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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.

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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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.

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from the respective cultures (3×3 dishes/experimental setting) using guanidinium isothiocyanate/phenol/chloroform (peqGOLD TriFast, peqlab, Erlangen, Germany). To remove contaminating DNA, 5 μg of total cell RNA was treated with Turbo DNA-free (Ambion, Austin, TX, USA) according to the manufacturer's instructions. RNA (4.5 μl; 2.25 μg input RNA) was reverse transcribed using the RevertAidTM H Minus First Strand cDNA Synthesis Kit primed with oligo-deoxythymidylic acid 18 primers (Fermentas, St. Leon-Rot, Germany); primers: S100B, forward 5′-GAGAGAGGGTGACAAGCACAA-3′, reverse 5′-GGCCATAAACTCCTGGAAGTC-3′ (169 bp, GenBank accession no. NM_013191); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5′-TTAGCACCCCTGGCCAAGG-3′, reverse 5′-CTTACTCCTTGGAGGCCATG-3′ (GAPDH, housekeeping gene, 531 bp, GenBank accession no. NM_017008). cDNA (2 μl) was then PCR amplified with *Taq*DNA polymerase (peqlab; *Taq*, *Thermus aquaticus* (a bacterium)). One-tenth of each reaction product was electrophoresed on a 1% agarose gel. The PCR product bands were quantified by densitometric analysis using a Biometra BioDocAnalyzer and the ratio of S100B expression to expression of the housekeeping gene GAPDH was calculated. Each experiment was repeated three times.

Protein extraction and S100B assay

Media were removed from the cultures, centrifuged at 4000×*g* at 4 °C and supernatants were frozen at −80 °C until further analysis. Cells were scraped from the surface of the culture dishes. The cell suspension was harvested in 300 μl sodium phosphate buffer (50 mM, pH 7.4) with protease inhibitors (Complete Mini™, Roche, Mannheim, Germany) and centrifuged at 2000×*g*. Subsequently, the cell pellet was homogenized at 4 °C using a Potter-Elvehjem glass-Teflon homogenizer, performing 10 strokes at 600 r.p.m. The homogenates were stored at −80 °C until further analysis. Protein was measured with the BCATM protein assay (Pierce, Rockford, IL, USA) according to the manufacturer's instructions using bovine serum albumin as standard.

S100B concentrations were measured by immunoluminometric sandwich assay using directly coated magnetic microparticles (LIAISON S100™, DiaSorin, Dietzenbach, Germany), as previously described (Steiner et al., 2006; Wunderlich et al., 2004). The minimal measurable concentration of S100B was 0.02 μg S100B/l. Intra- and inter-assay coefficients of variation were <5%. Measurements were performed in duplicate.

Cell integrity analysis

Cell integrity was assessed by double-labeling with propidium iodide (PI) and fluorescein diacetate (Keilhoff and Wolf, 1993). The technique is based on the ability of living cells to hydrolyze fluorescein diacetate (10 μg/ml phosphate-buffered saline, 5 min) by intracellular esterases, resulting in a green–yellow-colored fluorescence. Dead cells were labeled by PI (5 μg/ml phosphate-buffered saline, 5 min), which interacts with DNA to yield a red fluorescence of cell nuclei. Evaluation was carried out on a fluorescence microscope (Axiophot, Zeiss, Jena, Germany) equipped with phase-contrast, fluorescein, rhodamine and DAPI optics.

Incubation of OLN-93 cells with exogenous S100B

A concentrated stock of S100B (Merck/Calbiochem, Darmstadt, Germany) was made in phosphate-buffered saline. S100B was added to OLN-93 medium at final concentrations of 0, 0.5, 2.0 and

