Published in final edited form as:

J Neurosci Res. 2010 March ; 88(4): 758–763. doi:10.1002/jnr.22256.

Astrocytes Protect Oligodendrocyte Precursor Cells via MEK/ERK and PI3K/Akt Signaling

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Abstract

Accumulating evidence suggest that trophic coupling among different cell types in the brain is required to maintain normal CNS function. Here we show that astrocytes secrete soluble factors that can be oligodendrocyte-supportive. Oligodendrocyte precursor cells (OPCs) and astrocytes were prepared from neonatal rat brain and cultured separately. We conducted cell culture medium-transfer experiments to examine whether astrocytes secrete OPC-protective factors. Conditioned media from astrocytes protected OPCs against H₂O₂-induced oxidative stress, starvation, and oxygen-glucose deprivation. This protective effect may be mediated in part via ERK and Akt signaling pathways. Astrocyte-conditioned media upregulated the phosphorylation levels of ERK and Akt in OPC cultures. Blockade of ERK or Akt signaling with U0126 or LY294002 cancelled the OPC-protective effects of astrocyte-conditioned media. Taken together, these data suggest that astrocytes are an important source for oligodendrocyte-supportive factors. Coupling between these two major glial components in brain may be vital for sustaining white matter homeostasis.

Keywords

astrocyte; oligodendrocyte precursor cell; cell-cell interaction; white matter; stroke

Dynamic regions of ongoing new brain cell generation are present in adult mammalian brains. Active neuroblasts and neurogenesis has been demonstrated to persist in the subventricular and subgranular zones (Gross 2000; Zhang et al., 2005; Lee et al., 2006; Chopp et al., 2007). And there are widely distributed subpopulations of oligodendrocyte precursor cells (OPCs) that participate in white matter maintenance and repair by generating new oligodendrocytes (OLs) (Nishiyama et al., 1999, 2001; Chang et al., 2000; Levine et al., 2001). Emerging data now suggest that these active processes of brain cell turnover and genesis may be mediated by cell-cell interactions. In the so-called neurovascular niche, neurogenesis is sustained by the trophic coupling between brain endothelium and neuroblasts (Iadecola 2004; Greenberg and Jin 2005; Chopp et al., 2007; Zacchigna et al., 2008; Zlokovic 2008). Recently, we suggested that a similar oligovascular niche might also exist, wherein trophic support from brain endothelium help sustain OPCs and defend them against cellular stress and injury (Arai and Lo, 2009).

Although interactions between the vascular compartment and brain cells are fundamentally important, it is clear that astrocytes should also play a vital role in cell-cell signaling and homeostasis. Astrocytes mediate the coupling between neuronal activity and cerebral blood

flow that underlie the hemodynamic responses during brain activation (Takano et al., 2006; Koehler et al., 2009). Astrocytes modify brain endothelial cells to produce the blood-brain barrier phenotype (Abbott et al., 2006; Seifert et al., 2006). In gray matter, astrocytes interact with neurons to refine patterns of neuro-transmitter release and reuptake (Newman, 2003; Theodosios et al., 2008). In white matter, astrocytes help maintain the functional integrity of oligodendrocytes (OLs) (Noble et al., 1994; Yonezawa et al., 1996; Corley et al., 2001).

In this study, we tested the hypothesis that astrocytes provide trophic support for OPCs and help protect these important precursors against injury. We assessed the ability of astrocyte-conditioned media to protect OPCs against 3 different types of cellular insults: Oxidative stress induced by H₂O₂, starvation, and oxygen-glucose deprivation.

