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MICROGLIA PROLIFERATION AS A RESPONSE TO ACTIVATION IN THE FRESHWATER SNAIL *PLANORBARIUS CORNEUS:* A BrdU INCORPORATION STUDY*

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Invertebrate microglia constitute a class of cells resident in the ganglionic nervous system which are activated after tissue injury or by the presence of pathogens. The microglia activation response includes graduated morpho-functional and biochemical changes and cell proliferation. In this study we verified in the freshwater snail *Planorbarius corneus* that an activation caused by a traumatic event may induce microglia division. Cell proliferation was assessed immunocytochemically using BrdU incorporation technique and documented on both ganglionic sections and microglia cultured cells at different experimental conditions and times after activation. In addition, we studied the possibility of increasing microglia proliferation by adding to the cultured medium the Macrophage-Colony Stimulating Factor (M-CSF) that has been shown to stimulate specifically this process in vertebrates.

Keywords: Microglia – BrdU – proliferation – *Planorbarius corneus* – M-CSF

INTRODUCTION

In the ganglionic nervous system (GNS) of the freshwater snail *Planorbarius corneus* we previously showed a class of glia corresponding to the description of vertebrate microglia [11, 12] and now we are investigating different aspects about the nature and role of these cells. It has been shown that activation of microglia after CNS injury results in morphological and functional changes and in a rapid cell proliferation, probably in order to face the disrupting event with an appropriate cell support [1, 2, 5] but little is known about how this process is initiated and regulated. In this perspective, *in vitro* studies on vertebrates demonstrated the role of several growth factors and cytokines in stimulating or inhibiting microglia proliferation [4]. In this study, we wanted to verify if an experimental trauma might induce microglia proliferation in *P. corneus* GNS and also to test the action of Macrophage-Colony Stimulating Factor (M-CSF) in order to stimulate this process specifically.

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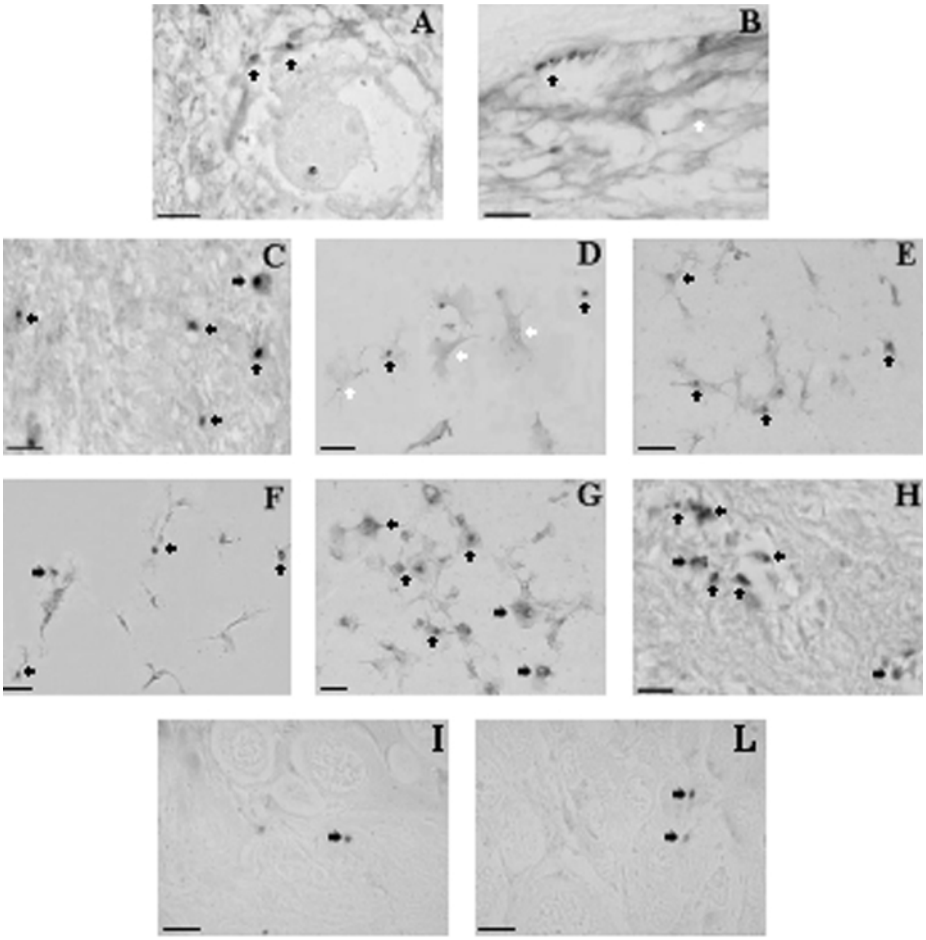