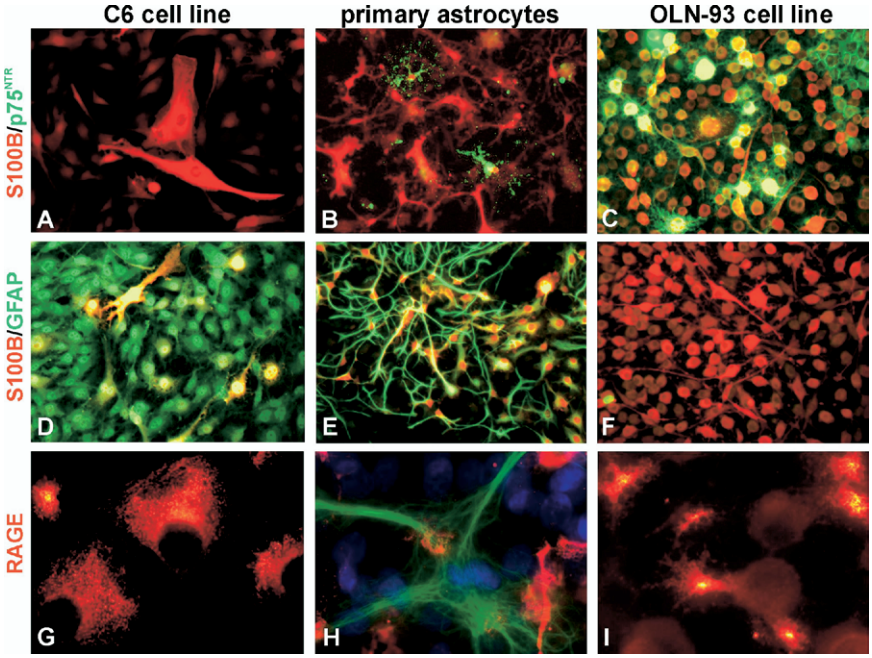

Fig. 1. Immunocytochemical characterization of C6, primary astrocyte and OLN-93 cultures. (A–C) (400× Magnification): S100B (red) was detected in all investigated cell types. C6 cultures (A) contained no $p75^{NTR}$-labeled (green) oligodendroglial cells, while primary astrocyte cultures (B) contained only few of them (<5%) indicating a high purity of these culture systems. S100B (red) was detected in most $p75^{NTR}$-labeled oligodendroglial OLN-93 cells (C). (D–F) (400× Magnification): Co-localization of S100B (red) and the astrocytic marker GFAP (green) was restricted to C6 cells (D) and primary astrocyte cultures (E). OLN-93 cells did not show GFAP-labeling, indicating that S100B expression was not caused by astrocytic cells in this culture system (F). (G–I) (1000× Magnification): Punctate immunolocalization of RAGE (orange red) was observed in subpopulations of all investigated cell types. Merged DAPI nuclear stain (blue), RAGE (red), and GFAP (green) indicated no expression of RAGE in GFAP-immunostained astrocytes (H). RAGE was mainly detected in the growth cone and processes of OLN-93 cells (I).

5.0 μg/l in order to determine whether exogenous S100B has a cytotoxic effect on OLN-93 cells. The range of S100B concentrations used in these experiments was comparable to that measured in the SGD experiment using OLN-93 cultures. Cell integrity was assessed after incubation for 6 or 24 h by double-labeling with PI and fluorescein diacetate (see 2.5).

Statistical analysis

The following quotient was calculated for each sample: S100B concentration divided by the total protein concentration, relative to control samples. In addition, the percentages of dead cells (PI-positive cells) were calculated. These data were normally distributed, as indicated by Kolmogorov-Smirnov tests. *t*-Tests or analysis of variance was employed and a probability level of $P<0.05$ was considered to be statistically significant.

RESULTS

S100B/RAGE immunolocalization and S100B gene expression

Positive S100B immunostaining was observed in both astrocyte culture systems (Fig. 1A, B) and in most $p75^{NTR}$-labeled oligodendroglial OLN-93 cells (Fig. 1C). The immunolocalization of S100B in OLN-93 cells was not caused by contamination with astrocyte-like cells, as revealed by co-labeling with GFAP (Fig. 1F).

The immunolocalization of RAGE (the surface receptor that is known to bind S100B) had a punctate pattern, which is typical for surface receptors, and was observed in subpopulations of astro- and oligodendroglial cells (Fig. 1G–I). However, astrocytes that were expressing GFAP appeared to be RAGE-negative (Fig. 1H). For OLN-93 oligodendrocytes, RAGE was mainly detected within the growth cone and processes (Fig. 1I).

