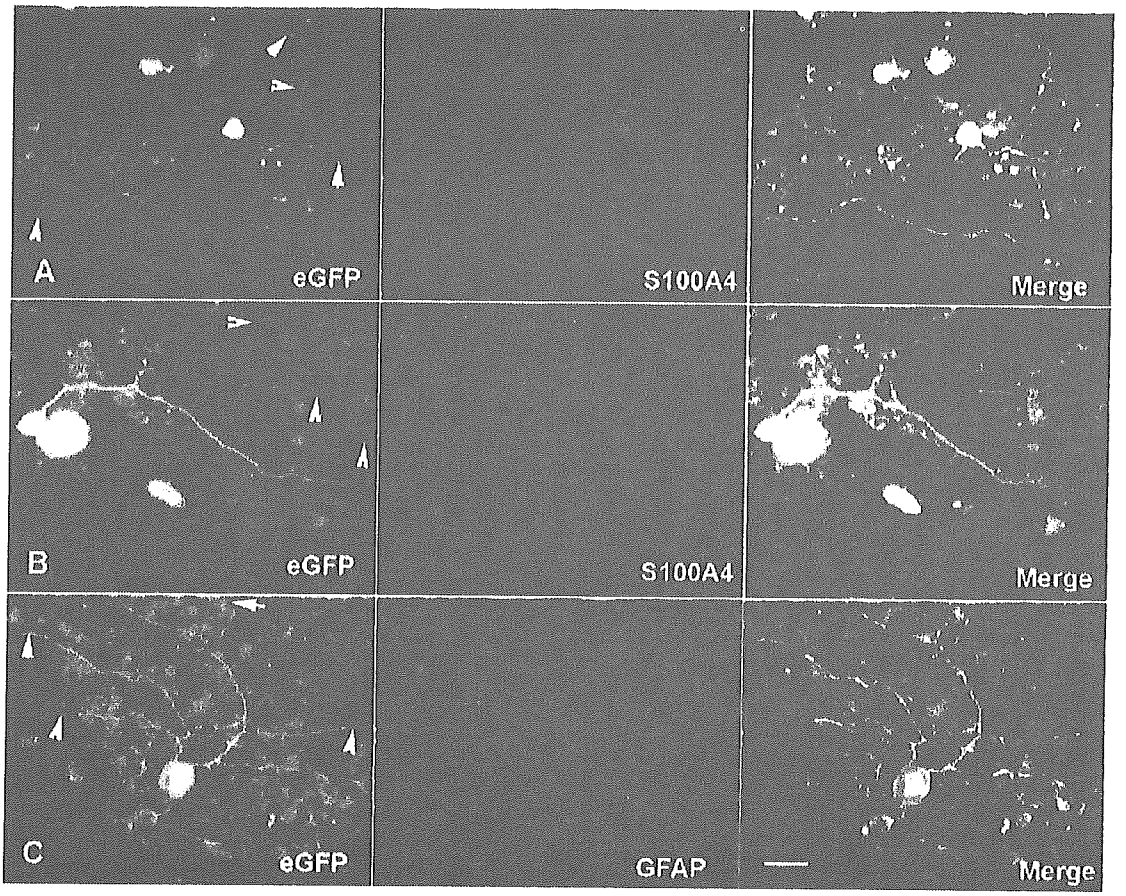

Fig. 3. Neurite growth from GFP-expressing dorsal root ganglion cells 24 hr after seeding on different types of white matter astrocyte cultures. **A:** White matter astrocytes treated with control siRNA. **B:** White matter astrocytes treated with S100A4 siRNA. **C:** White matter, S100A4-expressing astrocyte cultures treated with recombinant S100A4 (5 μM) 2 hr before dorsal root ganglion cells were seeded on the astrocytes. Note the thin and relatively few neurites in A, compared with the more intensive outgrowth in B and, in particular, in C. The middle panel shows labelling of astrocytes with antibodies to S100A4 (A,B) and glial fibrillary acidic protein (GFAP; C). The right panel shows merged pictures of GFP/antibody labelling. Scale bar = 50 μm.

S100A4 are incompletely known. The neuritogenic effect on hippocampal neurons was shown to involve activation of phospholipase C, protein kinase C, the extracellular regulated kinases Erk-1 and -2, as well as the levels of intracellular calcium (Novitskaya et al., 2000). To influence neurite extension, these events have to be transduced into changes in cytoskeleton organization. Studies on glioma cell migration in vitro have provided evidence for modifications of the actin cytoskeleton and several of its regulators by extracellular S100A4 in association with increased migration of these cells (Belot et al., 2002).

The neurite outgrowth-promoting effect of extracellular S100A4 in our cocultures may also, at least in part, be indirect via S100A4-mediated changes in the white matter astrocytes. S100A4 is a target for the CCN3 (NOV) protein, a member of the CCN family, which are matricellular proteins involved in the regulation of cell proliferation, differentiation, survival, adhesion, and migration (Lombet et al., 2003). CCN3 is widely expressed in the CNS and in primary astroglial cultures (Fu et al., 2004). CCN3 activity increases integrin ligand binding, which may result in increased production of MMPs (Lombet et al., 2003; Benini et al., 2005). In this way, extracellular CCN3–S100A4 interactions can lead to increased production of astroglial MMPs and facilitated neurite extension.

Finally, extracellular S100A4 may induce changes in intracellular functions of S100A4-expressing astrocytes, via a recently identified RAGE (receptor for advanced glycation endproducts)-mediated nuclear translocation of intracellular S100A4 (Hsieh et al., 2004). This process appears to be highly specific; it requires the extracellular activity of the same S100 protein that is present intracellularly. In the present situation, extracellular S100A4 may therefore alter the