MATERIALS AND METHODS

Cell culture

Oligodendrocyte precursor cells (OPCs) were prepared as previously described (van Leyen et al., 2008; Arai and Lo, 2009). Briefly, cerebral cortices from 1–2 day old Sprague-Dawley rats were dissected out, minced, and digested. Dissociated cells were plated in poly-D-lysine-coated 75-cm² flasks, and maintained in Dulbecco's Modified Eagle's medium containing 20% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin. After the cells were confluent (~10 days), the flasks were shaken for 1 hour on an orbital shaker (220 rpm) at 37°C to remove microglia. They are then changed to new medium and shaken overnight (~20 hr). The medium was collected and plated on non-coated tissue culture dishes for 1 hour at 37°C to eliminate contaminating astrocytes and microglia. The non-adherent cells were collected and replated in Neurobasal Media containing glutamine, 1% penicillin/streptomycin, 10 ng/mL PDGF, 10 ng/mL FGF, and 2% B27 supplement onto poly-DL-ornithine-coated plates. Four to 5 days after plating, the OPCs were used for the experiments. Primary astrocyte cultures were prepared from cerebral cortices of 2-day-old neonatal Sprague-Dawley rats as previously described (Arai et al., 2003). Briefly, dissociated cortical cells were suspended in Dulbecco's modified Eagle medium containing 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin and plated on 25 cm² flasks at a density of 600,000 cells/cm². Monolayers of type 1 astrocytes were obtained 12–14 days after plating. Nonastrocytic cells such as microglia and neurons were detached from the flasks by shaking and removed by changing the medium. Astrocytes were dissociated by trypsinization and then reseeded on 25-cm² flask at a density of 20,000 cells/cm². In this system, more than 95% of the cells were identified as type 1 astrocytes by GFAP staining and their flattened, polygonal morphology. NIH-3T3 mouse fibroblast cell lines were maintained in Dulbecco's Modified Eagle's medium containing 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin.

Preparation of astrocyte-conditioned media

When astrocytes were 90–95% confluent, the cells were once washed with PBS followed by culturing in Neurobasal Media containing glutamine, 1% penicillin/streptomycin and 2% AO-free B27 supplement for 24 hr. Then the culture medium was collected and filtered using 0.20 µm filters. The astrocyte-conditioned medium was kept in –80°C until use.

Immunocytochemistry

After OPCs reached 70–80% confluence, they were washed with PBS (pH 7.4), followed by 4% paraformaldehyde for 10 min. After being further washed three times in PBS, they were incubated with 1% bovine serum albumin in PBS containing 0.1% Triton X-100 for 1 hr. Then cells were incubated with primary antibodies against the OPC marker A2B5 (1:100)

and NG2 (1:100), or the astrocytic marker GFAP (1:100) at 4°C overnight. After washing with PBS, they were incubated with secondary antibodies conjugated with fluorescein isothiocyanate for 1 hr at room temperature. Finally, nuclei were counterstained with 4, 6-diamidino-2-phenylindole (DAPI). Cells were rinsed and coverslips were placed. Immunostaining was analyzed with a fluorescence microscope (Olympus BX51) interfaced with a digital charge-coupled device camera and an image analysis system.

Western blotting

OPC cultures were rinsed twice with ice-cold phosphate buffered saline and the cells were collected into cell lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerolphosphate, 1 mM Na_3VO_4 , 1 $\mu\text{g}/\text{mL}$ leupeptin, and 1 mM PMSF). Cell lysates were then homogenized and centrifuged at 10,000g for 10 min at 4°C and protein concentration in the supernatant was determined with the Bradford assay (Bio-Rad). Samples were heated with equal volumes of SDS sample buffer (Novex) and 10 mM DTT at 95°C for 5 min, then each sample (20 ng per lane) was loaded onto 4–20% Tris-glycine gels. After electrophoresis and transferring to polyvinylidene difluoride membranes (Novex), the membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 and 0.2% I-block (Tropix) for 90 min at room temperature. Membranes were then incubated overnight at 4°C with monoclonal anti-p-Akt antibody (1:1,000, Cell Signaling Technology), anti-Akt antibody (1:1,000, Cell Signaling Technology), anti-pERK1/2 antibody (1:1,000, Cell Signaling Technology), or anti-ERK antibody (1:1,000, promega) followed by incubation with peroxidase-conjugated secondary antibodies and visualization by enhanced chemiluminescence (Amersham).