RT-PCR analysis of S100B gene expression was carried out in order to determine if astrocyte- and oligodendrocyte-like cells are capable of producing S100B themselves. As shown in Fig. 2, S100B mRNA was detected in C6 cells, OLN-93 cells and primary astrocytes. In contrast, N_2A neuroblastoma cells did not show significant S100B expression.

Release of S100B from astrocytes and oligodendrocytes during metabolic stress

To analyze the kinetics and amount of S100B release under normal and metabolic stress conditions, C6 cells, primary astrocytes and OLN-93 cells were subjected to SGD for 6 and 24 h. Control cultures were left untreated. Quantification of extracellular S100B levels by immunoluminometric sandwich assay indicated a *basal release* of S100B from all tested cell types under *normal* conditions. According to our measurements, this release comprised less than 1% of the total intracellular S100B protein content. Six hours and 24 h of metabolic stress led to significantly elevated release of S100B in comparison to control

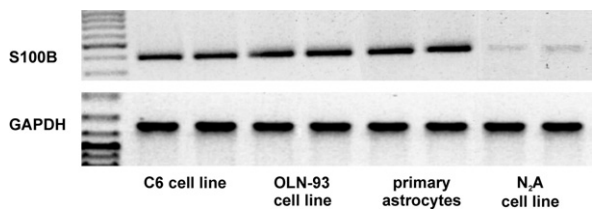

Fig. 2. S100B RT-PCR analysis in relation to expression of the housekeeping gene GAPDH. S100B mRNA was detected in C6 cells, OLN-93 cells and primary astrocytes, indicating S100B gene expression. In contrast, N_2A neuroblastoma cells did not show significant S100B expression.

