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MICROGLIAL STRESS INDUCIBLE PROTEIN 1 PROMOTES PROLIFERATION AND MIGRATION IN HUMAN GLIOBLASTOMA CELLS

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Abstract—Microglial activation is a key event in the progression and infiltration of tumors. We have previously demonstrated that the co-chaperone stress inducible protein 1 (STI1), a cellular prion protein (PrP^C) ligand, promotes glioblastoma (GBM) proliferation. In the present study, we examined the influence of microglial STI1 in the growth and invasion of the human glioblastoma cell line GBM95. We demonstrated that soluble factors secreted by microglia into the culture medium (microglia conditioned medium; MG CM) caused a two-fold increase in the proliferation of GBM95 cells. This effect was reversed when STI1 was removed from the MG CM. In this context, we have shown that microglial cells synthesize and secrete STI1. Interestingly, no difference was observed in proliferation rates when GBM cells were maintained in MG CM or MG CM containing an anti-PrP^C neutralizing antibody. Moreover, rec STI1 and rec $STI1_{\Delta 230-245}$, which lack the PrP^C binding site, both promoted similar levels of GBM95 proliferation. In the migration assays, MG CM favored the migration of GBM95 cells, but migration failed when STI1 was removed from the MG CM. We detected metalloproteinase 9 (MMP-9) activity in the MG CM, and when cultured microglia were treated with an anti-STI1 antibody, MMP-9 activity decreased. Our results suggest that STI1 is secreted by microglia and favors tumor growth and invasion through the participation of MMP-9 in a PrP^C-independent manner.

Key words: glioblastoma, microglia, STI1, migration, proliferation.

Mesodermal microglial progenitors appear in the developing neuroepithelium during the early stages of neurogenesis. In the brain parenchyma, motile ameboid cells proliferate and differentiate into ramified microglia after reaching their final location (Alliot et al., 1999; Lima et al., 2001; Dalmau et al., 2003; Streit and Xue, 2009). During development, the ameboid microglia, also called brain macrophages, participate in the formation of the complex network of connections in the adult brain (Mallat et al., 2005; Vilhardt, 2005). In adults, microglial cells compose the resident macrophage population of the CNS. In a pathologic context, microglial cells return to a macrophagic phenotype and are recruited to lesion sites. Microglial activation is a key event for the defense of the nervous system parenchyma from ischemic, neurodegenerative and inflammatory diseases (Vilhardt, 2005; Garden and Moller, 2006; Hanisch and Kettenmann, 2007; Kettenmann et al., 2011).

Glioblastoma (GBM) is the most common primary brain tumor, characterized by a high proliferative rate, aggressive invasiveness, insensitivity to radio- and chemotherapy as well as a short survival period. GBMs are considered to be one of the deadliest human cancers (Maher et al., 2001; Alves et al., 2011). Glioma tissue consists of tumor cells and up to 30% microglia or macrophages. However, little is known about the immune performance and interactions of microglia with GBMs. Currently, there is a growing body of evidence suggesting that microglia contribute to tumor progression (Badie and Schartner, 2000; Watters et al., 2005; Hanisch and Kettenmann, 2007). In particular, microglial cells acquire an interesting phenotype when in contact with gliomas. They are recruited to the glioma site and accumulate, primarily, at the margins of the site. Soluble factors secreted by the glioma induce changes in the microglial phenotype, leading to an activated form distinctive from the typical inflammatory phenotype. In fact, glioma-associated microglial activation does not necessarily induce the release of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin 1β (IL1β) or IL-6. However, gliomas can trigger other transcriptional changes, such as the up-regulation of metalloproteinase-2 (MMP-2) and MMP-9, which can lead to an invasion of tumor cells (Markovic et al., 2005; Hussain et al., 2006; Sliwa et al., 2007). In addition, Kim and collaborators observed that components of the extracellular matrix, such as laminin and proteoglycans prepared from glioma cell lines, suppress the induction of iNOS/nitric oxide (NO) and various pro-inflammatory molecules in lipopolysaccharides (LPS) stimulated microglial cells (Kim et al., 2006). Recently, Rodrigues and collaborators presented evidence supporting the idea that normal human monocytes ex-