Fig. 1. A and B. Anti-BrdU on ganglionic section of ganglia incubated in 3 μM BrdU in PBS. Bar = 15 μm. A) Immunopositive glial nuclei (black arrows) beside a negative neuron (n). B) Immunopositive glial nuclei (black arrow) and a negative one (white arrow). C. Histological sections immunostained with anti-BrdU from ganglia cultured for 24 h. Bar = 10 μm. Immunopositive glial nuclei are present (arrows) in neuropile. D. Immunoreaction on glial cells cultured for 24 h. Bar = 10 μm. Positive glial cells are present (black arrows) beside negative ones (white arrows). E. Immunoreaction on glial cells cultured for 48 h. Bar = 10 μm. An higher number of cells have incorporated the nucleotide (arrows). F and G. Immunoreaction on glial cells from ganglia cultured with M-CSF. Bar = 10 μm. F) Cells cultured for 24 h: some immunopositive nuclei are present (arrows). G) Cells cultured for 48 h: immunopositive nuclei are more numerous (arrows). H. Ganglia cultured in L-15 with M-CSF for 24 h. Numerous immunopositive glial nuclei are present (arrows). I and L. Histological sections immunostained with anti-BrdU from ganglia cultured for 48 h. Bar = 10 μm. Only some glial cells have incorporated the BrdU. I) Ganglia cultured in L-15 without M-CSF. L) Ganglia cultured in L-15 with M-CSF

MATERIALS AND METHODS

Cell proliferation is assessed using 5-bromo-2'-deoxi-uridine (BrdU) incorporation technique followed by immunodetection using an anti-BrdU monoclonal antibody. We performed several comparative experiments. In order to verify microglia proliferation following activation, ganglionic rings were dissected and simply maintained in physiological solution for 1 h followed by incubation in PBS with 3 μM BrdU at 20 °C for 1 h. After this short period of time (acute trauma experiment), they were immediately fixed and paraffin-embedded. In further experiments, we kept the dissected ganglia in a modified L-15 medium [6, 11] charged with 3 μM BrdU for a longer period of time (24 and 48 h, prolonged trauma). In this case, we analysed for BrdU incorporation in ganglionic sections and migrated cultured microglial cells. Finally, we checked the possibility of promoting microglia proliferation by adding to the culture medium the nucleotide and 50 ng/ml M-CSF at the same time. The ganglionic rings used for the immunohistochemical detection were fixed in 4% paraformaldehyde, paraffin-embedded and sections (7 μm thickness) were prepared and mounted on gelatin-coated slides. Microglial cell cultures were fixed in 4% paraformaldehyde for 5 min at 37 °C and immunostained immediately. Controls were made by replacing the primary antiserum with normal serum, and ganglia from animals untreated with BrdU were used as further controls.

RESULTS AND DISCUSSION

Our results show that, after experimental trauma, BrdU incorporation is detected both in glial cells inside the nervous parenchyma and in cultured migrated microglia. We never found neurons showing BrdU incorporation (Fig. 1A). After an acute injury, some microglial cells appear to have incorporated the synthetic nucleotide (Fig. 1B). The majority of them still appears at the original site around the neuronal cell bodies and close to the periganglionic sheath (Figs 1A and B). In another series of experiments we cultured the ganglia for a prolonged period of time after trauma and BrdU pulse exposure; we found the same amount of labelled microglial nuclei inside the ganglia, but several cells were moving towards the neuropile and nerve stumps (Fig. 1C). In addition, some of the microglial cells which had migrated into the culture dishes appear to have incorporated the nucleotide (Fig. 1D). In some cases, we transferred the ganglia into new dishes and left them for further 24 h (total 48 h from the trauma). In this case, we noted on these substrates a higher percentage of cells showing a BrdU incorporation (Fig. 1E). The addition of M-CSF to the medium containing the ganglia leads to a higher rate of migrated cells in respect to controls and causes a significant increase of BrdU immunopositive nuclei in the population of migrated cells (Fig. 1F) with the highest level reached at 48 h (Fig. 1G). Compared sections from ganglia cultured for 24 h in presence or in absence of the tested growth factor show a different number of labelled resident microglia nuclei that are more numerous in presence of M-CSF (Figs 1C and 1H). In contrast, ganglia

cultured for 48 h in presence or in absence of M-CSF show in both cases a decrease of resident microglia marked by the antibody (Figs 1I and 1L), caused by the migration of these cells into the dishes. From our data, we can postulate that in *P. corneus* microglia proliferation takes place as an early event, soon after the neurological trauma, and that it primarily served to neuroprotection. Cell proliferation is well known as a component in the activation of vertebrate microglia [1, 2, 5, 7], but little is known about the regulation of this process and the role of activating molecules released from injured neurons [5]. Several cytokines (IL1, IL3 and IL5) and other molecules like Colony Stimulating Factors (CSFs) have been shown to induce microglia proliferation [4, 10]. We can justify the need to increase the number of microglia cells only in consequence of a pathogenic event, with the larger amount of molecules produced, which will play usually a neuroprotective role. Because microglia have a potential neurotoxicity depending on different conditions, these cells are thus controlled by maintaining them quiescent and their number as low as possible [8]. Indeed, the features of the several factors released by activated microglia have the potential of either protecting damaged neurons or aggravating their state until neuronal death [4]. Moreover, in case of degenerating structures or presence of pathogens, an increased number of microglia is justified with the shifting of these cells to a higher number of macrophages involved in phagocytosis. Concerning the effects of M-CSF on *P. corneus* microglia proliferation, we can confirm that it acts not only in invertebrates as a strong mitogen, but also promotes the mobilisation of the activated cells. Neurons may secrete M-CSF in response to injury [3]. Several *in vitro* studies show that M-CSF is also expressed in the microglia following stimulation by neuronal cytokines; its expression and release would cause a further microglia migration and proliferation [9]. Finally, we cannot exclude that microglia proliferation may be seen, as noted in some cases [8], also as a consequence of the deficiency of inhibitor stimuli on microglia by neurons after their damage.

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