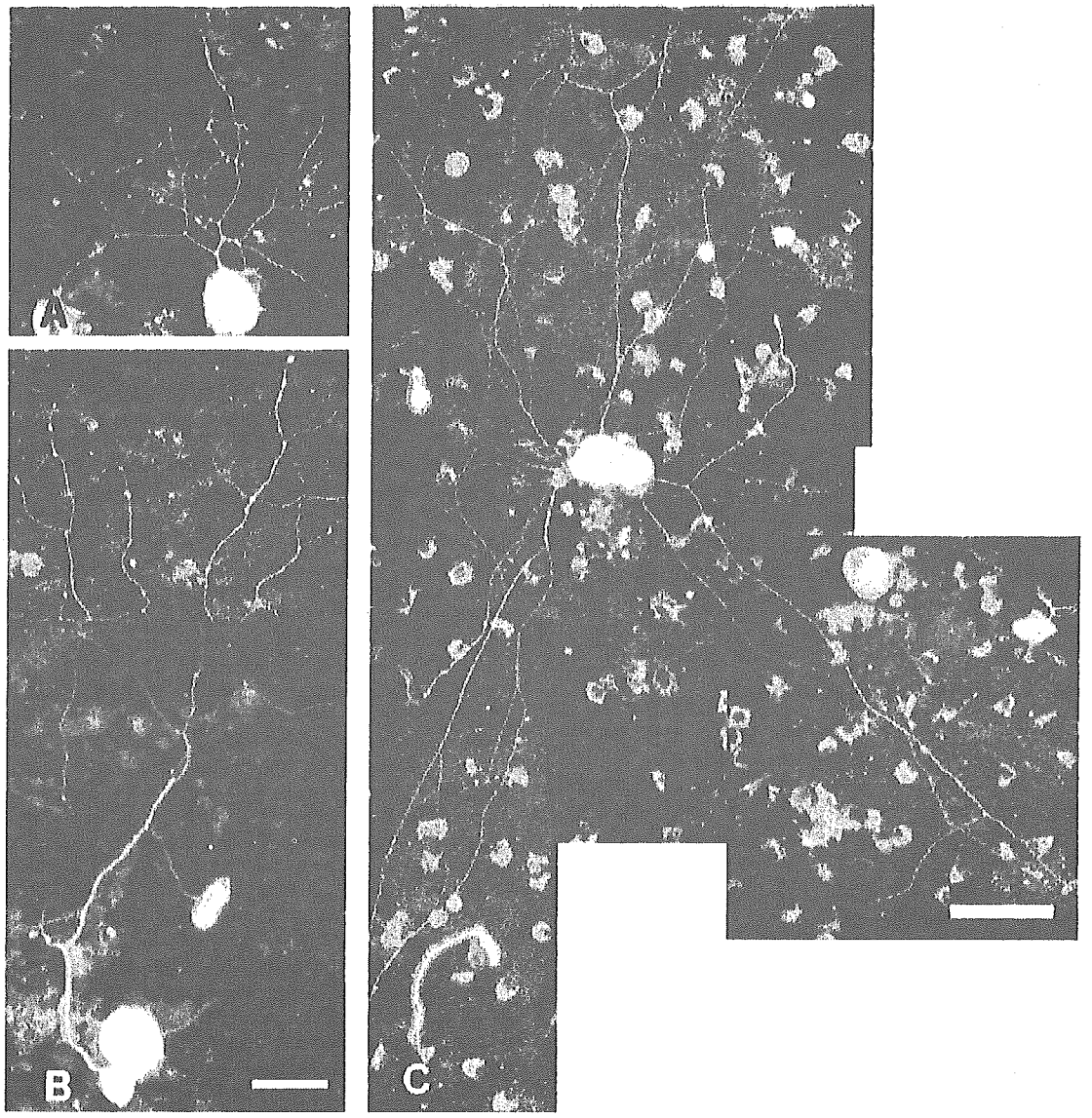

Fig. 4. Details of neurite growth from GFP-expressing dorsal root ganglion cells 18 hr after seeding on white matter astrocyte cultures. **A:** Control siRNA-treated cultures. **B:** S100A4 siRNA-treated cultures. **C:** S100A4-expressing cultures treated with recombinant S100A4 (5 μM) 2 hr before dorsal root ganglion cells were seeded on the astrocytes. Note the rich outgrowth of neurites following silencing of S100A4 and the even greater outgrowth in cultures treated with S100A4. Scale bar = 50 μm.

intracellular localization of S100A4 in white matter astrocytes and thereby influence the functional state of these cells.

Our in vitro system is clearly a simplification of the complex cellular relationships and interactions in vivo. Changes in, e.g., oligodendrocytes, microglia, and/or the blood–brain barrier, are likely to have a significant impact on the properties of white matter astrocytes and thereby alter their possibilities for supporting neurite growth. Nevertheless, our findings indicate that white matter astrocytes are able to support axonal growth. However, intracellular S100A4 reduces this potential, implying that the high levels of this protein in reactive astrocytes are likely to contribute to the nonpermissive properties of degenerating mature white matter. Interestingly, the presence of extracellular S100A4 overcomes the inhibitory influence of the same protein within white matter astrocytes. Thus, administration of S100A4 may help injured axons to negotiate the nonpermissive CNS environment.

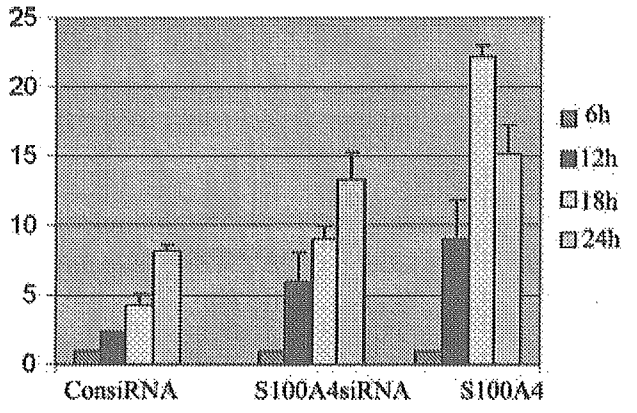

Fig. 5. Quantitative data of neurite outgrowth at 6, 12, 18, and 24 hr after seeding of dorsal root ganglion cells on control siRNA-treated, S100A4 siRNA-treated, and S100A4-treated white matter astrocyte cultures. There is a significantly increased outgrowth following treatment with S100A4 siRNA compared with control siRNA-treated cultures at 12, 18, and 24 hr. S100A4-treated cultures show significantly greater outgrowth compared with S100A4 siRNA treated cultures at 18 hr.

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Role of Intracellular S100A4 for Migration of Rat Astrocytes

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KEY WORDS
white matter; calcium-binding protein; microfilament; metalloproteinase; cell motility; siRNA; in vitro

ABSTRACT
S100A4 is a member of the EF-hand family of calcium-binding proteins, first identified in tumor cells, and implicated in tumor invasion and metastasis. Intracellular upregulation of S100A4 is associated with increased motility of tumor cells. Extracellular application of S100A4 increases the motility of glioma cells in vitro. We showed previously that astrocytes in spinal cord and brain white matter also express S100A4. This expression is markedly increased in reactive white matter astrocytes after injury. Here, we have explored how changes in intracellular S100A4 affect migration of astrocytes. We produced cultures of white matter, S100A4 expressing astrocytes, and developed a small interfering (si) RNA approach to specifically eliminate S100A4 expression in these cells, and compared the migration of astrocytes expressing S100A4 with astrocytes transfected with S100A4 siRNA. As a "positive control" we used S100A4 expressing C6 glioma cells. In contrast to malignant cells, S100A4 expressing astrocytes increased their migration capacity after S100A4 siRNA treatment. At the same time, and in parallel with increased migration, white matter astrocytes increased their expression of metalloproteinases MMP-9 and MT1-MMP. The addition of MMP-2/MMP-9 inhibitor resulted in a significant inhibition of migration in S100A4 siRNA-treated astrocytes. These findings indicate that S100A4 has a stabilizing function in reactive white matter astrocytes, a function that may contribute to the development of a rigid, growth-inhibitory glial scar.

INTRODUCTION

Astrocytes form a highly diversified group of cells, which are adapted to the regional functional context. Thus, it has long been recognized that astrocytes from white and gray matter are morphologically different and typically show different levels of the prototypic astroglial marker, glial fibrillary acidic protein (GFAP). In general, white matter astrocytes, so-called fibrous astrocytes, display relatively higher levels of GFAP than astrocytes from gray matter, protoplasmic astrocytes. We previously showed that white, but not gray matter astrocytes in vivo express S100A4 (Kozlova and Lukanidin, 1999; Åberg and Kozlova, 2000). This protein is strongly implicated in invasive and metastatic properties of several non-neural tumor cells (Barraclough, 1998; Jenkinson et al., 2004), but its role in normal astrocytes is unknown. However, S100A4 is markedly upregulated after injury to dorsal roots (Kozlova and Lukanidin, 1999), brain (Kozlova and Lukanidin, 2002) or spinal cord (K.H. Zhang et al., 2004), suggesting that this protein is involved in the development of a CNS environment, which is nonpermissive for regeneration (Sandvig et al., 2004).

