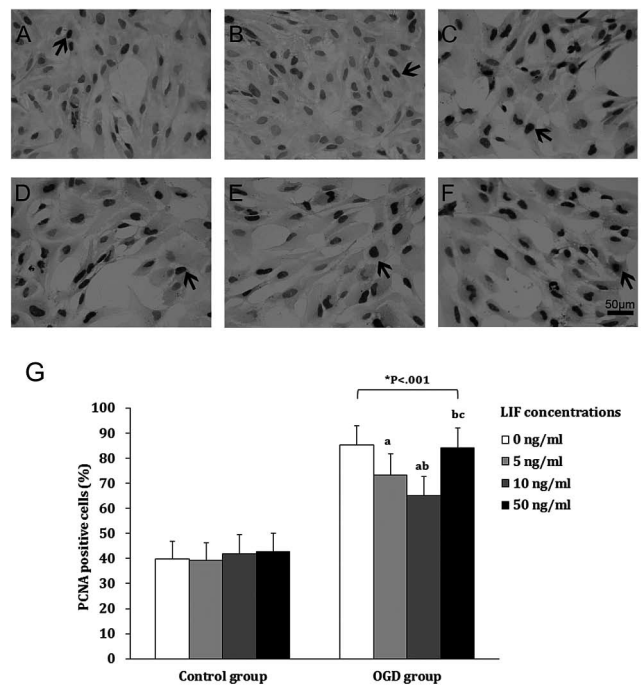


Fig. 3. PCNA immunocytochemistry staining of astrocytes under different culture conditions. (A) normal culture group without LIF treatment; (B) normal culture group with 10 ng/mL LIF treatment; (C) OGD culture group without LIF treatment; (D) OGD culture group with 5 ng/mL LIF treatment; (E) OGD culture group with 10ng/mL LIF treatment; (F) OGD culture group with 50 ng/mL LIF treatment. (G) depicts the comparison of the percentage of PCNA-positive cells under the various culture conditions. (*) significant difference $P<0.05$ among LIF concentrations within the OGD group using the Kruskall-Wallis test with a Mann-Whitney *U* test for pair-wise comparisons; furthermore the actual calculation to reach statistical difference $P<0.001$ had been marked within figure. When compared to LIF concentrations with corresponding groups, including 0 ng/mL (^{a}P), 5 ng/mL (^{b}P), and 10 ng/mL (^{c}P), respectively, which $P<0.01$ (0.05/4) indicates a significant difference. Arrows indicate astrocytes positive for PCNA immunostaining. Scale bar is 50 µm.

positive cell rate. Of the various LIF doses used in the OGD group, LIF at 10 ng/mL had the lowest EdU-positive cell rate (all $P<0.01$). In contrast, LIF did not affect the EdU-positive cell rate in the control group.

Figure 2 compares astrocyte PCNA mRNA expression levels in the control and OGD groups for each LIF concentration tested. The level of PCNA mRNA expression was significantly different between the control and OGD groups at all LIF concentrations (all $P<0.05$; not shown). In the OGD group, the PCNA mRNA expression level at LIF concentrations of 5 ng/mL and 10 ng/mL was lower than that at 0 ng/mL and 50 ng/mL (All P-values <0.01).