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Abbreviations: CM, conditioned medium; ERK1/2, extracellular signal-regulated kinases 1 and 2; FCS, fetal calf serum; GBM, glioblastoma; LPS, lipopolysaccharides; MAPK, mitogen-activated protein kinase; MG CM, microglia conditioned medium; MMP, metalloproteinase; NO, nitric oxide; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C-dependent; PrP^C, cellular prion protein; rec STI1, recombinant STI1; SDS, sodium dodecyl sulfate; STI1, stress inducible protein 1.

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posed to glioblastoma cells could assume an immunosuppressive phenotype similar to myeloid-derived suppressor cells *in vitro* (Rodrigues et al., 2010). Thus, it is possible that the glioma instructs microglia not to attack, but to promote tumor spreading within the brain parenchyma (Hanisch and Kettenmann, 2007).

Several cellular functions have been attributed to the cellular prion protein (PrP^C), which is highly expressed in the brain and has many ligands (Prusiner et al., 1998; Brown et al., 1998; Linden et al., 2008). In this context, the PrP^C can interact with the co-chaperone stress inducible protein 1 (STI1) and induce the activation of protein kinase A (PKA) and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinases 1 and 2 (ERK1/2), with the former promoting neuroprotection and the latter, neuritogenesis (Zanata et al., 2002; Lopes et al., 2005). We have also shown that STI1 is secreted by astrocytes and GBM (Erlich et al., 2007; Lima et al., 2007) and that it promotes survival and differentiation in astrocytes (Lima et al., 2007; Arantes et al., 2009). Interestingly, STI1 inhibited the proliferation of both wild-type and PrP^C-null astrocytes in a protein kinase C-dependent (PKC) manner (Arantes et al., 2009), whereas it induced GBM proliferation through the activation of MAPK/ERK1/2 and phosphatidylinositol 3-kinase (PI3K) (Erlich et al., 2007).

The present study aimed to investigate the influence of STI1 in the microglia-GBM interaction. Here, we have demonstrated that microglia secrete soluble factors that induce tumor growth and invasion. We have identified STI1 as one of the factors secreted by microglia. In addition, our results show that microglial STI1 promotes tumor proliferation, modulates MMP-9 activity and favors the migration of GBM95 cells in a PrP^C-independent manner.

EXPERIMENTAL PROCEDURES

Reagents

All culture media components were obtained from Invitrogen-Life Technologies (Carlsbad, CA, USA). Recombinant STI1 (rec STI1, His_6-STI1), and rec $STI1_{\Delta 230-245}$ (His_6-$STI1\Delta_{230-245}$) were purified as previously described (Zanata et al., 2002). Both recombinant proteins were quantified for bacterial LPS contamination by the Limulus Amebocyte Lysate test (Cambrex, Walkersville, MD, USA), and samples were purified by detoxi-gel endotoxin removing gel (DGRG; Thermo Scientific Pierce, Rockford, IL, USA) before use. Quantification assays showed an LPS concentration of 0.6 EU per ml of STI1 solution (Arantes et al., 2009).

Polyclonal anti-STI1 antibody raised in rabbits (Zanata et al., 2002) was produced by Bethyl (Montgomery, TX, USA). The anti-PrP^C monoclonal antibody, 3F4, was acquired from DAKO (Glostrup, Denmark). The anti-PrP^C monoclonal antibody, 6H4, was obtained from Prionics (Schlieren, Zurich, Switzerland), and a polyclonal antibody against recombinant PrP^C produced in $Prnp^{0/0}$ mice (Chiarini et al., 2002) was provided by Dr. Vilma Martins (A. C. Camargo Hospital, São Paulo, Brazil). Mitomycin C and 4′-6-diamino-2-phenylindole (DAPI) were acquired from Sigma (St. Louis, MO, USA). [^{3}H]-thymidine was obtained from IPEN/CNEN (Rio de Janeiro, RJ, Brazil). Glucose was purchased from Merck (Darmstadt, Germany), and Fungizone was purchased from Bristol-Meyers Squibb (Princeton, NJ, USA). All culture plates and flasks were obtained from techno plastic products (TPP; Trasadingen, Switzerland). The Alexa Fluor® 488 monoclonal antibody and the peroxidase-conjugated secondary antibody were obtained from Molecular Probes (Carlsbad, CA, USA). The secondary antibody conjugated with FITC (goat anti-rabbit) and LPS from *Salmonella enterica* were obtained from Sigma (St. Louis, MO, USA). Protease and phosphatase inhibitory cocktails were obtained from Roche (South San Francisco, CA, USA), and the Amicon apparatus was obtained from Amicon (Houston, TX, USA). Protein A Sepharose was obtained from GE Healthcare (Piscataway, NJ, USA).