S100A4 belongs to the EF-hand calcium-binding protein family, has intracellular as well as extracellular activities, and interacts with several target proteins to modify their functions. The levels of S100A4 protein expression in many tumor cells correlate with their degree of malignancy and motility (see, e.g., Cho et al., 2003; Hernan et al., 2003; Lee et al., 2004; Moriyama-Kita et al., 2004). S100A4 modulates shape and motility of tumor cells via interaction with non-muscle myosin (Takenaga et al., 1994a,b, 1997; Ford et al., 1997; Kriajevska et al., 1994, 1998, 2000; Chen et al., 2001; S. Zhang et al., 2005). Moreover, administration of S100A4 to glioma cells in vitro increased their motility, indicating that extracellular S100A4 also enhances tumor cell motility by affecting the cytoskeleton (Belot et al., 2002). However, the role of intracellular S100A4 for migration of astrocytes is unknown.

To elucidate this issue, we produced white matter astroglial cultures with abundant strongly S100A4 expressing astrocytes. We compared the migration of S100A4 expressing astrocytes with astrocytes in which S100A4 protein expression was silenced with S100A4 siRNA. We unexpectedly found that downregulation of this protein dramatically increased the migration capacity of astrocytes in a transwell migration assay. To substantiate these findings we used S100A4 positive glioma cells in the same migration assay, and found that their migratory capacity is reduced after silencing with S100A4 siRNA. Thus, upregulation of S100A4 in reactive astrocytes appears to confer stabilization to these cells.

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MATERIALS AND METHODS

Astroglial Cell Cultures

The animal procedures were approved by the Uppsala county regional committee for research on animals. The detailed procedure for preparing cultures of white matter astrocytes is described elsewhere (Kozlova and Takenaga, 2005). Briefly, white matter astrocytic cultures were made from 4-day-old rat pups. The animals were deeply anesthetized on ice and decapitated. The brain from a sacrificed pup was immediately removed and placed in a Petri dish with cold phosphate-buffered saline (PBS). A 2-mm-thick coronal slice at the level of rostral hippocampus was made and placed in a Petri dish with Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen) containing 10% fetal calf serum (FCS; Gibco, Invitrogen) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin.. The corpus callosum area was carefully dissected under high magnification in the dissecting microscope, and used for obtaining white matter astrocytes. The tissues were rinsed with PBS containing 0.2% glucose (PBS/glucose), resuspended in PBS/glucose containing 10 mg/ml trypsin (Sigma-Aldrich, St. Louis, MO), 1 mg/ml DNase (Worthington Biochemicals, Lakewood, NJ) and 5 mg/ml $MgSO_4$, incubated for 3 min at 37°C, and then carefully washed three times with PBS/glucose. After removing the last wash solution, the tissues were suspended in DMEM containing 0.5 mg/ml DNase. Beginning with the 18-G needle, the tissues and the DNase solution were drawn up and expelled back into the tube for a total of 15 times, and this procedure was repeated with the 20- and 23-G needles for fifteen times each. The resulting cell suspension was centrifuged at 1,200*g* for 1 min, and the pellet was resuspended in 1 ml of a 1:1 mixture of DNase solution and PBS/glucose/$MgSO_4$. The cell suspension was transferred onto the discontinuous Percoll (Amersham Biosciences, Piscataway, NJ) gradient that had been made by overlaying 2.5 ml of 30% Percoll on 2.5 ml of 60% Percoll in PBS/glucose. After centrifugation at 2,000*g* for 10 min at 4°C, the astrocyte-enriched fraction that migrated to the medium/30% Percoll interface was carefully aspirated with a Pasteur pipette, and suspended in 10 ml of PBS/glucose. The cells were centrifuged at 2,000*g* for 10 min at 4°C, resuspended in DMEM/10% FCS/3% glutamine glucose, plated at a concentration of 1×10^6 cells/ml in culture flasks (T25), and then cultured for 10 days. For subculturing or preparing for immunocytochemical and biochemical analyses, the cells were incubated with 0.05% trypsin/0.53 mM EDTA (National Veterinary Institute, Uppsala, Sweden) for 3 min at 37°C. After adding an equal volume of DMEM/10% FCS, the cells were detached from the culture flasks by smacking the side of the flasks, centrifuged, and resuspended in DMEM/10% FCS/glucose.

Abbreviations

BrdU	bromodeoxyuridine
BSA	bovine serum albumin
DMEM	Dulbecco's modified Eagle's medium
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
ECM	extracellular matrix
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GFAP	glial fibrillary acidic protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
IR	immunoreactivity
MMP	matrix metalloproteinase
MT1-MMP	membrane type 1-matrix metalloproteinase
PBS	phosphate-buffered saline
RT-PCR	reverse transcription-polymerase chain reaction
siRNA	short interference RNA
TIMP	tissue inhibitor of matrix metalloproteinase
TRITC	tetramethylrhodamine isothiocyanate

C6 Glioma Cell Cultures

Rat C6 glioma cells (Namba et al., 2000) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Enhanced green fluorescent protein (EGFP) expressing C6 (C6/EGFP) cells were established by introducing a pCI-neo/E9/EGFP plasmid with the Lipofectin (Invitrogen) method and selecting with 400 μg/ml G418 (Sigma-Aldrich) followed by cloning. Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2.

siRNA Treatment

siRNA-mediated silencing of endogenous expression of S100A4 in C6 cells and astrocytes was performed using 21 nucleotide siRNA duplexes (Ambion, Austin, TX). The sequence of sense and antisense oligonucleotide was 5′-GGGUGACAAGUUCAAGCUGtt-3′ and 5′-CAGCUUGAACUUGUCACCCtc-3′, respectively. S100A4 siRNA was transfected with Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Briefly, one day before transfection, astrocytes and C6 cells were resuspended in 12-well plates or on poly-L-lysine-coated coverslips in appropriate growth medium and then grown overnight. On the day of the experiment, siRNA-Lipofectamine 2000 complexes were prepared and transfection was performed according to the manufacturer's instructions. The medium for astrocytic cultures was changed to Opti-MEM (Invitrogen) 20 min before transfection. The siRNA complexes were added dropwise while gently rocking the 12-well plates. Cells were transfected with S100A4 siRNA for at least 5 h at 37°C before switching to fresh Opti-MEM containing 20% FCS, and subsequently incubated overnight. The medium was then changed to DMEM supplemented with 3% glutamine, antibiotics (see above) and 10% FCS. The amounts of siRNA, Lipofectamine 2000, and siRNA transfection medium were proportionally scaled up to the surface area of the cell culture. As a control for S100A4, siRNA, BLOCK-iT Fluorescent Oligo (Invitrogen) or Silencer Negative Control #1 siRNA (Ambion) was used. For Western blot and PCR analyses, astro-

cytes were transfected with the siRNA in 60-mm-diameter culture dishes (Nunc). For immunocytochemistry, astrocytes that had been transfected with siRNA 2 days earlier were detached, immediately replated on glass coverslips and incubated for 24 h before immunohistochemical staining was performed. Transfection efficiency was about 90% for astrocytes as well as C6 cells.

