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Galectin-3 enhances angiogenic and migratory potential of microglial cells via modulation of integrin linked kinase signaling

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Abstract

Focal cerebral ischemia initiates self-repair mechanisms that include the production of neurotrophic factors and cytokines. Galectin-3 is an important angiogenic cytokine. We have previously demonstrated that expression of galectin 3 (Gal-3), a carbohydrate binding protein is significantly upregulated in activated microglia in the brains of rats subjected to focal ischemia. Further blocking of Gal-3 function with Gal-3 neutralizing antibody decreased the microvessel density in ischemic brain. We currently show that Gal-3 significantly increases the viability of microglia BV2 cells subjected to oxygen glucose deprivation (OGD) and re-oxygenation. Exogenous Gal-3 promoted the formation of pro-angiogenic structures in an *in vitro* human umbilical vein endothelial (HUVEC) and BV2 cell co-culture model. Gal-3 induced angiogenesis was associated with increased expression of vascular endothelial growth factor. The conditioned medium of BV2 cells exposed to OGD contained increased Gal-3 levels, and promoted the formation of pro-angiogenic structures in an *in vitro* HUVEC culture model. Gal-3 also augmented the *in vitro* migratory potential of BV2 microglia. Gal-3 mediated functions were associated with increased levels of integrin-linked kinase (ILK) signaling as demonstrated by the impaired angiogenesis and migration of BV2 cells following targeted silencing of ILK expression by SiRNA. Furthermore, we show that ILK levels correlate with the levels of phos-AKT and ERK1/2 that are downstream effectors of ILK pathway. Taken together, our studies indicate that Gal-3 contributes to angiogenesis and microglia migration that may have implications in post stroke repair.

Keywords

Survival; Angiogenesis; Migration; Galectin-3; ILK signaling; Ischemic injury

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1. Introduction

Following cerebral ischemia, expression of neurotrophic factors, cytokines, and chemokines increase significantly. Many of these factors contribute to neuronal survival, migration, and angiogenesis that can promote self-repair (Jablonska and Lukomska 2011; Dempsey and Kalluri 2007; Lamberts et al., 2012; Yan et al., 2009).

Angiogenesis and migration are tightly linked processes that are stimulated by specific tropic factors, chemokines, and cytokines (Bussolino et al., 1997; Comte et al., 2011; Hess and Allan 2011; Ribatti et al., 2003; Thored et al., 2007; Vagima et al., 2011; Wang et al., 2011; Xiong et al., 2010). Particularly, the carbohydrate-binding proteins including galectin-3 (Gal-3) play a major role in cell growth, survival, angiogenesis, and motility (Boscher et al., 2012; Cay 2012; Nangia-Makker et al., 2000; Markowska et al. 2010, Newlaczyk and Yu 2011). Gal-3 is shown to induce endothelial cell morphogenesis, migration, and angiogenesis in various types of tumors (Nangia-Makker et al., 2000; Markowska et al., 2011; Yu, 2010). Role of galectins in post ischemic angiogenesis and brain plasticity is not completely understood. Recent study reports that galectin-1 enhances the production of the cytokine BDNF and improves functional outcome in rats following cerebral ischemia (Qu et al., 2010). Increased Gal-3 was also observed after renal ischemia reperfusion in the rat (Vansterthem et al., 2010; Fernandes Bertocchi et al., 2008); liver ischemia and reperfusion (Lee et al., 2002), and in microglia during neonatal hypoxic-ischemic brain injury (Doverhag et al., 2010). Early changes following ischemic injury involve activation of microglia that produce numerous growth factors and cyto/chemokines some known to be neuroprotective, and some neurotoxic (Davalos D et al., 2005, Venneti S, et al., 2006; Weinstein JR et al., 2010; Sahota and Savitz 2011). The main source of Gal-3 appears to be the microglia (Walther et al., 2000; Yan et al., 2009; Doverhag et al., 2010; Satoh et al., 2011; Lalancette-Hébert et al., 2012). We observed Gal-3 up-regulation in astrocytes as well, and showed that exogenous Gal-3 increases proliferation of endothelial and neural progenitor cells, and enhances microvessel density in ischemic rat brain (Yan et al., 2009).