Animals

This study was approved by the Ethics Committee of the Health Sciences Center (Centro de Ciências da Saúde—CCS) at the Federal University of Rio de Janeiro (Universidade Federal do Rio de Janeiro—UFRJ) (Protocol No. DAHEICB 015) and by the Brazilian Ministry of Health Ethics Committee (CONEP No. 2340). The "Principles of laboratory animal care" (NIH publication No. 85–23, revised 1996) guidelines were strictly followed for all experiments. All efforts were made to minimize the number of animals used and their suffering. Swiss mice were obtained from the Biomedical Sciences Institute (Instituto de Ciências Biomédicas—CCS, UFRJ, Brazil). PrP^C-null mice ($Prnp^{0/0}$) and their respective controls (wild-type, $Prnp^{+/+}$) were provided by Dr. Vilma R. Martins (A. C. Camargo Hospital, São Paulo, Brazil). The animals were descendants from the Zrchl line (Büeler et al., 1992). $Prnp^{+/+}$ were generated by continuously crossing descendants from an initial 129/Sv and C57BL/6J mating.

Cell cultures

Microglial cultures. Microglial cell cultures were obtained as described previously (Théry et al., 1991; Lima et al., 2001). Briefly, floating microglial cells were isolated from 2-week-old primary glial cultures prepared from the cerebral cortex of newborn mice and grown in Dulbecco's modified Eagle medium/F12 supplemented with glucose (33 mmol L^{-1}), glutamine (2 mmol L^{-1}), sodium bicarbonate (3 mmol L^{-1}), penicillin/streptomycin (0.5 mg mL^{-1}), Fungizone (2.5 L g ml^{-1}) and fetal calf serum 10% (FCS, v/v) at 37 °C in a humidified 5% CO_2 and 95% air atmosphere. The microglial cultures were assessed by immunocytochemistry using isolectin-B4 and an anti-CD68 antibody, and cells presented more than 99% purity.

Astrocyte primary cultures. Astrocyte primary cultures were prepared as previously described (Faria et al., 2006; Lima et al., 2007) from the cerebral cortex of newborn mice. Briefly, single cell suspensions were obtained by dissociating cells from the cerebral cortex in DMEM-F12 supplemented with the same compounds as in the microglia medium. The cells were plated on pre-coated poly-L-lysine plates (5 μg ml^{-1}) and grown in a DMEM/F12 enriched media with 10% FCS. The cultures were incubated at 37 °C in a humidified 5% CO_2 and 95% air atmosphere. The medium was changed every 2 days until the cells reached confluence. The astrocyte primary cultures were assessed by immunocytochemistry using an anti-GFAP antibody and cells presented more than 95% purity.

Neuron primary cultures. Neuron primary cultures were prepared from the cerebral cortex of E18 mice as previously described (Lima et al., 2007). Briefly, single cell suspensions were obtained by dissociating cells from the cerebral cortex in DMEM-F12 supplemented with the same compounds as in the microglia medium. The neurons were incubated at 37 °C in a humidified 5% CO_2 and 95% air atmosphere. The neuronal primary cultures were assessed by immunocytochemistry using an anti-βIII-tubulin antibody and cells presented purity higher than 95%.

Peritoneal macrophage cultures. Peritoneal macrophages were obtained from Swiss mice by an i.p. injection with 2 ml