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Research article

Astroglioma conditioned medium increases synaptic elimination and correlates with major histocompatibility complex of class I (MHC I) upregulation in PC12Cells

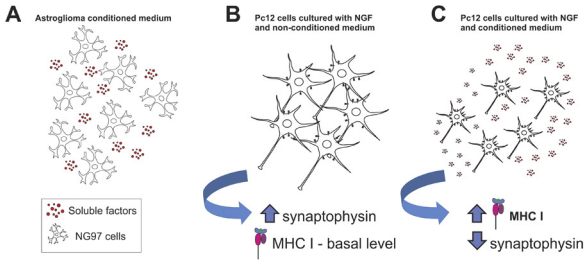
Rodrigo Fabrizzio Inácio[a], Renata Gacielle Zanon[b], Mateus Vidigal de Castro[a], Henrique Marques de Souza[c], Marcio Chaim Bajgelman[d], Liana Verinaud[a], Alexandre Leite Rodrigues de Oliveira[a,*]

[a] Department of Structural and Functional Biology, Institute of Biology, University of Campinas, Campinas, São Paulo, Brazil
[b] Department of Human Anatomy, Institute of Biomedical Sciences, Federal University of Uberlândia, Uberlândia, MG, Brazil
[c] Department of Biochemistry and Tissue Biology, Institute of Biology, University of Campinas, Campinas, São Paulo, Brazil
[d] Brazilian National Laboratory for Biosciences, Research Center in Energy and Materials, Campinas, São Paulo, Brazil

HIGHLIGHTS

- PC12 cells may be considered as an alternative model for studying MHC I regulation and synaptic plasticity.
- Synapse formation in PC12 cells is affected by MHC I upregulation.
- Glioma derived soluble factors reduce synapse formation in PC12 cells.

GRAPHICAL ABSTRACT



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ABSTRACT

Astrocytes are multifunctional glial cells that actively participate in synaptic plasticity in health and disease. Little is known about molecular interactions between neurons and glial cells that result in synaptic stability or elimination. In this sense, the main histocompatibility complex of class I (MHC I) has been shown to play a role in the synaptic plasticity process during development and after lesion of the CNS. MHC I levels in neurons appear to be influenced by astrocyte secreted molecules, which may generate endoplasmic reticulum stress. *In vitro* studies are of relevance since cell contact can be avoided by the use of astrocyte conditioned medium, allowing investigation of soluble factors isolated from cell direct interaction. Thus, we investigated synaptic preservation by synaptophysin and MHC I immunolabeling in PC12 neuron-like cells exposed to NG97 astroglioma conditioned medium (CM). For that, PC12 cells were cultured and differentiated into neuron-like profile with nerve growth factor. MHC I was induced with interferon beta treatment (IFN), and the effects were compared to PC12 exposure to NG97 CM. Overall, the results show that NG97 CM increases, more than IFN alone, the expression of MHC I, negatively influencing synaptic stability. This indicates that glial soluble factors influence synapse elimination, compatible to *in vivo* synaptic stripping process, in a cell contact independent fashion. In turn, our results indicate that deleterious effects of astroglioma are not only restricted to rapid growth ratio of the tumor, but also correlated with secretion of stress-related molecules that directly affect neuronal networks.

* Corresponding author at: Department of Structural and Functional Biology, Institute of Biology, University of Campinas, Rua Monteiro Lobato, 255; CP 6109, CEP 13083-970, Campinas, Sao Paulo, Brazil.
E-mail address: alroliv@unicamp.br (A.L.R.d. Oliveira).

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1. Introduction

In recent decades, numerous studies have pointed to glial cells as active participants in synapse plasticity of the nervous system [2,5,18–20]. In this context, astrocytes play prominent role during the course of diseases and following trauma, contributing to the rewiring of the central nervous system (CNS). Araque et al. [1] proposed the concept of tripartite synapse, where cytoplasmic projections of astrocytes would be the third element of the synaptic components. Beside such function, astrocytes participate in the immune response [7] and they are activated by inflammation [15].

To date, little is known about the molecular mechanisms behind astrocyte/neuron communication. In this sense, Oliveira et al. [14] reported that MHC class I expression by neurons influences synaptic pruning from the surface of neurons following peripheral nerve axonal transection. This is in line with the work by Boulanger and Shatz [3] implicating MHC I molecule in the final stages of development and organization of the visual system in newborn mice.

The above described non-canonical functions of MHC I may also involve glial cells, especially astrocytes. In a recent study, Song et al. [16] described that MHC I expression by neurons directly influences astroglial reactivity and secretion of neurotoxic substances, contributing to motoneuron loss in an animal model of amyotrophic lateral sclerosis (ALS). In line with such pro-inflammatory pattern of reactive astrocytes, similar deleterious effects may be triggered by astroglial tumors.