Gal-3 acts through various signaling pathways including survival and integrin signaling pathways (Balan et al. 2012; Newlaczyk and Yu, 2011; Cay 2012; Matarrese et al., 2000; Filer et al., 2009; Lei et al., 2009). Recent study has shown that Gal-3 modulates bFGF and VEGF mediated $\alpha v \beta 3$ integrin signaling (Markowska et al., 2010). Integrin Linked Kinase (ILK), a serine/threonine kinase is known to promote endothelial cell migration, proliferation and tube formation *in vitro*, and up-regulates VEGF levels in human scar fibroblasts and gastric cancer (McDonald et al., 2008; Mi et al., 2011). The effects ILK is mediated by its down stream signaling pathways, including mitogen activated protein kinase (MAPK-ERK1/2), and phosphoinositol-3 kinase (PI3K-Akt) (Eke et al., 2009; Krasilnikov., 2012; Legate et al., 2006; Wani et al., 2010; Yoganathan et al., 2000). The role of Gal-3 and the mechanism with which it mediates its function under ischemic conditions is not well understood.

In this study, we show that Gal-3 increases the viability of microglia BV2 cells subjected to OGD/re-oxygenation through activation of phos-Akt. Furthermore, Gal-3 promoted the

formation of pro-angiogenic structures and *in vitro* migratory potential of BV2 microglia. These actions of Gal-3 were mediated through integrin-linked kinase (ILK) signaling as shown by impaired angiogenesis and migration of BV2 cells following siRNA mediated silencing of ILK expression. Taken together, our studies support a role for Gal-3 in promoting angiogenesis and microglial cell migration, the critical processes that potentially contribute to post stroke repair.

2. Results

2.1 Gal-3 increases the survival of BV2 microglial cells exposed to OGD

Ischemic micro-environment can lead to decreased cell viability. Although controversial, the beneficial effects of microglia have been recently recognized. We examined the protective effects of Gal-3 on BV2 microglia under *in vitro* ischemia/re-oxygenation injury. The viability of BV2 microglia was significantly increased by Gal-3 treatment in a dose dependent manner. Gal-3 (5 µg/ml) resulted in increased number of viable cells subjected to OGD, by about 30-35 % as compared to untreated cells (Figure 1A). It is well established that the PI3K/Akt signaling plays an important role in promoting cell survival. We therefore investigated whether this pathway mediated the protective effects of Gal-3 on BV2 cells in OGD/re-oxygenated conditions. Western blot analysis showed increased levels of active phosphorylated-AKT in Gal-3 treated cells in a dose dependent manner (Figure 1B). Calcein-AM viable cell assay further confirmed that Gal-3 increases the BV2 cell viability by about 30% (Figure 1C-D). These results suggest that Gal-3 protects BV2 microglia cells against OGD/REOX-induced cell injury and AKT activation is involved in this process.

2.2 Gal-3 promotes *in vitro* angiogenic potential in HUVEC cell culture model

Angiogenesis is an important element of the injury repair processes in which inter-cellular interactions are involved. Here we examined the direct effects of Gal-3 on stimulation of proangiogenic structures formation in an *in vitro* microglia-HUVEC 3D co-culture model. Our results demonstrate that Gal-3 significantly increases the formation of pro-angiogenic structures (8-9 per field) in OGD exposed cells as compared to control group (3-4 per field) (Figure 2A). Quantification of number of pro-angiogenic structures revealed increases in pro-angiogenic structure formation in presence of Gal-3 (40-50% increase, $p < 0.05$) (Figure 2B). Increased angiogenic potential was associated with increased expression of VEGF as shown by immunofluorescence staining (Figure 2C). Of note, the functional benefits provided by microglial cells are mediated, in part, through secreted factors. We further examined if conditioned media (CM) of OGD exposed BV2 cells secrete Gal-3, and if this enhances angiogenic potential of HUVEC cells *in vitro*. Indeed, our results show that CM of OGD exposed cells contain about 2-3 fold higher levels of Gal-3 (Figure 2D-E) and promotes formation of pro-angiogenic structures (30-35% increase, $p < 0.05$) from HUVEC cells (Figure 2F-G).