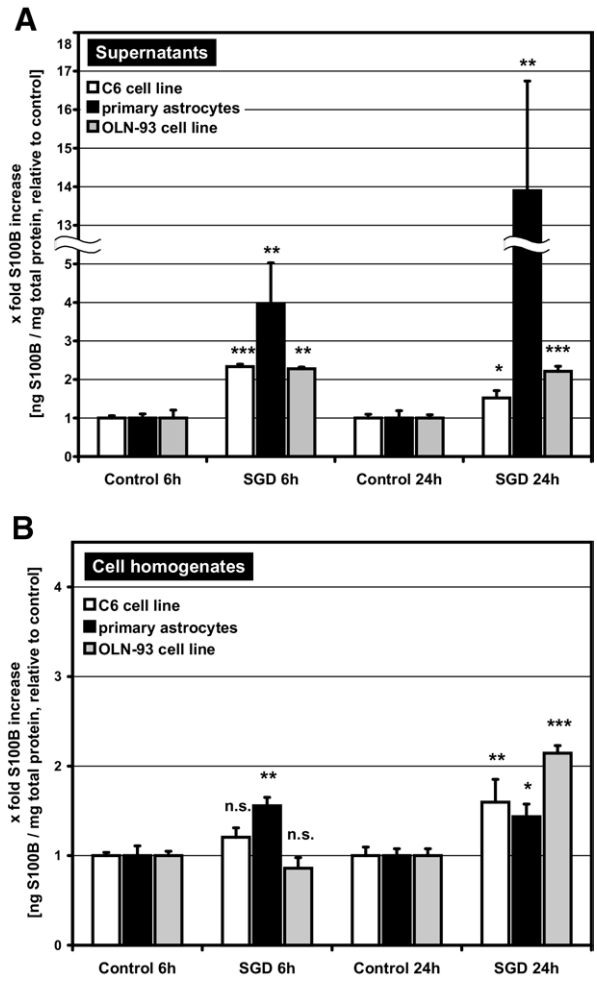

Fig. 3. S100B protein concentrations in supernatants and cell homogenates of C6, primary astrocyte and OLN-93 cultures were measured by immunoluminometric sandwich assay (LIAISON S100™, DiaSorin, Dietzenbach, Germany). (A) Time course of S100B release [fold S100B release relative to control] into the culture supernatant after serum- and glucose-deprivation (SGD) for 6 and 24 h. Control cultures were left untreated. Metabolic stress induced an increased release of S100B from all cultures after 6 and 24 h of metabolic stress, which was particularly pronounced in primary astrocytes but was similar in the C6 and OLN-93 cell lines. (B) Time course of intracellular S100B concentration, measured in cell homogenates [fold S100B increase, relative to control] after SGD for 6 and 24 h. Control cultures were left untreated. Metabolic stress induced an elevated intracellular content of S100B at 24 h in all culture systems, indicating active S100B synthesis. This effect was most prominent in OLN-93 cells. Six hours of SGD led to a significant increase of S100B in primary astrocytes, which seemed to respond earlier than C6 and OLN-93 cells to SGD stimulation. *Annotation:* Data are given as mean±S.E.M. from $n=15$ cultures per treatment. Experiments were repeated two times with similar results. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, n.s.=not significant in comparison with controls.

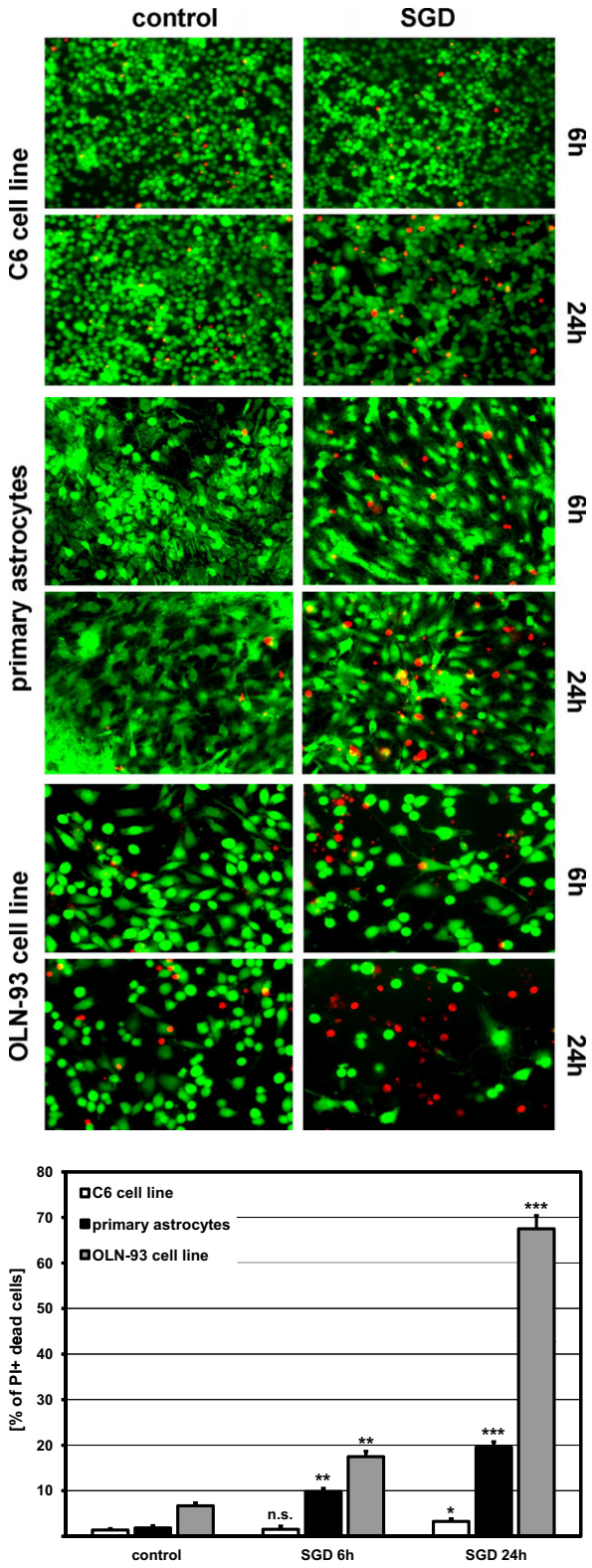

Fig. 4. The cell integrity in C6, primary astrocyte and OLN-93 cultures was assessed by double-labeling with PI (red) and fluorescein diacetate (green). Photographs display characteristic examples of the degeneration process in all three culture systems after SGD for 6 or 24 h, in comparison with untreated control cultures (200× magnification). The percentage of dead cells (PI/PI positive) is shown in the diagram for each cell culture type, for untreated cells and after 6 or 24 h of SGD. *Annotation:* Data are given as mean±S.E.M. from $n=10$ cultures per treatment. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, n.s.=not significant in comparison with controls. Both the photographs and the quantitative evaluation show a particularly pronounced effect of SGD on OLN-93 cells, leading to increased cell death. In contrast, C6 cells were the most robust cell type. Primary astrocytes were intermediate concerning their sensitivity to cytotoxic effects of SGD.