Migration Assay

Cell motility was measured by using 8-μm pore size FluoroBlok transwell chambers (BD Sciences Falcon). Cells were collected by a brief treatment with trypsin/EDTA solution, washed once with serum-containing DMEM, centrifuged, resuspended in DMEM containing 0.1% bovine serum albumin (BSA), and then placed in the inserts at a concentration of 5×10^4 cells/200 μl. In the lower compartment of the chamber, 750 μl of DMEM containing 10% FCS or 10 μg/ml of laminin from Engelbreth-Holm-Swarm murine sarcoma (basement membrane) (Sigma-Aldrich) was added as a chemoattractant. After incubation at 37°C, astrocytes that migrated through the pores to the lower chamber were stained with 1 μM Calcein AM (Molecular Probes, Eugene, OR), and examined under an inverted fluorescence microscope. C6/EGFP cells were directly observed under the microscope. The number of cells, which had migrated through the membranes, was counted on the pictures of randomly selected fields taken at 40× magnification and printed to a final magnification of 130×. In some experiments, MMP-2/MMP-9 inhibitor II (Calbiochem) was added to the medium in the upper and the lower compartments at a concentration of 200 nM before running the migration assays. All migration assays were repeated at least three times.

Apoptosis Assay

Apoptosis of white matter astrocytes pre-treated with control siRNA (100 nM) or S100A4 siRNA (100 nM) for 3 days was assessed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining using ApopTag Fluorescein In Situ Apoptosis Detection Kit (Serologicals, Chemicon, Temecula, CA).

CELL PROLIFERATION ASSAY

Proliferation of white matter astrocytes pre-treated with control siRNA (100 nM) or S100A4 siRNA (100 nM) for 3 days was measured with Cell Proliferation ELISA, BrdU (colorimetric) Kit (Roche Diagnostics GmbH). Briefly, cells (1×10^4 cells/well) in 96-well plate (Falcon) were labeled with 10 μM 5-bromo-2′-deoxyuridine (BrdU) for 4 h and then fixed and denatured. Incorporated BrdU was detected by incubating the denatured cells with monoclonal anti-BrdU-POD (peroxidase) followed by the peroxidase substrate reaction. The reaction product was quantified by measuring the absorbance at 450 nm using a scanning multiwell spectrophotometer (Bio-Rad, Hercules, CA).

TABLE 1. Oligonucleotide Primer Sequences Used for RT-PCR

Gene	Oligonucleotides	Sequence
MMP-2	Sense	5′-CTGGGTCTATTCTGCCAGCACTCTG-3′
	Antisense	5′-AGCCAGTCTGATTTGATGCTTCCAA-3′
MMP-9	Sense	5′-AGTTTGGTGTCGCGGAGCAC-3′
	Antisense	5′-TACATGAGCGCTTCCGGCAC-3′
MT1-MMP	Sense	5′-GTGCCCTATGCCTACATCCG-3′
	Antisense	5′-TTGGGTATCCGTCCATCACT-3′
TIMP-1	Sense	5′-CTGGCATCCTCTTGTTGCTA-3′
	Antisense	5′-AGGGATCGCCAGGTGCACAA-3′
TIMP-2	Sense	5′-AGACGTAGTGATCAGGGCCA-3′
	Antisense	5′-GTACCACGCGCAAGAACCAT-3′
GAPDH	Sense	5′-ACCACAGTCCATGCCATCAC-3′
	Antisense	5′-TCCACCACCCTGTTGCTGTA-3′

Immunoblot

Cells were lysed in 2% Triton X-100, 1% Nonidet P-40 (NP-40), 20 mM Tris-HCl, pH 7.4, 300 mM NaCl, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 10,000*g* for 10 min at 4°C, the supernatant was used for immunoblot analysis. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to a Hybond-N membrane (Amersham Biosciences). Primary antibodies used were against: β-actin (mouse monoclonal, Sigma-Aldrich, 1:1,000), GFAP (rabbit polyclonal, Dak°Cytomation, Glostrup, Denmark, 1:100) and S100A4 (rabbit polyclonal, Takenaga et al., 1994c). The membrane was incubated with the first antibodies, washed extensively with TBS-T, and then with species appropriate horseradish peroxidase conjugated secondary antibodies. Immunodetection of GFAP and β-actin was carried out with the enhanced chemiluminescence (ECL) system (Amersham Biosciences).

RT-PCR

Total RNA was prepared with TRIzol reagent (Invitrogen) according to the manufacturer's recommendation and 1 μg of total RNA was used as a template for cDNA synthesis. RNA was transcribed into cDNA at 37°C for 1 h in 20 μl of RT buffer consisting of 1× first-strand buffer, 1 μg oligo$(dT)_{12\text{-}18}$ primers (Invitrogen), 100 U Moloney murine leukemia virus reverse transcriptase (Gibco-BRL), 2 mM dNTPs, 0.1 M dithiothreitol (DTT), and 40 U RNase inhibitor (TOYOBO Biochemicals, Osaka, Japan). The resulting cDNA was used for the amplification of the target cDNAs using the appropriate sense and antisense primers, and rTaq DNA polymerases (TOYOBO). The sense and the antisense primers used are shown in Table 1. The PCR conditions were 94°C for 10 s, 55°C for 5 s, 72°C for 1 min, and the cycles were repeated 30 times except for glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 25 cycles). Ali-

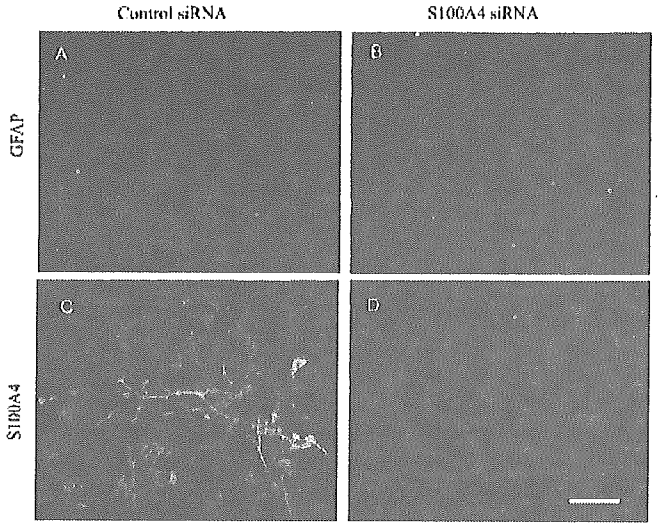

Fig. 1. Immunoreactivity for GFAP and S100A4 in white matter astrocytes. The cells were stained with anti-GFAP and anti-S100A4 antibodies three days after treatment with control small interfering RNA (siRNA) (**A, C**) or S100A4 siRNA (**B, D**). There is an increased immunoreactivity for GFAP (cf. A and B), concomitantly with the complete elimination of S100A4 expression after S100A4 siRNA transfection (D). Scale bar = 50 μm.

quots of the PCR products were fractionated by electrophoresis in 1% agarose gels and visualized on a transilluminator after staining with ethidium bromide. We used GAPDH expression as an internal control to estimate the expression levels of matrix metalloproteinase (MMP)-2, MMP-9, membrane type (MT)1-MMP, tissue inhibitor of metalloproteinases (TIMP)-1, and TIMP-2.