Determination of cell proliferation

Cell proliferation was assessed by WST reduction assay (Dojindo). The cells were incubated with 5% WST solution for 1.5 hr at 37°C. Then the absorbance of the culture medium was measured with a microplate reader at a test wavelength of 450 nm and a reference wavelength of 630 nm.

Statistical analysis

Statistical significance was evaluated using the unpaired t-test to compare differences between two groups, and ANOVA followed by Bonferroni tests for multiple comparisons. Data are expressed as mean $\pm$ SD. $P < 0.05$ was considered significant. All experiments were repeated at least three times in triplicate.

RESULTS

Astrocyte-conditioned Media Protect Oligodendrocyte Precursor Cells Against H_2O_2 -induced Oxidative Stress

Immunocytochemical staining confirmed that our culture system of oligodendrocyte precursor cells (OPCs) was not contaminated by astrocytes (Fig. 1). With this pure-OPC culture system, we examined whether astrocytes are protective to OPCs against H_2O_2 -induced oxidative stress. We used a media transfer system that was previously used to dissect trophic coupling between OPC and cerebral endothelium (Arai and Lo, 2009). Cultured rat astrocytes were grown separately and astrocyte-conditioned media were obtained. Control media were prepared from empty wells without astrocytes. H_2O_2 was used as an oxidative stress inducer. 24-hour- H_2O_2 treatment induced OPC death assessed by cell morphology observations and a WST assay (Figs. 2 and 3A). OPCs treated with astrocyte-conditioned media showed significant resistance to H_2O_2 -induced injury (Figs. 2 and 3A). However, because the number of OPC cells increased in the presence of astrocyte-

conditioned media even in the absence of H₂O₂ insult, it was not clear whether this result was due to “protection” or “proliferation”. Therefore, the OPC survival ratio was recalculated based on the data from control media or astrocyte-conditioned media alone. When normalized for this baseline, astrocyte-conditioned media still showed a slight OPC protective effect (Fig. 3B), suggesting that astrocyte-conditioned media may have both “protective” and “proliferative” effects in OPC cultures.

Because any cultured cell can produce many types of trophic factors, it was important to ask whether this type of conditioned-media-OPC-protection was specific for astrocytes. Therefore, conditioned media from NIH-3T3 cells (a fibroblast cell line) was also tested. This fibroblast conditioned media had no detectable effects on OPC survival (Fig. 3C), suggesting that the astrocyte findings were not an artifact of our cell culture system.

Astrocyte-conditioned Media Protect Oligodendrocyte Precursor Cells Against Serum-starvation and Oxygen/Glucose Deprivation

Although H₂O₂ is a well-characterized reagent to induce oxidative stress in cell culture, it is still a somewhat artificial stress. To further investigate OPC protection by astrocytes, we tested this idea in two other OPC injury models: Deprivation of supplemental factors in the culture media (starvation) and oxygen-glucose deprivation (OGD). Maintaining OPCs in vitro requires supplemental factors in the culture media such as FGF and PDGF. Removing these factors from culture media induced OPC death over a course of 5 days (Fig. 4A). Treatment with astrocyte-conditioned media significantly improved OPC survival under these starvation conditions, and in fact partially promoted OPC proliferation as well (Fig. 4A). Similarly, astrocyte-conditioned media was also OPC protective against 4 hr OGD and 20 hr reoxygenation/recovery (Fig. 4B).

OPC-protective Effects of Astrocyte-conditioned Media are Mediated by MEK/ERK and PI3K/Akt Pathways

Both MEK/ERK and PI3K/Akt are known as to be pro-survival cell signaling pathways (Stariha and Kim 2001; Cui and Almazan 2007; Zhang et al., 2008). Hence, we asked whether the OPC protection in our system involved these two pathways. Twenty-minute exposure to astrocyte-conditioned media induced the phosphorylation of ERK and Akt in cultured OPCs, with no change in total ERK and Akt levels (Fig. 5A). The MEK inhibitor U0126 and the PI3K inhibitor LY294002 blocked these ERK and Akt responses, respectively (Fig. 5A). To assess causality, we next asked whether blockade of ERK or Akt signaling in OPCs can negate the protective effects of astrocyte-conditioned media. U0126 or LY294002 was applied to OPCs 30 min before injury with H₂O₂. The OPC-protective effects of astrocyte-conditioned media were significantly cancelled by the pre-treatment of either U0126 or LY294002, indicating that both MEK/ERK and PI3K/Akt are involved (Fig. 5B).