cultures. This effect was similar in the C6 and OLN-93 cell lines (C6: 2.3-fold after 6 h, 1.5-fold after 24 h; OLN-93: 2.3-fold after 6 h, 2.2-fold after 24 h; Fig. 3A) and was particularly pronounced in primary astrocytes (4.0-fold after 6 h, 13.9-fold after 24 h; Fig. 3A). Relative to the total cellular protein content, the intracellular S100B concentration also increased after SGD for 24 h in all culture systems, indicating activated S100B synthesis induced by metabolic stress (Fig. 3B). This effect was particularly pronounced in OLN-93 cells (OLN-93: 2.2-fold; C6: 1.6-fold; primary astrocytes: 1.4-fold). Six hours of SGD induced a significant increase in S100B in primary astrocytes (1.6-fold), which seem to respond earlier than C6 and OLN-93 cells to SGD stimulation (Fig. 3B).

To determine the degree of cell death and concomitant non-specific release of cellular proteins due to membrane leakage, we performed parallel measurements of cell integrity by double-labeling with PI and fluorescein diacetate after SGD for 6 or 24 h, in comparison with untreated control cultures (Fig. 4). Both the photographs and the quantitative evaluation show a particularly pronounced effect of SGD on OLN-93 cells. In contrast, C6 cells were the most robust cell type. Primary astrocytes were intermediate concerning their sensitivity to metabolic stress. The percentage of PI positive cells after 6 h was 1.5%, 9.8% and 17.5% for the C6, primary astrocyte and OLN-93 culture system and 3.3%, 19.5% and 67.5% after 24 h, respectively (Fig. 4, diagram).

Effect of exogenous S100B on OLN-93 cells

OLN-93 cells were incubated for 6 or 24 h with exogenous S100B. The concentrations used (0, 0.5, 2.0 or 5.0 μg/l) were comparable to those measured in supernatants of OLN-93 cultures with or without SGD. Double-labeling with PI and fluorescein diacetate did not reveal dose-dependent cytotoxic effects of exogenous S100B itself, which were similar to those of SGD (Fig. 5 in comparison with Fig. 4, diagram).

DISCUSSION

S100B has been widely used as an astrocytic marker in animal models and studies of human brain diseases. However, our recent investigations on human brain tissue revealed an additional immunolocalization of S100B in oligodendrocytes or immature O2A glial progenitor cells (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;

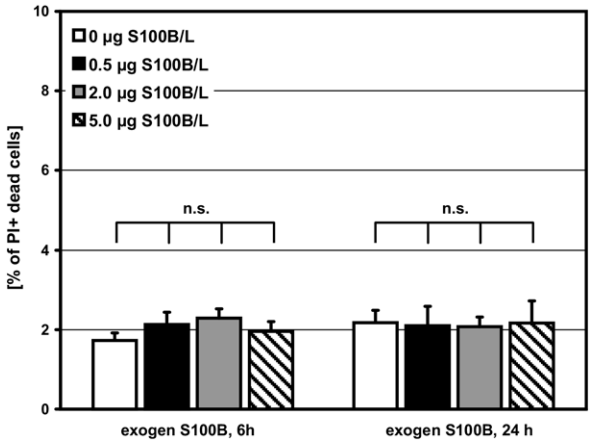

Fig. 5. Cell integrity of OLN-93 cells was assessed by double-labeling with PI and fluorescein diacetate after incubation with 0, 0.5, 2.0 or 5.0 µg/l exogenous S100B for 6 or 24 h. Exogenous S100B alone did not seem to contribute to the cell death sensitivity of OLN-93 cells within this concentration range. *Annotation:* Data are given as mean±S.E.M. from $n=5$ cultures per treatment. n.s.=No significant differences in cell integrity.