Immunofluorescence

White matter astrocytes on glass coverslips were washed three times with PBS and fixed for 30 min with 4% formaldehyde (w/v) in PBS. After washing, the cells were permeabilized with 1% Triton X-100, incubated with 3% BSA in PBS to block nonspecific binding, and then incubated with polyclonal anti-GFAP antibody (DakoCytomation; 1:200) for 1 h. After extensive washing with PBS, the primary antibody was localized with FITC conjugated swine anti-rabbit IgG (DakoCytomation; 1:200). For labeling F-actin, the cells were also incubated with 0.1 μg/ml TRITC-conjugated phalloidin (Sigma-Aldrich) for 30 min. After rinsing, the coverslips were mounted in 50% glycerol in PBS containing 100 mM propyl-gallate to prevent photobleaching.

To verify the efficiency of transfection and silencing of S100A4 expression, coverslips with fixed white matter astrocytes were also double labeled with rat monoclonal anti-GFAP antibodies (Zymed Labs; 1:100), and rabbit polyclonal anti-S100A4 antibodies. S100A4 antibodies were raised against recombinant S100A4 protein and affinity-purified (Takenaga et al., 1994c). Secondary antibodies were Texas Red conjugated donkey anti-rat IgG (Jackson ImmunoResearch, West Grove, PA; 1:100),

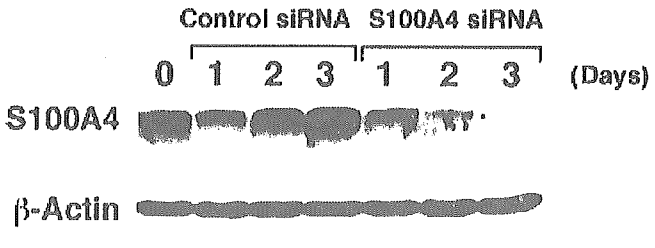

Fig. 2. Time course of the effect of S100A4 small interfering RNA (siRNA) on the expression of S100A4 in white matter astrocytes. White matter astrocytes were transfected with 100 nM control siRNA or S100A4 siRNA by the Lipofectamine 2000 method. At 1–3 days after the transfection, cell extracts (40 μg of protein) were prepared and subjected to immunoblot analysis of S100A4. β-actin was used as a loading control. The expression of S100A4 declines gradually, and is almost completely abolished on day 3 after S100A4 siRNA transfection. Transfection with control siRNA does not affect S100A4 expression.

and FITC-conjugated goat anti-rabbit IgG (Sigma-Aldrich; 1:500), respectively. The immunolabeling was viewed and photographed in a Nikon Eclipse fluorescence microscope equipped with filter for simultaneous examination of FITC and TRITC fluorescence.

RESULTS

The objectives of our studies were to (1) determine whether intracellular S100A4 regulates the migratory properties of white matter astrocytes, and, if this is the case, (2) to explore whether this regulation involves metalloproteinase activity. We produced cultures from the cerebral white matter (corpus callosum) of postnatal day 4 (P4) old rats, i.e., the stage when S100A4 positive astrocytes appear in vivo (Åberg and Kozlova, 2000). By developing the siRNA technique specifically to eliminate S100A4 protein expression, we were therefore able to examine the role of S100A4 for the migratory capacity of white matter astrocytes.

Astrocytes generated from the corpus callosum expressed high levels of S100A4 (cf. Figs. 1A,C and 2). Transfection of these cells with S100A4 siRNA resulted in a gradual depletion of S100A4 immunoreactivity (IR) with an almost complete disappearance of S100A4-IR on day 3 after transfection (Figs. 1D and 2). Concomitantly, there was an increase in their expression of GFAP-IR and protein (Figs. 1B and 3).

Migration Assay

White matter astrocytes showed limited migration toward laminin or FCS in a transwell migration assay. After S100A4 silencing, migration toward these factors was markedly increased compared with cells treated with control siRNA (Figs. 4 and 6A). This difference was particularly dramatic when laminin was used as a chemotaxic agent (Figs. 4B,D and 6A). These results were in sharp contrast to those obtained with C6 glioma cells, which decreased their migration capacity after siRNA mediated downregulation of their S100A4 expression

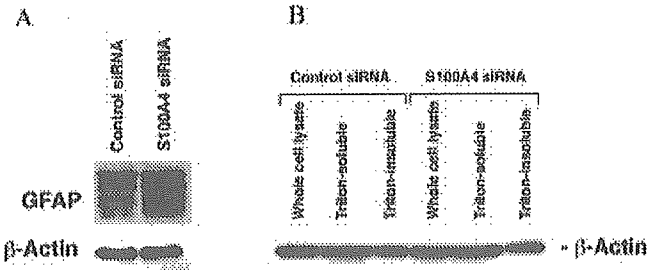

Fig. 3. **A:** Immunoblot analysis of GFAP expression. Whole cell extracts (40 μg of protein) from white matter astrocytes were subjected to immunoblot analysis of GFAP expression. β-actin was used as a loading control. There is a marked increase in GFAP expression in white matter astrocytes after transfection with S100A4 small interfering RNA (siRNA). **B:** Immunoblot analysis of the amount of polymerized F-actin in S100A4 siRNA-transfected white matter astrocytes. White matter astrocytes were transfected with 100 nM control siRNA or S100A4 siRNA. Three days after the transfection, cells were directly lysed in SDS-sample buffer (whole cell extracts) or fractionated into Triton X-100-soluble and insoluble fractions. Equivalent amounts of Triton X-100-soluble and -insoluble fractions were applied onto the gels. The protein amount of whole cell lysates loaded was one-half the amount of Triton-soluble plus -insoluble fractions. Unpolymerized and polymerized F-actin in Triton-soluble and -insoluble fractions, respectively, was probed with β-actin antibodies. There is no difference in the amount of F-actin in Triton-insoluble fractions of control siRNA and S100A4 siRNA-treated cells.

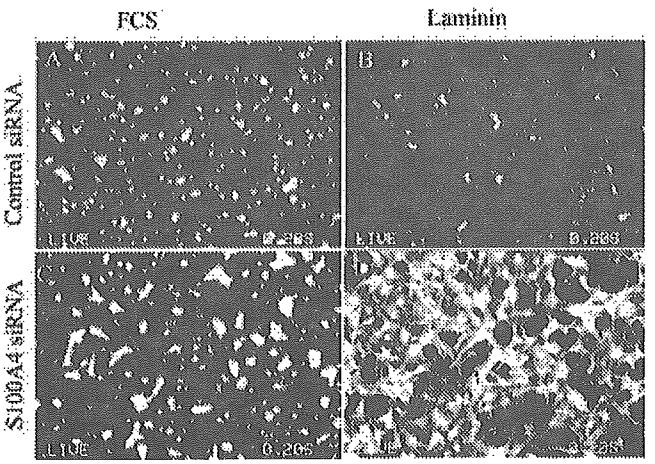

Fig. 4. Migration of control and S100A4 siRNA-transfected white matter astrocytes in a transwell migration assay. White matter astrocytes were transfected with 100 nM control small interfering RNA (siRNA) (**A,B**) or S100A4 siRNA (**C,D**). Three days after the transfection, cells were detached from the culture dishes and subjected to migration assays using the chamber equipped with FluoroBlok culture inserts. As chemoattractants, 10% FCS (A,B) or 10 μg/ml of laminin (C,D) was added to the lower compartment of the chamber. After incubation for 10 h at 37°C, white matter astrocytes that migrated through the pores to the lower chamber were stained with 1 μM Calcein AM for 10 min, and then observed under an inverted fluorescence microscope. C6/EGFP cells were directly observed under an inverted fluorescence microscope. Cells transfected with S100A4 siRNA show more extensive migration, particularly when laminin is used as chemotactic agent.