2.3 Gal-3 promotes *in vitro* migration of microglia (BV2) cells

Cell migration is an integral part of angiogenesis and injury repair. Although, recent studies in rodent models suggest that microglia migrate to injured site and limit brain damage after stroke, it remains unclear which factors promote microglia migration. Therefore we

examined the effects of chemotactic Gal-3 on *in vitro* migration of BV2 cells. In a scratch-induced migration assay, BV2 cells treated with Gal-3 migrated to fill the injured area after 8 hours following injury. However, untreated cells showed less migratory potential as indicated by an unfilled wound area (Figure 3A). We further quantified the BV2 cell migration using Boyden chamber transwell migration assay. As shown in figure 3B, the ability of BV2 cells to migrate through matrigel was significantly increased by Gal-3 treatment (40-50%, $p<0.05$), when compared with the untreated cells.

2.4 Gal -3 increases the levels of ILK and phos-ERK1/2 and phos-AKT

Direct interaction between integrins and Gal-3 has been recognized. Integrin-linked kinase (ILK), a serine/threonine protein kinase is shown to promote anti-apoptosis, invasion/migration, and angiogenesis. Therefore, we examined whether the effects of Gal-3 is mediated through regulation of ILK. Results from western blot analysis show that exposure of BV2 cells to Gal-3 increased the levels ILK by 2-3 fold. However, pre-treatment with ILK specific siRNA, blocked the up-regulation of ILK1 following Gal-3 treatment (Figure 4A-B). This increase in ILK was directly associated with the increase in phos-ERK1/2 and Phos-AKT that are down stream effectors of ILK signaling pathways. Silencing of ILK with siRNA pre-treatment resulted in decreased Phos-Erk1/2 and Phos-Akt suggesting that effects on ERK and AKT is mediated through ILK (Figure 4C).

2.5 Gal-3 mediates pro-angiogenic and migratory effects through ILK signaling

Our data shows that Gal-3 increases ILK levels. Therefore, we sought to determine whether Gal-3 mediated angiogenesis and migration specifically depend on ILK upregulation, using RNA interference approach. Indeed, silencing of ILK by siRNA prior to Gal-3 treatment resulted in decreased number of pro-angiogenic structures (Figure 5A) and decreased migratory potential of BV2 cells (Figure 5B). These data demonstrate that Gal-3 mediates its function through increased levels of ILK.

3. Discussion

Ischemic injury changes microenvironment by altering inflammatory cytokines. Understanding the functional consequences and molecular mechanisms of these cytokines may provide clues for improving therapeutic strategies for post stroke repair. Post ischemic repair requires concerted actions of survival, neo-angiogenesis, and recruitment of progenitor cells and microglia to injured site. Although the cytokine Gal-3 is well recognized to modulate multiple physiological functions, its role in ischemic brain plasticity and repair remains largely unknown. Our previous study (Yan et.al., 2009) has demonstrated that Gal-3 is up-regulated following ischemic injury, and blockade of Gal-3 activity decreases the micro vessel density. Furthermore, exogenous Gal-3 increases the proliferation of neural progenitor and endothelial cells. In addition, recent study by Dr. Kriz's group (Lalancette-Hébert et.al., 2012) has demonstrated that lack of Gal-3 inhibits microglia proliferation and activation leading to increased infarct size. These emerging studies strongly suggest that Gal-3 is a critical component of ischemic injury repair. However, study by Doverhag et.al., 2010 show increased Gal-3 expression after neonatal hypoxic-ischemic brain injury in activated microglia, and also report that Gal-3-deficient mice are protected

from hypoxic injury, suggesting that Gal-3 may have detrimental effects in neonatal stroke. Certainly more studies are required to better understand the role of Gal-3 in events associated with ischemic stroke injury and repair. Our current study provides evidence that Gal-3 increases both angiogenesis and migration of microglia exposed to *in vitro* ischemia. Moreover, we identified ILK signaling as a possible downstream mechanism involved in Gal-3 action.

