Neuroscience 154 (2008) 496–503

S100B IS EXPRESSED IN, AND RELEASED FROM, OLN-93 OLIGODENDROCYTES: INFLUENCE OF SERUM AND GLUCOSE DEPRIVATION

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Abstract—S100B (member of a family of proteins that are 100% soluble in ammonium sulfate at neutral pH) has been widely used as astrocyte marker in animal models and in human brain diseases. Recent studies revealed S100B-immunopositivity in oligodendrocytes and O2A oligodendroglial progenitor cells. It is unknown, however, if oligodendrocytes produce S100B themselves, or if the S100B-immunolabeling is caused by binding or absorption of the protein.

To address this question, S100B expression and protein release were analyzed in a highly pure oligodendrocytic OLN-93 cell line (from rat), in the astrocytic C6 cell line (from rat) and primary astrocytes. S100B was gene expressed in all cultures, as revealed by reverse transcriptase polymerase chain reaction (RT-PCR) analysis. OLN-93 cells and glial fibrillary acidic protein (GFAP)-negative astrocytes expressed the multiligand receptor for advanced glycation end products (RAGE). S100B protein levels were determined in supernatants and cell homogenates by immunoluminometry under normal conditions and after serum and glucose deprivation (SGD). SGD led to a several-fold increased release of S100B (after 6 and 24 h), which was particularly pronounced in primary astrocytes. Increased S100B in cell homogenates was most notable in OLN-93 cells under SGD, indicating activated S100B synthesis. These cells also showed the highest percentage of dead cells, as determined by propidium iodide-positivity, after SGD. Incubation with 0.5, 2 and 5 μg/l exogenous S100B was not toxic to OLN-93 cells.

In conclusion, OLN-93 cells produce more S100B under SGD than astrocytes and are more susceptible to cell death upon SGD, which provokes leakage of S100B. Our data indicate active S100B secretion from astrocytes under SGD since highly elevated levels of S100B were detected in the supernatant despite a low percentage of dead cells. The experimental results provide further evidence for a production/release of S100B in/from oligodendrocytes, e.g. in metabolic stress conditions like cerebral ischemia. Studies on S100B in bodily fluids should be carefully interpreted in order to avoid misleading hypotheses concerning the specific involvement of astrocytes, due to the various cellular sources of S100B. **© 2008 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: astrocytes, oligodendrocytes, OLN-93, RAGE, S-100B, S100 beta.

S100B (10.5 kDa; gene locus on human chromosome 21q22.3; 100% soluble in ammonium sulfate at neutral pH) is a calcium, copper and zinc ion binding protein that is a member of the S100-calmodulin-troponin superfamily, and was primarily found in high abundance within the nervous system (Moore, 1965; Moore and Perez, 1967). A number of intracellular growth-associated target proteins have been identified for S100B, such as growth-associated protein 43, the regulatory domain of protein kinase C, the anti-apoptotic factor Bcl-2 (product of the B-cell lymphoma/leukemia 2 gene) and the tumor-suppressor protein P53 (with a molecular mass of 53,000 Da) (Donato, 2001). S100B also regulates protein ubiquitination and the assembly of cytoskeleton components such as microtubules, glial fibrillary acidic protein (GFAP) and vimentin (Bianchi et al., 1994; Donato, 2001; Nowotny et al., 2003). Additionally, S100B is a secretory protein, and it exhibits cytokine-like activities that mediate interactions among glial cells and between glial cells and neurons. Nanomolar levels of S100B stimulate neurite growth and promote neuronal survival, while micromolar levels result in opposite effects (van Eldik and Wainwright, 2003). This cytokine-like activity is transduced, in part, by the receptor for advanced glycation end products (RAGE) and nuclear factor kappa B (Donato, 2001).