(Figs. 5 and 6B). There were no differences in the number of TUNEL positive (Fig. 7) or BrdU-labeled cells (Fig. 8) between control and S100A4 siRNA-treated astrocytes, thus ruling out the possibility that the increased migration of S100A4 siRNA-treated astrocytes was due to differences in cell death or proliferation.

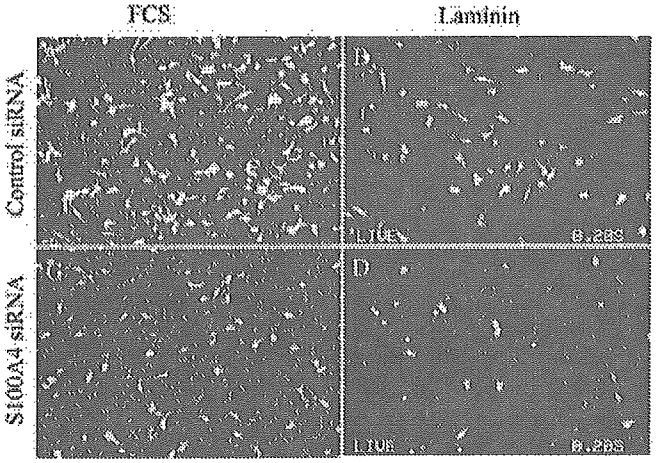

Fig. 5. Migration of control and S100A4 siRNA-transfected C6/EGFP cells in a transwell migration assay. For methodology, see Fig. 4. C6/EGFP cells show less migratory activity after transfection with S100A4 small interfering RNA (siRNA) to FCS (**A,C**), as well as laminin (**B,D**).

Actin Cytoskeleton

The downregulation of S100A4 protein expression was associated with a change in the arrangement of the actin cytoskeleton of white matter astrocytes. In control siRNA-treated white matter astrocytes, phalloidin labeled actin was diffusely distributed throughout the cytoplasm, with occasional thin actin filaments (Fig. 9A). However, after S100A4 siRNA transfection, the actin cytoskeleton appeared in the form of typical stress fibers (Fig. 9B). These changes in actin cytoskeleton occurred without any changes in the total amount of actin and F-actin (polymerized actin in Triton-insoluble fraction) (Fig. 3B).

Metalloproteinases

We examined the expression of matrix metalloproteinase (MMP)- 2, 9 and membrane type (MT)1-MMP, as well as the tissue inhibitors of MMPs (TIMP)-1 and -2, using RT-PCR. MMP-9 and MT1-MMP mRNAs showed increased expression in white matter astrocytes treated with S100A4 siRNA compared with corresponding control siRNA-treated cells (Fig. 10). Addition of MMP-2/MMP-9 inhibitor resulted in inhibition of migration toward laminin in both control siRNA-treated and S100A4 siRNA-treated astrocytes, with the most marked inhibition of S100A4 siRNA-treated cells (Fig. 11).

DISCUSSION

Astrocytes in white, but not gray matter, express the calcium-binding protein S100A4 under normal conditions, and this protein is rapidly and markedly upregulated in white matter astrocytes after injury (Kozlova and Luka-

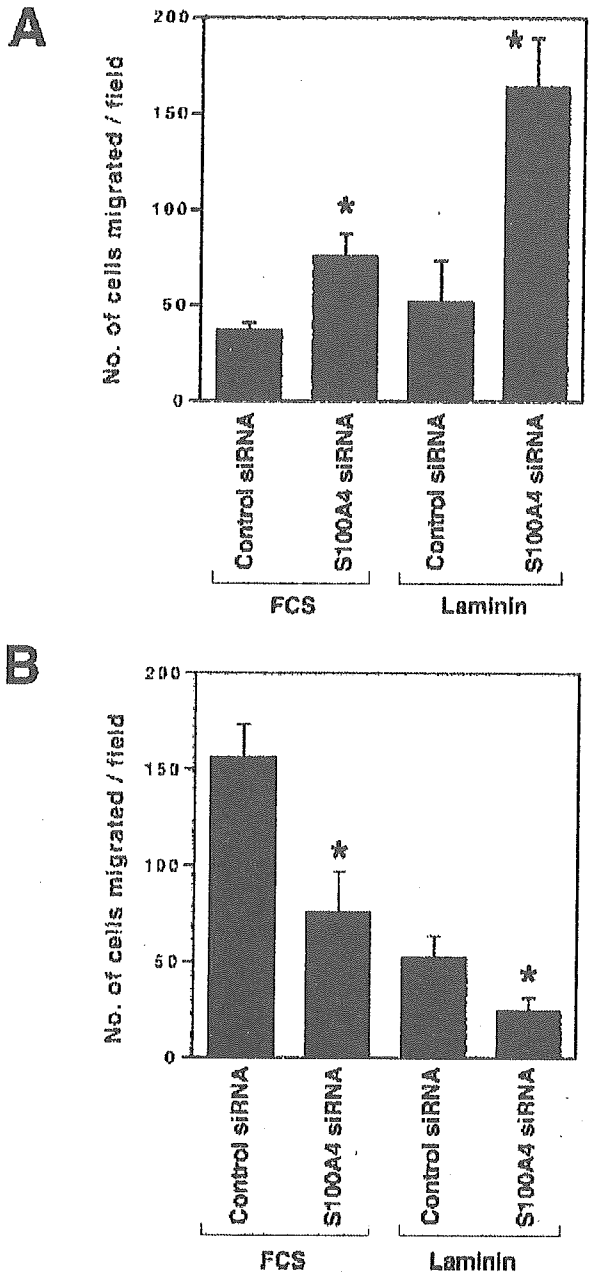

Fig. 6. Quantitative data on migration of control and S100A4 siRNA-transfected white matter astrocytes (**A**) and C6/EGFP cells (**B**). The number of cells, which had migrated through the membrane toward FCS or laminin was counted on the pictures of randomly selected fields taken at a total magnification of 130×. The migratory activity is significantly increased in white matter astrocytes after S100A4 small interfering RNA (siRNA) transfection, particularly with laminin as chemotactic agent (A), whereas C6/EGFP cells display the opposite behavior (B). * $P < 0.05$

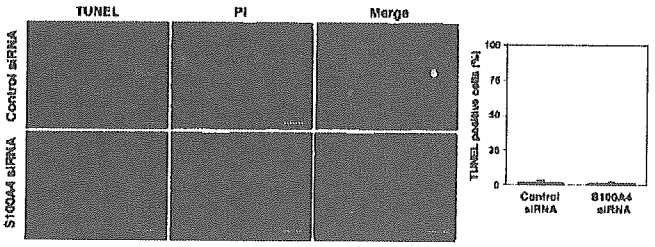

Fig. 7. Apoptosis in small interfering RNA (siRNA)-transfected white matter astrocytes. White matter astrocytes were transfected with 100 nM control siRNA or S100A4 siRNA. Three days after transfection, apoptosis was assessed by TUNEL staining (green). The quantitative data are shown in the graph. There are no significant differences in apoptosis between control and S100A4 small interfering RNA (siRNA)-transfected cells. PI, propidium iodide labeling. Scale bar 200 = μm.