Gal-3 produced by microglia might stimulate the autocrine and paracrine signaling pathways involved in post stroke repair. Particularly, extracellular or secreted Gal-3 enhances cellular survival, migration, and angiogenesis (Nangia-Makker et.al., 2008). Our current data show that Gal-3 protects BV2 microglia exposed to *in vitro* ischemic conditions, through activation of phos-AKT, a pro-survival factor. Thus, promoting the survival of microglia may be the first beneficial step as microglia is the major source of cytokines needed to establish the neurovascular niche in the ischemic brain. Increased survival of BV2 presumably leads to increased Gal-3 production that in turn contributes to enhanced angiogenesis. In support of this, our data show that exogenous Gal-3 as well as BV2 conditioned media containing Gal-3 significantly increases *in vitro* pro-angiogenic structure formation. Recent studies in rodent models suggest that microglia rapidly migrate to injured site and limit brain damage after stroke (Davalos et al., 2005, Eyo et.al., 2012). It is likely that Gal-3 promotes migration of microglia, as suggested by our *in vitro* migration studies. Chemotactic property of Gal-3 was further supported by its ability to promote endothelial cell migration and angiogenesis (Nangia-Makker et.al., 2000). It is intriguing that Gal-3 facilitates plethora of functions including cell survival, migration, and angiogenesis following injury. This multifunctional property of Gal-3 may be attributed to its ability to interact with a large number of ligands (Krzejak et.al., 2004).

Signaling pathways stimulated by Gal-3 are not fully defined. Recent study has shown that Gal-3 modulates integrin signaling and PI3K/AKT pathways (Melo et.al., 2011). Our current study shows that Gal-3 acts via ILK, a multifunctional serine/threonine kinase which regulates several downstream targets that promote proliferation, survival and migration. Importantly, ILK modulates hypoxia-inducible factor 1 alpha mediated VEGF expression and is essential for endothelial cell migration, tube formation, and tumor angiogenesis (Eke et.al., 2009; Tan et al., 2004). Furthermore, increased ILK expression is linked to induction of angiogenesis after myocardial infarction (Xie et.al., 2011). Of note, a transient increase in ILK was detected after focal cerebral ischemia (Saito et.al., 2004). In support of this, our studies show that Gal-3 increases ILK level under ischemic conditions. We further show that silencing of ILK inhibits the activation of downstream signaling targets p-Akt and p-ERK1/2, leading to curtailed angiogenesis and microglia migration. Thus, activation of ILK signaling pathway by Gal-3 might be responsible for enhanced neovascularization and migration following cerebral ischemia.

Conclusions

We have demonstrated that Gal-3 protects microglia cells under *in vitro* ischemic conditions. We have further shown that Gal-3 promotes *in vitro* migratory and angiogenic potential through ILK signaling pathway. Our previous work (Yan et.al., 2009) and the results of the

present study together suggest that Gal-3 bridges multiple functions under ischemic conditions. Thus, Gal-3 may be an attractive target for post stroke recovery.

4. Experimental procedures

4.1 Cell culture

Murine BV-2 microglia cell line developed by Dr V. Bocchini (Blasi et al. 1990) was a generous gift from Dr Grace Y Sun (University of Missouri, Columbia, MO). Human Umbilical Vein Endothelial Cells (HUVEC) was purchased from American Type Culture Collection (ATCC; Manassas, VA). The BV2 cells were grown in DMEM medium containing 10% fetal bovine serum, 0.1mM non-essential amino acids, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. HUVEC cells were grown in a complete medium with growth supplements (Cell Applications Inc, San Diego, CA). All cells were grown at 37°C in 5% CO₂. Recombinant Galectin-3 (R&D Systems, Minneapolis, MN) was added to media as needed.