Elevated levels of S100B in cerebrospinal fluid and peripheral blood have been observed in Alzheimer's disease, stroke, traumatic brain injury, meningoencephalitis, mood disorders and schizophrenia (Lins et al., 2005; Pelinka et al., 2004; Peskind et al., 2001; Schroeter et al., 2002; Steiner et al., 2006; Wunderlich et al., 2004). In these contexts, high S100B levels in body fluids have been thought to be the result of elevated secretion from astro-

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Abbreviations: C6, an astrocytic cell line (from rat); DAPI stain, a nuclear staining with 4′,6-diamidin-2′-phenylindol-dihydrochloride; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; LDH, lactate dehydrogenase; OLN-93, an oligodendrocytic cell line (from rat); O2A progenitor cells, immature oligodendrocytes; $p75^{NTR}$, p75 neurotrophin receptor; PI, propidium iodide; RAGE, receptor for advanced glycation end products; RT-PCR, reverse transcriptase polymerase chain reaction; SGD, serum and glucose deprivation; S100B, member of a family of proteins that are 100% soluble in ammonium sulfate at neutral pH.

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doi:10.1016/j.neuroscience.2008.03.060

cytes or leakage from damaged cells. This idea may have arisen from postmortem studies on Alzheimer's disease and Down's syndrome that described predominant S100B immunostaining in reactive astrocytes surrounding cortical neuritic plaques (Griffin et al., 1989; Sheng et al., 1994; van Eldik and Griffin, 1994; Mrak et al., 1996). Additionally, cell culture experiments showed that S100B can be produced by astrocytes, and that the release of S100B is activated by metabolic stress, such as oxygen-, serum- and glucose-deprivation, and is suppressed by glutamate (Gerlach et al., 2006; Tramontina et al., 2006). However, our recent studies on human brain tissue revealed immunolocalization of S100B in oligodendrocytes and immature oligodendroglial O2A progenitor cells, especially in the white matter (Steiner et al., 2007a,b). These findings are in accordance with immunohistochemical studies in animals (Deloulme et al., 2004; Dyck et al., 1993; Hachem et al., 2005; Rickmann and Wolff, 1995; Romero-Aleman Mdel et al., 2003; Vives et al., 2003) and early observations by those that discovered the protein family, who found higher levels of S100 in white matter relative to cortical brain regions (Moore and Perez, 1967). It is unknown, however, if oligodendrocytes produce S100B themselves, or if the immunodetection of S100B is caused by binding or absorption of the protein.

OLN-93 is a permanent oligodendrocytic cell line that was derived at a late stage of differentiation from spontaneously transformed cells in a primary rat brain glial culture (Richter-Landsberg and Heinrich, 1996). These cells show characteristics of immature oligodendrocytes, and their antigenic and morphological properties resemble a highly pure culture of primary oligodendrocytes. Thus, OLN-93 cells appear to be a suitable model for the study of S100B in oligodendrocyte-like cells in comparison with astrocyte cultures. The following issues were addressed in the present study: (i) Are OLN-93 and C6 (an astrocytic cell line (from rat)) cells capable of S100B gene expression? (ii) Is the cellular production and release of S100B protein increased in these cultures by metabolic stress, as was previously described in primary astrocytes by Gerlach et al. (2006)?

EXPERIMENTAL PROCEDURES

Cell culture

Primary cortical astrocyte cultures from Wistar rats were prepared by removing the cortex of newborn rats (0–1 day old), cleaning the meninges and placing blocks of this tissue in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 1% (volume per volume) penicillin/streptomycin (growth medium). After mechanical dispersion, aliquots of the cell suspension (2 ml) were plated in Petri dishes at a final density of 2.5×10^5 cells per ∅35 mm Petri dish. After 14 days, the cells were used in the experiments described below.

C6 glioma cells were obtained from the European Collection of Cell Cultures (Porton Down, Wiltshire, UK). The same passage number (passage 6) was used for all experiments, in order to achieve standardized conditions by excluding passage-related changes in cell character. Cryopreserved C6 cells were defrosted, resuspended in Roswell Park Memorial Institute medium 1640 medium supplemented with 10% fetal calf serum, 1% L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin (growth medium), and transferred to culture flasks. After 3 days, cells were removed from the flasks by mild trypsinization (5 min; trypsin/EDTA: 0.05%/0.002%) and replated on ∅35 mm Petri dishes (50,000 cells/dish). After 6 days, experiments were performed.