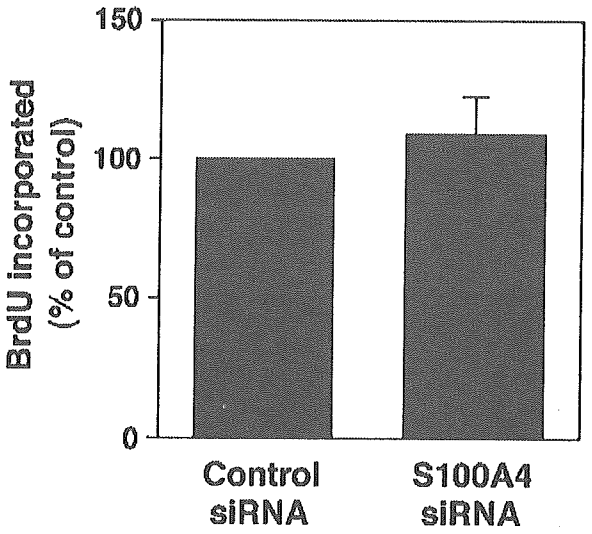

Fig. 8. Proliferation of small interfering RNA (siRNA)-transfected white matter astrocytes. White matter astrocytes were transfected with 100 nM control siRNA or 100 nM S100A4 siRNA, and cell proliferation was measured 3 days after transfection with Cell Proliferation ELISA, BrdU (colorimetric) Kit. Incorporated BrdU was detected by incubating the denatured cells with monoclonal anti-BrdU-POD (peroxidase) followed by the peroxidase substrate reaction. The reaction product was quantified by measuring the absorbance at 450 nm, using a scanning multiwell spectrophotometer. There are no significant differences in proliferation between control and S100A4 siRNA-transfected cells.

nidin, 1999, 2002; Åberg and Kozlova, 2000). The functions of S100A4 in white matter astrocytes are unknown, however. Since S100A4 has been closely associated with tumor cell motility, we asked whether upregulation of this protein in white matter astrocytes increases their motility. To elucidate this issue, we developed an in vitro system for producing abundant white matter S100A4-expressing astrocytes. Using the S100A4 siRNA approach, we were able to remove S100A4 expression from cultured astrocytes and compare the migratory capacity of S100A4-positive and -negative astrocytes. Our findings indicate that S100A4 reduces the migratory capacity of white matter astrocytes.

Although downregulation of S100A4 expression resulted in increased migratory capacity of white matter astrocytes, the overall level of actin did not change. However, there was a striking change in the arrangement of the actin cytoskeleton in parallel with loss of S100A4 protein. Control siRNA-transfected white matter astrocytes showed a diffuse actin pattern or thin actin filaments, whereas S100A4 siRNA-transfected cells were

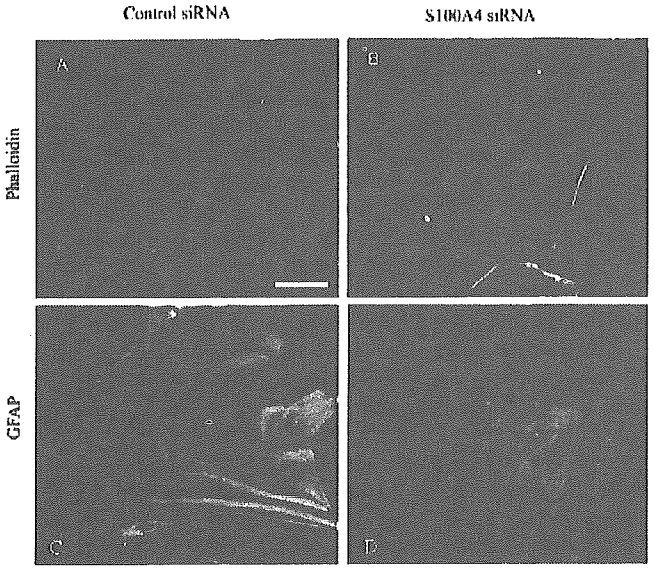

Fig. 9. Actin stress fibers in S100A4 siRNA-transfected white matter astrocytes. White matter astrocytes were transfected with 100 nM control siRNA (**A,C**) or S100A4 siRNA (**B,D**). Three days after transfection, cultures were fixed with formaldehyde and stained for F-actin with TRITC-phalloidin (0.1 μg/ml; **A,B**) and GFAP (**C,D**). Transfection with S100A4 small interfering RNA (siRNA) results in a more extended shape of white matter astrocytes, and prominent actin stress fibers appear in these cells (cf. A,B). Scale bar = 5 μm.

elongated with a distinct network of actin stress fibers. These changes are in line with the increased migratory capacity of the S100A4 silenced astrocytes.

White matter astrocytes displayed a distinct increase in GFAP immunoreactivity (IR) and protein levels after transfection with S100A4 siRNA. GFAP-IR was predominantly localized in astroglial processes, whereas S100A4 was present in the cytoplasm and proximal part of processes (cf. Kozlova and Lukanidin, 1999; Kozlova, 2003). Astrocytes with homozygous deletion of the GFAP gene are less motile in vitro compared with wild-type cells (Lepekhin et al., 2001). The increased GFAP expression after S100A4 silencing may therefore help astroglial motility, e.g., by transiently stabilizing the shafts of delicate lamellipodia during rapid translocations of the cells.

S100A4 silenced astrocytes increased their migration two to three times in the migration assay on laminin compared with S100A4-positive astrocytes. This increase in migration capacity was associated with increased expression of MT1-MMP and MMP-9 mRNAs. Although MMP-2 and TIMPs are expressed at similar levels in control and S100A4 siRNA-treated astrocytes, it is known that MT1-MMP and TIMP-2 are required for activation of proMMP-2 (Nagase, 1998). Therefore, it seems likely that MMP-2 is also activated in S100A4 siRNA-treated astrocytes compared with control siRNA-treated cells. After the addition of an MMP-2/MMP-9 inhibitor, migration of control as well as S100A4 siRNA-treated astrocytes decreased, with the most prominent effect in S100A4 siRNA-treated cells. These findings indicate that the increased migration of S100A4 silenced