4.2 Oxygen and Glucose Deprivation (OGD)

Cells were grown in complete media supplemented with glucose (4.5 g/ml) for 18 hours in normoxic conditions (5% CO₂ and 21% O₂). To initiate OGD, the culture medium was replaced by glucose-free medium (DMEM with L-glutamine and no glucose, GIBCO), and cells were transferred to a humidified incubation chamber flushed by a gas mixture of 95% N₂ and 5% CO₂ at 37 ° C for 4 hours in an incubator (Serico CB, Binder GmbH, Tuttlingen). Control cells were incubated for 4 hours in 5% CO₂ and 21% O₂ in a media identical to the OGD media except for the addition of glucose. Following OGD, cells were subjected to re-oxygenation by feeding with glucose-supplemented (4.5 g/ml) DMEM medium, and returned to the incubator under normoxic condition at 37°C in 5% CO₂ and 21% O₂ for 18 hours.

4.3 Measurement of cell viability

To examine the cytoprotection by Gal-3, cell viability was evaluated by a standard colorimetric assay for mitochondrial reductase catalyzed reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT assay kit, ATCC, Manassas, MD). Briefly, 5×10³ BV2 cells per well were plated in triplicate on 96 well plates and cultured for 18 hours in 37°C incubator. Cells were then exposed to OGD for 4 hours and re-oxygenated for 18 hours in 37°C with 5% CO₂ and 21% O₂. Cells were treated with various concentrations of Gal-3 (0.1, 1, and 5 µg/ml) at the start of re-oxygenation. BV2 cells that were exposed to OGD but not treated with Gal-3 were used as controls. The absorbance of released purple formazan that indicates cell viability was measured at 570 nm in a microtiter plate reader (Spectramax Plus 384, Molecular Devices, Sunnyvale, CA). Cell viability was also quantified using calcein-AM fluorescence assay as per manufacturer's instruction (Life Technologies, CA). Briefly, BV2 cells exposed to 4 hour OGD and 18 hours re-oxygenation were either untreated or treated with Gal-3 (5 µg/ml) and then incubated with 1 µg/ml calcein-AM at 37°C for 30 min. Calcein AM is a non-fluorescent compound and gets converted to the green fluorescent calcein when hydrolyzed by intracellular esterases in live cells. Calcein fluorescence correlating viable cells was visualized using a Nikon

fluorescence microscope (TE-2000U, Nikon, Melville, New York). Images were collected and, viable fluorescent cells per field were counted using MetaMorph image-processing software (Universal Imaging, Downingtown, PA). Results are presented as mean values $\pm$ SD of triplicates.

4.4 Production of conditioned media (CM)

BV2 cells were grown to 75% confluence and the complete medium was switched to serum and glucose-free medium (DMEM with L-glutamine and no glucose, GIBCO). Cells were then subjected to OGD as described above. After 18 hours of re-oxygenation, the CM was collected, filtered (0.2 μ m polyethersulfone membrane, Thermo Fisher Scientific), concentrated to 20-fold using filters with a 10 kd cutoff (Millipore, Bedford, MA), and used for western blot analysis.

4.5 Western blot analysis

Cells were lysed in protein extraction RIPA buffer (Boston Bioproducts) containing protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO). Cell lysates were briefly sonicated and centrifuged at 13,000 rpm for 10 min at 4°C. Thirty μ g of total protein was separated by SDS-PAGE and probed with respective antibodies; 1:1000 for phospho/total AKT and ERK, and ILK (Cell Signaling, Danvers, MA); 1:500 for actin, (Sigma Aldrich, St. Louis, MO); followed by secondary antibody conjugated to horseradish peroxidase for 1 hour. Signals were developed using Super Signal West Pico Chemi-luminescent Substrate (Thermo Scientific, Rockford, IL). Total AKT/ERK and Actin were used as loading controls. The experiment was repeated three times and representative data are presented.