DISCUSSION

Within the concept of neurovascular unit, different cell types interact closely not just for coordinated brain function, but also for cellular survival (Park et al., 2003; Ward and Lamanna 2004; Lok et al., 2007). Recently, it was shown that brain endothelial cells secreted trophic factors that help defend neurons against stress and injury, thus providing a basis for cellular cross-talk in gray matter (Dugas et al., 2008; Guo et al., 2008). Here, we showed that astrocytes may be an important source of secreted factors that support OPCs, thus providing an analogous model for cellular defense in white matter. In this study, we subjected cultured rat OPCs to three different insults: Hydrogen peroxide (H₂O₂), supplemental factor deprivation (starvation), and OGD (in vitro ischemia). OPCs treated

with conditioned-media from astrocytes showed significant resistance against all three insults, suggesting that astrocytes may defend OPCs under a broad range of pathologic conditions.

Our findings may be significant for several reasons. First, OPCs are known to comprise an endogenous pool for replacement of OLs throughout the adult brain (Nishiyama et al., 1999, 2001; Chang et al., 2000; Levine et al., 2001). Hence, interactions between these two major classes of glia in mammalian brain may provide a key substrate for sustaining white matter homeostasis. Secondly, it was recently proposed that OPCs may also have the potential of maturing into some subsets of cortical projection neurons (Rivers et al., 2008). So the ability of astrocytes to protect OPCs may have broader relevance for gray matter physiology and pathology as well. Thirdly, our studies here identify ERK and Akt signaling as two candidate pathways that underlie this protective coupling from astrocyte to OPC. Both of these pathways are known to be “pro-survival” (Stariha and Kim 2001; Cui and Almazan 2007; Zhang et al., 2008), so these findings make sense. But more importantly, they provide potential mechanisms that one might be able to target when the need to rescue OPCs components arises. Finally, our data here suggest that astrocytes may comprise a target for gene therapies. This glial population is widely distributed in mammalian brain. If it is true that they comprise a critical source for soluble signaling to other brain cells (neurons, OLs, OPCs etc), then targeting them with beneficial pro-survival genes may be a potential therapeutic approach against a wide spectrum of CNS diseases including stroke, brain injury and neurodegeneration.

Taken together, our findings support the concept of cytoprotective signaling between astrocytes and OPCs. But there are a few caveats that require further consideration. First of all, we did not identify the soluble factor(s) that mediate this astrocyte-OPC signaling. But because PI3K/Akt and MEK/ERK signaling pathways were involved, it might be reasonable to assume the involvement of growth factors. Astrocytes can certainly release many types of growth factors (Abbott et al., 2006). For example, basic fibroblast growth factor (bFGF, also known as FGF-2) can promote OPC proliferation (Eccleston and Silberberg 1985; Pfeiffer et al., 1993). Another example would be neuregulins (NRGs), which are secreted by several types of brain cells including astrocytes (Raabe et al., 1998). NRGs have been demonstrated to promote the survival of OL lineage cells (Canoll et al., 1996; Flores et al., 2000). It is likely that no one single factor is involved, and it would be interesting for future studies to carefully dissect the network of interacting factors that subserve astrocyte-OPC crosstalk. Second, our data suggest that both protective and proliferative effects may be present. Within the limitations of our model systems, we can quantitatively separate out protection versus proliferation. This is an important caveat. Further studies are required to dissect out how astrocytes may induce both effects in OPCs. Third, we have only focused on “soluble factors” for astrocyte-OPC coupling. However, in vivo, astrocytes are found in close apposition to OLs (Butt et al., 1995). Direct cell-cell signaling via gap junctions may also be relevant in this context (Orthmann-Murphy et al., 2008). Others have suggested that astrocytes may attenuate OL death through an $\alpha 6$ integrin-laminin-dependent mechanism (Corley et al., 2001). How matrix as well as trophic signaling works together will have to be examined in future studies. Finally, we collected astrocyte-conditioned media from only “healthy” astrocytes. Reactive astrocytosis occurs in response to many central nervous system diseases. Reactive astrocytes secrete nitric oxide, TNF- α , matrix metalloproteinases and other factors that can cause neuronal death (Chen and Swanson 2003; Swanson et al., 2004). Conversely, reactive astrocytes may also upregulate the production of beneficial factors such as endogenous antioxidant factors and angiogenic factors (Zhang and Chopp 2002; Chen and Swanson 2003). It was recently reported that astrocytes prepared from ALS-linked mutant SOD1 mice release toxic factors that killed motor neurons (Nagai et al., 2007). So, “sick” astrocytes lead to sick OPCs and white matter