Gliomas are relatively common cerebral tumors, corresponding to more than 70% of CNS primary neoplasia. Among the side effects of fast tumor growth, contributing to high lethality, are of note cerebral edema, herniation and hypertension. Astrocytoma, which derives from astrocytes, usually presents rapid growth rate and leads to profound alterations of neural circuits. Nevertheless, the delicate interaction between astrocytes and neurons, particularly the impact of soluble substances secreted by such glial cells on synapses and neuronal MHC I expression are difficult to investigate *in vivo*. In turn, the use of *in vitro* systems become useful. For that, establishment of glial and neuronal cell lines may allow detailed evaluation of processes involving synapse formation and elimination, indicating future therapeutic targets to preserve CNS from tumor deleterious effects.

A human malignant continuous cell line, named NG97, was established and sequentially sub-cultured for several generations in standard culture medium, presenting no signs of senescence. The NG97 cell line has a doubling time of about 24 h and immunocytochemistry analysis of glial markers demonstrated expression of glial fibrillary acidic protein (GFAP) and S-100 protein. NG97 cells grow as three sub-populations with distinct morphological appearance and certainly constitute a glial-committed cell line [10,13]. Due to its fast growth rate and relatively easy culturing conditions, NG97 cells were used herein in order to provide conditioned medium containing astrocyte-like derived substances, supposedly active on neurons.

The employment of neuron-like cells, that establish synapses and make complex circuits is a convenient choice for *in vitro* studies, granting reproducibility, lowering costs and reducing animal use. The PC12 cell line fits well such purposes, working as neurobiological and neurochemical systems [9]. Since PC12 cells produce synaptic contacts, it allows studying synapse plasticity in response to drug treatment and different culture conditions. Interestingly, PC12 cells, when exposed to nerve growth factor (NGF), cease to multiply and sprout dendrite-like processes, similar to sympathetic neurons in primary cell cultures, producing synaptic contacts. PC12 cells synthesize and store the catecholamine neurotransmitters dopamine and norepinephrine. Importantly, NGF differentiated PC12 cells express several neuronal proteins, including MHC I, that can be actively regulated, for *example*, by interferon beta (IFN) [11]. Also, PC12 express CD3ζ, a fundamental subunit of the MHC receptor in T cells, which is required for signaling transduction [11].

Based on recent literature about the interaction and communication between neurons and astrocytes, the present work investigated changes in synaptic stability in PC12 cells subjected to NG97 conditioned medium and compared such effects with exposure to the pro-inflammatory cytokine IFN beta. The results herein indicate that astrocyte-derived factors increase MHC I expression by neuron-like cells, which in turn reduce density of synapses, observed by downregulation of synaptophysin, similarly to IFN treatment. Overall, the present results show that regulation of CD3ζ/MHC I directly affects neurite arborization and synapse stability in PC12 cells. Also, the data herein reinforce the importance of glial cells to neural circuits preservation and indicate that astrocytes and astroglial tumors secrete molecules that contribute to downsizing of neural connections, possibly affecting CNS functionality.

2. Material and methods

2.1. Immortalized cell cultures

The NG97 cell line was obtained from a human astrocytoma tumor by Grippo et al. [10], being kept at 37 °C under 5% CO_2 atmosphere in T75 polystyrene flasks in 20 mL of DMEM supplemented with 2.5 g/mL of glucose, 10% of bovine fetal serum and 1% of antibiotics (penicillin/streptomycin).

PC12 cells were obtained from ATCC (*American Type Culture and Collection; ID: CRL-1721*™) and donated by the Department of Chemistry of São Paulo University (USP, Ribeirão Preto). PC12 culture flasks were kept at 37 °C under 5% CO_2 atmosphere in T75 polystyrene flasks in 20 mL of DMEM supplemented with 2.5 g/mL of glucose, 10% of bovine fetal serum and 1% of antibiotics (penicillin/streptomycin).

2.2. Lentiviral vector construction, virus production and establishment of genetically modified PC12 cell lines

The lentivector plasmid harboring CD3ζ was generated by cloning the CD3ζ-GFP fragment, isolated from plasmid pd1eGFP-N1 CD3ζ (kindly provided by Hélène Boudin, University of Nantes, France), into FUGW lentivector plasmid [12]. The pd1eGFP-N1 CD3ζ plasmid was digested with NotI, following T4 DNA polymerase reaction, digestion with BglII and gel isolation to obtain the insert. The FUGW plasmid was then digested with EcoRI, following T4 DNA polymerase, BamHI digestion, treatment with Calf Intestinal Alkaline phosphatase and gel isolation to obtain the vector. The insert flanked by a blunt end and BglII site was cloned into the vector flanked by a blunt end and BamHI site generating the CD3ζ-eGFP lentivirus.

The lentivector plasmid harboring shRNA to silence CD3ζ was generated cloning annealed oligonucleotides encoding shRNA sequences to pLKO lentivector plasmid [17], which was previously digested with EcoRI and AgeI. Virus were generated and titrated at Viral vector Laboratory at LNBio – CNPEM.

2.3. Culture treatment with interferon beta (IFN beta)

The experiments were carried out with both cell lines (PC12 and NG97) cultured separately being the culture medium changed every two days for up to ten days. The effects of interferon beta treatment as well as NG97 conditioned medium were evaluated after five and ten days of culturing. PC12 cells were cultured (1×10^3 cells/well) and differentiated in 24 well-plates. Cultures were maintained over 5 and 10 days, and part of them were exposed to different concentrations of IFN beta.