4.6 *In vitro* angiogenesis assay

To analyze *in vitro* angiogenesis and interactions between microglial and endothelial cells in formation of tubular networks, three dimensional tube formation assay was performed as described in our previous report (Arscott et.al., 2009). Briefly, 15 μ l of matrigel with properties of extra cellular matrix (BD Biosciences) was distributed per well in a 96-well plate and allowed to gel at room temperature. BV2 cells exposed to OGD for 4 hours were harvested for angiogenesis assay. HUVECs (7×10^3 cells/well) suspended in 100 μ l of DMEM supplemented with 2% FBS were added to each well and co-cultured in triplicate with 7×10^3 of either untreated or Gal-3 (5 μ g/ml) treated BV2 cells at 37°C under reoxygenated conditions. In another set of experiments, HUVEC cells resuspended in 100 μ l of conditioned medium from either control (grown in presence of glucose and normoxic condition) or OGD exposed BV2 cells were plated in triplicate on to matrigel containing wells. After 24 hours, proangiogenic tube structures were observed under phase contrast microscope (Nikon Eclipse TE-2000U, Nikon, Melville, New York). Images were captured and the total number of proangiogenic structures of five randomly chosen microscopic fields was measured. Results are presented as mean values $\pm$ SD of triplicates.

4.7 Immunofluorescence staining

BV2 Microglia grown on coverslips were exposed to OGD for 4 hours and re-oxygenated for 18 hours with or without Gal-3 (5 μ g/ml). Cells were then fixed in 4% paraformaldehyde

for 15 min at room temperature. Cells were then incubated with a blocking solution for 1 hour, followed by application of mouse anti-VEGF monoclonal antibody (1:100 in the blocking buffer; Santa Cruz Biotechnology, CA), at 4°C overnight. Cells were then incubated with goat anti-mouse IgG conjugated to Alexa Fluor 488 (1:500) for 1 hour at 37°C. Images were taken using Retiga-2000R camera (QImaging, Surrey, BC Canada) connected to an inverted fluorescence microscope (Eclipse TE-2000U, Nikon, Melville, New York). Images were analyzed using MetaMorph software.

4.8 Scratch-induced cell migration assay and transwell cell migration assay

BV2 cells exposed to OGD for 4 hours were harvested and used for migration assays. For scratch induced migration assay, cells were plated in a 24 well plate in the absence or presence of Gal-3 (5 µg/ml) and allowed to attach for 4-6 hours and then cell monolayers were injured with a sterile micropipette tip. Cells were photographed immediately after injury and at 8 hours after injury using a phase contrast microscope. Cell migration was further quantified using Boyden chambers containing matrigel coated cell culture inserts (BD Biosciences, Bedford, MA) with 8 µm pores in 24 wells. A total of 3×10^4 BV2 cells were placed in the upper compartment and the lower compartment was filled with 500 µl growth medium containing Gal-3 (5 µg/ml). Control set of chambers received BSA instead of Gal-3. After 8 hours of incubation, the cells that had migrated from the upper chamber through the matrigel to the lower surface towards Gal-3 were stained with cresyl violet and solubilized in extraction buffer. Optical densities (OD) values at 540 nm were plotted. Results are presented as mean values $\pm$ SD of triplicates.

4.9 ILK silencing with siRNA

BV2 cells were seeded at 1×10^5 cells per well in 6-well plates and incubated with 1 ml DMEM supplemented with 10% Fetal Bovine Serum at 37 °C for 18 h. Next day cells were transfected with 50 nM of either control siRNA or ILK siRNA (Santa Cruz Biotechnology, CA) using lipofectamine 2000 transfection reagent (Invitrogen, CA) and incubated at 37°C for 24 hours. Control or ILK siRNA pre-treated BV2 cells were exposed to OGD for 4 hours and cells were then re-oxygenated, and grown for 24 hours in absence or presence of Gal-3 (5 µg/ml). Cells were then harvested for western blot analysis, and *in vitro* angiogenesis and migration assays.

Statistical Analysis—All studies were performed in triplicate and results are expressed as mean $\pm$ standard deviation (SD). Statistical significances were determined by Student's t test or by ANOVA. A value $p < 0.05$ was considered statistically significant.