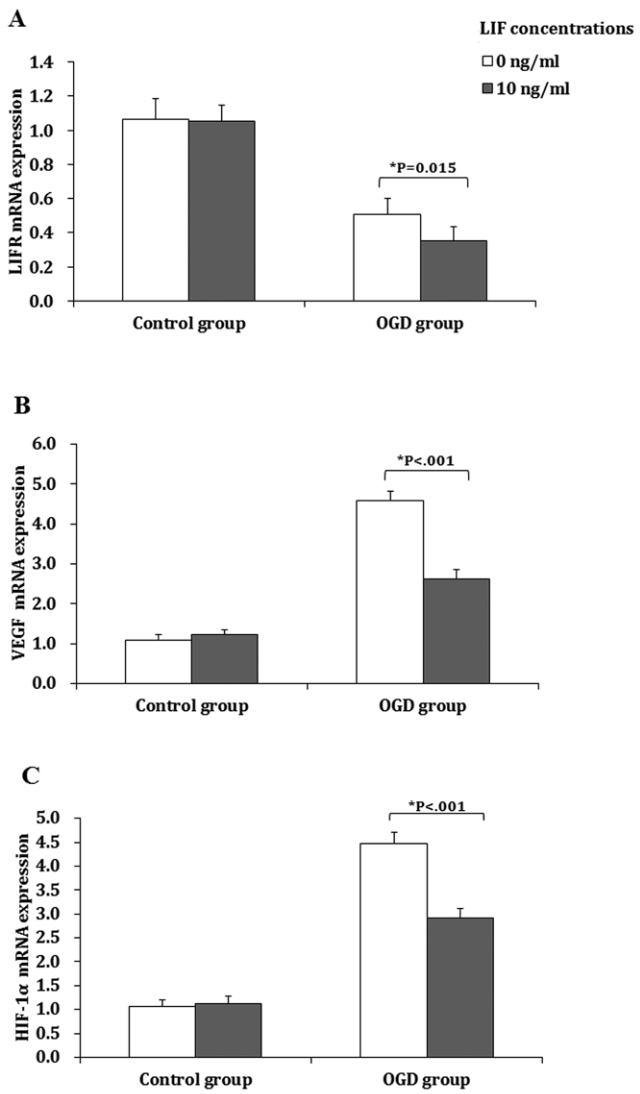


Fig. 4. Expression of LIFR, HIF-1α, and VEGF mRNA in astrocytes under different culture conditions. Data are presented as mean ± SD by group for each mRNA as follows: LIFR (A), VEGF (B), and HIF-1α (C). Comparisons between LIF concentrations were made using a Mann-Whitney U test for given control or OGD groups, respectively. (*) expression of LIFR, HIF-1α, and VEGF mRNA in astrocytes was significantly different between LIF concentrations within OGD group (all P-values <0.05).

Figure 3A–F shows PCNA immunochemistry staining of astrocytes under different culture conditions. Figure 3 G presents the quantitative data for PCNA-positive cells. Consistent with the EdU-positive cell rates, the OGD groups treated with various concentrations of LIF had significantly higher PCNA-positive cell rates than the corresponding control group (all $P<0.05$; not shown). Similarly, LIF did not affect the PCNA-positive cell rate in the control groups, while LIF at 10 ng/mL resulted in the lowest PCNA-positive cell rate of all the OGD groups (all $P<0.01$).

Figure 4A–C compares the expression of LIFR, VEGF, and HIF-1α mRNA in astrocytes in 4 different groups: control, control + LIF 10 ng/mL, OGD, and OGD + LIF10 ng/mL. The mean expression levels of LIFR, VEGF, and HIF-1α mRNA differed significantly different between the 4 groups (all P-values <0.001). Cells in the OGD groups (OGD and OGD+LIF 10 ng/mL) expressed lower levels of LIFR mRNA but higher levels of HIF-1α mRNA than did the controls (control and control + LIF10 ng/mL) (all P-values <0.01; not shown in figure). VEGF mRNA was expressed at higher levels in cells of the OGD groups (OGD and OGD + LIF 10 ng/mL) than that in controls (control and control + LIF10ng/mL) (all P-values <0.01). However, VEGF mRNA expression decreased in the OGD group when combined with LIF. The expression level of VEGF mRNA in astrocytes treated with both OGD and LIF (10 ng/mL) was significantly lower than of astrocytes treated with OGD alone (4.48 ± 0.23 vs. 4.58 ± 0.24; $P<0.01$) (Fig. 4).

Figure 5A shows photos of western blots. Figure 5B presents the quantitative data for western blotting. The OGD groups treated with or without 10 ng/mL LIF expressed significantly higher levels of VEGF protein than the corresponding control groups (all $P<0.05$; not shown). Comparing OGD groups, 10 ng/mL LIF significantly suppressed the VEGF protein expression induced by OGD ($P<0.05$).

DISCUSSION

We cultivated primary rat astrocytes under oxygen and glucose deprivation conditions (1% O_2 and glucose withdrawal) for 6 h and successfully induced astrocyte proliferation, mimicking astrogliosis after hypoxia and ischemia injury, as demonstrated by Schmid-Brunclik and coauthors (2008). Increased proliferation of astrocytes after OGD was confirmed by increased EdU incorporation, which indicates DNA replication, and by increased expression of PCNA mRNA and protein. LIF administration at relatively low concentrations resulted in decreased astrocyte proliferation, which was accompanied by decreased expression of LIFR, HIF-1α, and VEGF mRNA, as well as decreased VEGF protein expression.