dysfunction. How this balance between positive versus negative signaling will have to be rigorously dissected.

In conclusion, our data suggest that astrocytes may secrete soluble factors that broadly protect OPCs against cellular stress and injury, in part by activating pro-survival ERK and Akt pathways. Further investigations are warranted to dissect the coupling and signaling mechanisms between these two glial components under normal and diseased conditions.

Acknowledgments

Contract grant sponsor: The Deane Foundation; Contract grant sponsor: The American Heart Association; Contract grant sponsor: NIH.

We thank Dr. Larry A. Feig for kindly providing NIH-3T3 cells.

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Fig. 1. Immunostaining demonstrate that our OPC cultures express the expected markers A2B5 (green) and NG2 (red). The lack of GFAP (red) show that these cultures are not contaminated by astrocytes. Nuclei are stained with DAPI (blue).

Fig. 2. Representative images of OPC cultures show that H₂O₂ treatment (10 μ M for 24 hr) induce cell damage in OPCs. Co-treatment with astrocyte-conditioned media (Astro-CM) blocks the H₂O₂-induced cell death.

Fig. 3.

(A) A cell survival (WST) assay shows that H₂O₂ treatment induces OPC death in a concentration-dependent manner. Astrocyte-conditioned media (Astro-CM) are OPC-supportive both under normal and stressed conditions. The assay was conducted 24-hr after H₂O₂ and/or Astro-CM treatment (see Materials and Methods). ★P < 0.05 vs Control Media in each H₂O₂ condition. (B) The OPC survival from 3A were recalculated by normalizing against baseline of Control media or Astro-CM alone without H₂O₂ injury. These renormalized data suggest that Astro-CM may have both “protective” and “proliferative” effects in OPC cultures. ★P < 0.05 vs Control Media in each H₂O₂ condition. (C) Conditioned media from NIH-3T3 cells (mouse fibroblast) do not promote OPC proliferation nor protect OPCs against H₂O₂-induced stress. NIH-3T3-conditioned media were prepared using the same procedures as Astro-CM.

Fig. 4.

(A) Removing supplements from OPC culture media causes OPC death in a time-dependent manner. Conditioned media from astrocyte (Astro-CM) support the OPC survival under starvation conditions. $\star P < 0.05$ vs Control Media in each starvation condition. (B) Astro-CM also show OPC-protective effects against oxygen-glucose deprivation (OGD)-induced stress. $\star P < 0.05$.

Fig. 5.

(**A**) Conditioned media from astrocytes (Astro-CM, 20 min treatment) increase phospho-ERK levels and phospho-Akt levels in recipient OPCs. A specific MEK inhibitor U0126 and a specific PI3K inhibitor LY294002 blocks Astro-CM-induced ERK phosphorylation and Akt phosphorylation, respectively. U0126 (10 μ M) and LY294002 (10 μ M) were added to OPCs 30 min before Astro-CM treatment. (**B**) Both U0126 and LY294002 block the ability of Astro-CM to protect OPCs against H₂O₂ (10 μ M)-induced cell damage. $\star P < 0.05$ vs H₂O₂ & Astro-CM.