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Abbreviations

Gal-3 Galectin 3

ILK Integrin Linked kinase
OGD Oxygen Glucose Deprivation

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Figure 1. Protective effects of Gal-3 on BV2 microglia cells exposed to OGD

(A). BV2 cells were subjected to 4 hour OGD and re-oxygenated (REOX) for 18 hours by feeding with glucose-supplemented (4.5 g/ml) DMEM medium. At the start of REOX, cells were treated with control BSA or Gal-3 at different concentration (0.1, 1, 5 µg/ml). At the end of 18 hours, the cell viability was determined by MTT assay. Optical density (OD) representing the change in cell viability is presented. Values represent mean $\pm$ S.D; n = 3. *p < 0.05 versus BSA treated control samples. **B**. Western blot analysis showing dose dependent increased levels of phosphorylated AKT in Gal-3 treated BV2 cells. **C**. Representative photomicrographs of Calcein-AM stained OGD exposed control BV2 cells, and OGD exposed Gal-3 (5 µg/ml) treated BV2 cells. **D**. Percent change in cell viability is presented. Values represent mean $\pm$ S.D; n = 3. *p < 0.05 versus control samples.

Figure 2. Gal-3 promotes *in vitro* angiogenic potential

Gal-3 increased the formation of proangiogenic structure *in vitro*. HUVECs were co-cultured with OGD exposed BV2 cells either with or without Gal-3 (5 µg/ml) for 24 hours on matrigel basement. (A) Representative photomicrographs of pro-angiogenic structure formation in co-culture experiments. (B) Closed rings arising from HUVEC sprouting (pro-angiogenic structure) were quantified in five randomly selected fields (mean ± S.D.; n = 5; *, p < 0.05). (C) Gal-3 treatment of BV2 cells is associated with increased expression of VEGF as shown by immunofluorescence staining. BV2 Microglia grown on coverslips were exposed to OGD and re-oxygenated for 18 hours with or without Gal-3 (5 µg/ml). Expression of VEGF was examined by immunofluorescence staining. (D-E). Western blot analysis showing that the conditioned medium (CM) of OGD exposed BV2 cells contain about 2-3 fold higher levels of Gal-3. (F). Representative photomicrographs of proangiogenic structure formation by HUVEC cells treated with CM (G). Closed rings arising from CM treated HUVEC sprouting (pro-angiogenic structure) were quantified in five randomly selected fields (mean ± S.D.; n = 5; *, p < 0.05).

Figure 3. Gal-3 promotes *in vitro* migration of microglia (BV2) cells

(A). Gal-3 (5µg/ml) enhances *in vitro* migratory potential of OGD exposed BV2 cells as shown by scratch-induced migration assay. (B). Boyden chamber transwell migration assay indicating the effects of Gal-3 on migratory potential of BV2 cells. The ability of BV2 cells to migrate through matrigel coated filters was significantly increased by Gal-3 treatment (40-50%), when compared with the untreated cells. Absorbance correlating with the number of cells invaded were read at 540 nm (mean $\pm$ S.D; n = 3; *, p < 0.05).

Figure 4. Gal-3 increases the levels of Interleukin kinase (ILK) and its downstream effectors phosph-AKT and Phos-ERK1/2

(A) A representative blot of western blot analysis showing up-regulated expression of ILK in Gal-3 treated BV2 cells. Pre-treatment with ILK specific siRNA silences Gal-3 induced ILK expression (B) Quantitative analysis by densitometry are shown (mean $\pm$ S.D.; $n = 3$; *, $p < 0.05$). (C) Western blot analysis showing the increased levels of phosphorylated AKT and ERK1/2 in Gal-3 treated BV2 cells. Pre-treatment with ILK specific siRNA decreases Gal-3-ILK induced phosph-AKT and Phos-ERK1/2 levels.

Figure 5. Gal-3 mediates pro-angiogenic and migratory effects through ILK signaling
BV2 cells were transfected with 50 nM of either control or ILK siRNA (Santa Cruz Biotechnology, CA) and incubated at 37 °C for 24 hours. Control or ILK siRNA treated BV2 cells were exposed to 4 hours OGD and re-oxygenated, and grown for 24 hours in presence of Gal-3 (5 µg/ml) and then cells were harvested for *in vitro* angiogenesis (A) and migration (B) assays as described in experimental procedure. Results are presented as mean values $\pm$ SD of triplicates.