Our data add to the convincing evidence that subjection of astrocytes to OGD for 6 h is a strong stimulus for proliferation; preventing astrocytic proliferation, especially within the first 6 h of ischemic injury, thus may be beneficial to reduce scarring (Schmid-Brunclik et al 2008).

We investigated how different concentrations of LIF affect astrocyte proliferation. Our results showed that LIF (5, 10, or 50 ng/mL) had no effect on astrocytes in normal culture. Lower concentrations of LIF (5 and 10 ng/mL) inhibited the astrocyte proliferation induced by OGD, with dramatic reductions in proliferation at a concentration of 10 ng/mL. Gadient and others (1998) demonstrated that LIF increases astrocyte survival but does not influence astrocyte proliferation, and that the addition of LIF significantly reduces the proliferative response triggered by EGF. High doses of LIF (10 nM) showed further inhibitory effects and did not appear to be toxic. IL-6 and another Il-6–related cytokine, ciliary neurotrophic factor (CNTF), enable resting astrocytes to become proliferative if glial mitogens are present. Levison and coworkers (2000) has shown that IL-6–type cytokines enhance the proliferation of EGF-stimulated "old" astrocytes (cultivated *in vitro* for 10 weeks) but mildly inhibits EGF-stimulated "young" astrocytes (cultivated *in vitro* for 3 weeks). A recent study has shown that the combination of EGF, LIF, and TGFß1 reconstitutes astrogliogenesis but, interestingly, LIF (10 ng/mL) suppresses the proliferative effects of EGF and TGFβ1 on astrocytes (Bain et al. 2010). Together with our present findings, these observations indicate that for astrocytes cultivated in vitro for 3 weeks, LIF (10 ng/mL) inhibits EGF-stimulated astrocyte proliferation.

The effects of LIF on myelination have shown a nonlinear concentration-response relationship, which is not unusual for cytokines in many biological processes. In one study, a significant concentration-dependent increase in myelination was observed upon LIF treatment (Stankoff et al. 2002). However, in another study, LIF treatment was reported to inhibit myelin formation (Park et al. 2001). This controversy was resolved by studying a wide range of LIF concentrations, and the results revealed that myelination was significantly increased by low concentrations of LIF (<5 ng/mL) but inhibited by higher concentrations (>5 ng/mL). Together with our data, these findings suggest that the effect of LIF on astrocyte proliferation is concentration-dependent.

In vivo studies investigating the effect of LIF on astrocyte proliferation are rare. Although Watanabe