Vives et al., 2003) and an earlier study by Richter-Landsberg and Heinrich (1995), who observed S100-immunoreactivity in both astrocytic and oligodendrocytic rat primary cell cultures. The immunodetection of S100B may relate to the synthesis of S100B in oligodendrocytes, or to the binding or absorption of S100B released from astrocytes. So far, however, no study has reported on S100B gene expression and protein release from oligodendrocytic cells.

OLN-93 cells have been chosen as a model to study S100B in highly pure oligodendrocytic cells in comparison with C6 and primary astrocyte cultures. In summary, S100B was immunolocalized to all investigated cell types. RAGE, the surface receptor that is known to bind S100B, was localized to subpopulations of astrocytes and oligodendroglial cells, as previously described (Ponath et al., 2007; Toth et al., 2006). RT-PCR analysis was carried out in order to determine whether the positive S100B-immunostaining was caused by extracellular binding of the S100B protein or due to endogenous S100B expression. We demonstrated that astrocyte- and oligodendrocyte-like cells are capable of intracellular S100B gene expression.

Less than 1% of the intracellular S100B protein content was released in all tested culture systems under *normal conditions*. Metabolic stress led to a several-fold increased release of S100B (after 6 and 24 h) that was particularly pronounced in primary astrocytes. Increased S100B in cell homogenates was observed, especially in OLN-93 cells under SGD, indicating activated S100B synthesis. These cells also showed the highest percentage of PI-positive dead cells after SGD. Exogenous S100B itself did not seem to contribute to the cell death sensitivity of OLN-93 cells.

OLN-93 cells produce more S100B under SGD than astrocytes and are more susceptible to cell death, which likely leads to the leakage of S100B from dying cells. In contrast, we provide indirect evidence that S100B is secreted from astrocytes under SGD, since high amounts of S100B were detected in the supernatant, despite a relatively low percentage of PI-positive dead cells (Figs. 3 and 4). These results are in accordance with those of Gerlach et al. (2006), who concluded that S100B is actively secreted from astrocytes based on the observation that the relative release of lactate dehydrogenase (LDH) into supernatants was lower than the release of S100B after metabolic stress. Other studies have also used this indirect method to prove basal and stimulated "secretion" of S100B from C6 cells and primary astrocytes (de Almeida et al., 2007; Leite et al., 2006; Nardin et al., 2007; Pinto et al., 2000; Tramontina et al., 2006; van Eldik and Zimmer, 1987). Measuring cell integrity via double fluorescence staining with PI and fluorescein diacetate, as in the present study, is conceptually similar to the LDH assay (Keilhoff and Wolf, 1993). One major problem is that the exact mechanism of S100B secretion has not been identified, even in astrocytes. Suitable direct techniques to assess the secretion of S100B need to be developed.

CONCLUSION

In summary, we and others have shown that C6 cells and primary astrocytes are capable of producing and releasing S100B. Here, we report that S100B is also expressed in, and released from, OLN-93 oligodendrocytes. The present study indicates that oligodendrocytes may contribute to elevated S100B levels in the brains and bodily fluids of patients suffering from various brain diseases.

However, S100B-immunopositivity has also been observed in other neural cell types of the CNS, including ependymal cells, choroid plexus epithelium, and even in a few neurons (Steiner et al., 2007a). In addition, extracranial sources of S100B have been identified, such as adipocytes, chondrocytes, lymphocytes, melanocytes, the myocardium, vascular endothelial/smooth muscle cells, satellite cells of dorsal root ganglia and Schwann cells (Donato, 2001; Netto et al., 2006; Steiner et al., 2007a). There are also physiological stimuli that promote the release of S100B into bodily fluids and are not related to CNS diseases. Examples of these stimuli are physical exercise (Dietrich et al., 2003; Schulpis et al., 2007), stress (Margis et al., 2004; Scaccianoce et al., 2004), fasting (Netto et al., 2006), critical illness (Routsi et al., 2006), cardiac arrest (Piazza et al., 2005) and extracranial injuries without brain injury (Savola et al., 2004; Unden et al., 2005). Thus, studies on S100B in bodily fluids should be carefully interpreted in order to avoid misleading hypotheses concerning the involved cellular pathology.

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