N_2A neuroblastoma cells were obtained from the European Collection of Cell Cultures. The same passage number (passage 4) was used for all experiments in order to achieve standardized conditions by excluding passage-related changes in cell character. Cryopreserved N_2A cells were defrosted, resuspended in DMEM supplemented with 10% fetal calf serum, 1% L-glutamine, 1% NEAA (non-essential amino acids), 50 U/ml penicillin and 50 μg/ml streptomycin (growth medium), and transferred to culture flasks. After 3 days, cells were removed from the flasks by mild trypsinization (5 min; trypsin/EDTA: 0.05%/0.002%) and replated on ∅35 mm Petri dishes (50,000 cells/dish). After 6 days, respective experiments were performed.

The permanent oligodendroglial cell line OLN-93 was obtained from the Richter-Landsberg laboratory (Richter-Landsberg and Heinrich, 1996). For the respective experiments, cryopreserved OLN-93 were defrosted, resuspended in DMEM supplemented with 10% fetal calf serum, 50 U/ml penicillin and 50 μg/ml streptomycin (growth medium), and transferred to culture flasks. After 1 week, cells were removed from the flasks by mild trypsinization (5 min; trypsin/EDTA: 0.05%/0.002%) and replated on ∅35 mm Petri dishes (30th passage, 50,000 cells/dish). After 3 days, the fetal calf serum concentration of the growth medium was reduced to 0.5%, and the respective experiments were performed 72 h later.

All cultures were plated on poly-D-lysine-coated dishes and maintained at 37 °C in a humidified atmosphere under 5% CO_2 in air, and were fed twice per week by changing 1 ml of medium.

For serum and glucose deprivation (SGD), the normal growth medium was replaced by serum- and glucose-free DMEM for 6 or 24 h. In control cultures, the normal growth medium was replaced by DMEM containing serum and glucose. Fifteen dishes were used for each experimental setting. All cultures were kept at 37 °C in a humidified atmosphere under 5% CO_2 in air (normoxic conditions) for the duration of the experiment.

Immunocytochemistry

C6, primary astrocyte and OLN-93 cultures were thoroughly washed twice with phosphate-buffered saline (pH 7.4), then fixed for 30 min in 4% buffered paraformaldehyde and incubated at room temperature with one of the following antibodies diluted in phosphate-buffered saline with 0.3% Triton X-100 and 1% normal goat serum for 3 h: (i) polyclonal rabbit anti-recombinant-S100B (DAKO, Glostrup, Denmark) 1:100; (ii) monoclonal mouse anti–p75 neurotrophin receptor ($p75^{NTR}$, clone 192, Chemicon, Hampshire, UK) 1:100; (iii) monoclonal mouse anti-GFAP (clone GA5, Chemicon) 1:1000; (iv) polyclonal rabbit anti-RAGE (ab3611, Abcam, Cambridge, UK) 1:200. The used S100B-antibody is highly specific, as revealed by preabsorption and immunoblotting in one of our previous studies (Steiner et al., 2007a). In addition, according to the manufacturer, no cross-reactivity with S100A1, S100A2, S100A4 or S100A6 has been observed.

Following incubation with primary antibodies, the cultures were washed in phosphate-buffered saline (3×5 min) and incubated for 3 h with the respective secondary antibodies (Molecular Probes, Göttingen, Germany) at a 1:500 dilution: Alexa Fluor 546 (goat anti-rabbit-IgG; red fluorescence) and Alexa 488 (goat anti-mouse-IgG; green fluorescence). The specimens were examined using a fluorescence microscope (Axiophot) equipped with phase-contrast, fluorescein, rhodamine and 4′,6-diamidin-2′-phenylindol-dihydrochloride (DAPI) optics. The specificity of the immunoreactions was controlled by the application of buffer instead of the primary antiserum. These negative controls were free of specific immunostaining.

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