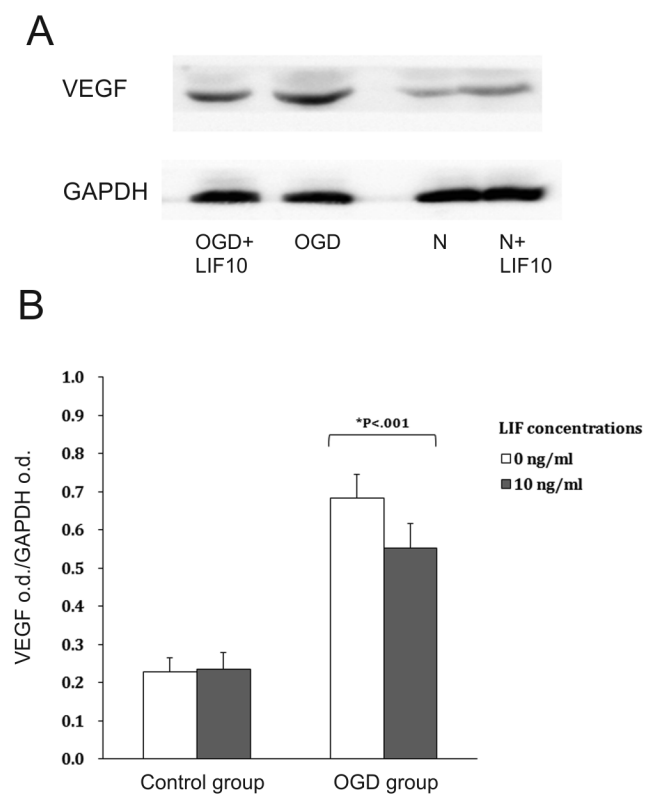


Fig. 5. Expression of VEGF protein in astrocytes under different culture condition. (A) The Western blots photos. Quantitation results were shown in (B). (B) Data are presented as mean ± SD by LIF concentration for control and OGD groups (n=8 for each condition). Differences between concentrations within groups were compared using a Mann-Whitney U test. $^{*}P<0.05$ indicates a significant difference when compared to no LIF treatment in the OGD group.

and colleagues (2004) reported that LIF administration in neonatal rats induced cortical astrogliosis, their study did not confirm that the observed increase in GFAP-positive cells was directly the result of astrocyte proliferation; it is possible that LIF enhanced the survival of astrocytes rather than induced their proliferation, as demonstrated by Gadient and others (1998). The investigation by Watanabe and coauthors (2004) showed that higher doses of LIF (1.0–0.5 mg/kg) led to severe weight loss and even death. However, a daily intraperitoneal dose of 25 μg/kg in mice has been shown to be well tolerated, without obvious systemic side effects (Azari et al. 2003). Based on these studies, we conclude that relatively low doses of LIF should be used for investigating its protective and inhibitory effects *in vivo*.

To investigate the mechanism whereby LIF inhibits astrocyte proliferation after OGD, we investigated the effect of LIF on the expression of LIFR, VEGF, and HIF-1α mRNA. Our data confirm that OGD upregulates VEGF and HIF-1α mRNA expression in primary astrocytes. Our data show, for the first time, that OGD downregulates the expression of LIFR mRNA. Suzuki and coauthors (2005) also showed that LIFR expression was significantly decreased after focal cerebral ischemia in rats, but this effect was observed mainly in neurons; the expression of LIFR protein was well preserved in the high-dose LIF group. Treatment of rat cerebral microvessel endothelial cells (RBE4) with 10 ng/mL LIF for 2 h also downregulated LIFR expression (Yu et al. 2007). Investigators demonstrated that in mouse embryonic stem cells under conditions of hypoxia, HIF-1α exerts an inhibitory effect on LIFR transcription by directly binding to the reverse hypoxia-responsive element located in the LIFR promoter (Jeong et al. 2007).

Studies on the regulation of retinal vascular development have shown that LIF secreted from endothelial cells acts cooperatively with oxygen as a negative feedback signal. The authors of these studies propose that LIF inhibits VEGF expression and astrocyte proliferation by inducing astrocytes to differentiate and express glial fibrillary acidic protein, thus counteracting their response to hypoxia (Kubota et al. 2008, Kubota and Suda 2009). Our data show that LIF does not affect VEGF expression in astrocytes under normal conditions but decreases the high level of VEGF induced by OGD, albeit to levels that are still higher than normal. VEGF is also a survival factor in many cells, and different levels of VEGF have different effects on astrocytes. We propose that LIF treatment may thus allow for the fine-tuning of VEGF protein expression, which could have advantages over complete inhibition of VEGF expression for preventing injury progression and promoting recovery. Our results indicate that LIF might only inhibit excessive astrocyte proliferation. Such a mechanism makes sense in light of the beneficial roles of astrogliosis, such as inducing production of various neurotrophic factors that assist in neuron survival.

Our study has some limitations. Observing the effects of a wider range of LIF concentrations would be useful for understanding the mechanism of LIF action. Further investigations are needed to elucidate the mechanism of LIF action on VEGF expression and astrocyte proliferation. It will be important to study the *in vivo* effects of LIF on astrogliosis to determining appropriate doses and exposure times for the therapeutic use of LIF. We used only one fixed time point of OGD 6 h and therefore did not obtain any data with regard to how OGD downregulates LIFR mRNA over time.

CONCLUSION

Our results show that relatively low concentrations of LIF inhibit astrocyte proliferation induced by OGD. Since LIF can promote myelination and inhibit astrocyte proliferation *in vitro*, we believe that LIF is a potential candidate for the treatment of PWMI.

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