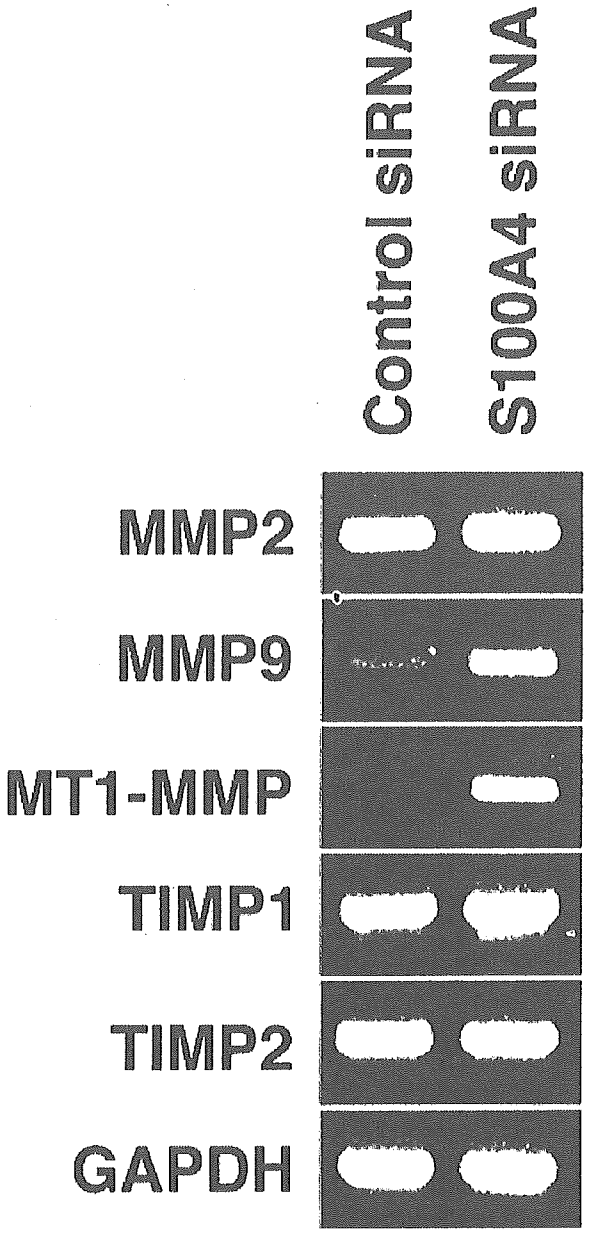

Fig. 10. RT-PCR analysis of the expressions of metalloproteinases and their inhibitors in S100A4 small interfering RNA (siRNA)-transfected white matter astrocytes. White matter astrocytes were transfected with 100 nM control siRNA or S100A4 siRNA. Three days after the transfection, total RNA was prepared using the Trizol method. After reverse transcription, the resulting cDNAs were applied to PCR using rat MMP-2, MMP-9, MT1-MMP, TIMP-1, and TIMP-2-specific primer pairs listed in Table 1. GAPDH was used as an internal control to estimate the expression levels of the genes. The expression of MT1-MMP and MMP-9 are markedly increased in S100A4 siRNA-transfected (right) compared with S100A4 expressing (control siRNA-treated; left) white matter astrocytes.

astrocytes on laminin is, at least in part, mediated by MMP-2 and MMP-9.

Because of the surprising finding that downregulation of S100A4 increased migration of white matter astrocytes, we used S100A4 expressing C6 glioma cells as con-

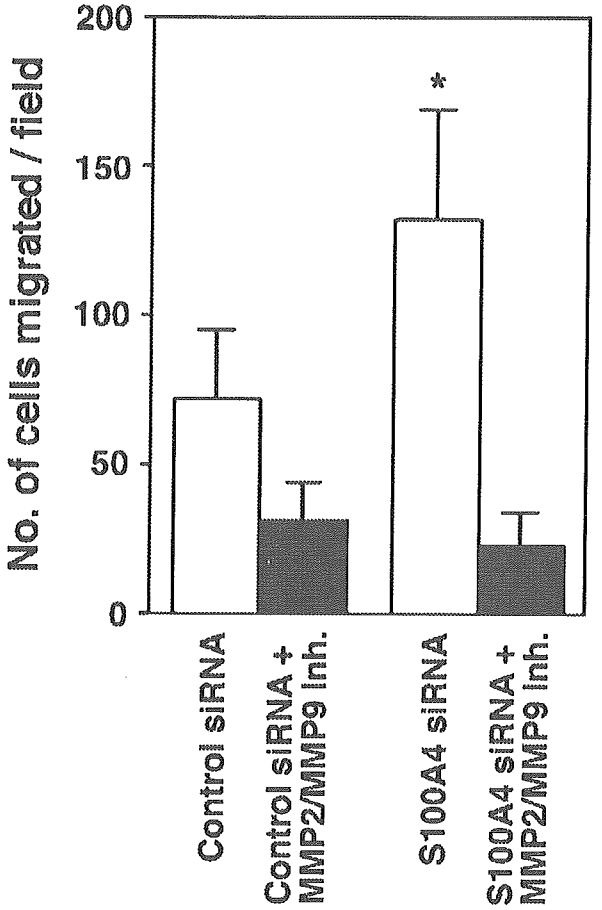

Fig. 11. Effect of MMP-2/MMP-9 inhibitor on migration of control and S100A4 small interfering RNA (siRNA)-transfected white matter astrocytes. White matter astrocytes were transfected with 100 nM control siRNA or S100A4 siRNA. Three days after transfection, cells were subjected to migration assay in the presence or absence of 200 nM MMP-2/MMP-9 inhibitor II. As a chemoattractant, 10 μg of laminin was used. $^*P < 0.04$. Scale bar = 200 μm.

trols for the reliability or our transwell migration assay. As expected from current data on the role of S100A4 in tumor cell motility (for review, see Helfman et al., 2005), silencing S100A4 expression in C6 glioma cells decreased their migration. This decrease may be associated with downregulation of MMP expression, since MMPs appear to be involved in glioma cell migration (see, e.g., Bellail et al., 2004). However, since our focus is on the role of S100A4 in white matter astrocytes, we did not pursue this issue within the frame of the current study.

MT1-MMP, MMP-2, and MMP-9 have been shown to promote migration and invasion of different types of cells in vitro and in vivo via processing of laminin (Adair-Kirk et al., 2003; Iida et al., 2004; Koshikawa et al., 2004a,b). In our migration assay we used laminin-1, which is composed of subunits α1, β1, and γ1. In the adult brain, laminin is present in the basement membranes of vasculature, choroid plexus, and the pial membrane (Miner and Yurchenco, 2004). Non-basement laminin isoforms within the CNS parenchyma have also been described, but these findings have not been confirmed (Yin et al., 2003; Tom et al, 2004). Specifically, laminin isoforms 8 and 10 are present in the endothelial basement membrane of cerebral blood vessels (Sixt et al., 2001), whereas laminin-1 and -2 isoforms are components of the parenchymal basement membrane (Jucker et al., 1996; Sasaki et al., 2002), which is continuous with the pial basement membrane (see Sixt et al., 2001). Astroglial processes are in contact with the parenchymal as well as pial basement membranes, and therefore able to interact with laminin-1 subunits under normal as well as pathophysiological conditions in vivo.

MMPs have been shown to be important for migration of glial precursors. Oligodendrocyte-type 2 astrocyte (O-2A) progenitors migrate in the developing and adult CNS (Armstrong et al., 1990). This behavior depends on the presence of a metalloendoprotease, which is downregulated when these progenitors differentiate to astrocytes, and their motility in white matter becomes restricted (Amberger et al., 1997). This change in motility coincides in time with the appearance of S100A4 expressing astrocytes in the postnatal developing spinal cord, i.e., shortly after myelination starts. In the context of our current findings, this suggests that the induction of S100A4 expression may contribute to restrict astroglial motility in parallel with myelination.

In summary, we demonstrate that, contrary to what would be expected from the current knowledge about tumor cells, S100A4 actually reduces the migratory capacity of white matter astrocytes.

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Protocol

A procedure for culturing astrocytes from white matter and the application of the siRNA technique for silencing the expression of their specific marker, S100A4

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Abstract

White matter astrocytes have physiological functions which are distinct from those of astrocytes in gray matter. White matter becomes highly non-permissive to neurite growth after injury, but the role of white matter astrocytes in this process is incompletely understood. Current protocols for making primary astroglial cultures are inadequate for exploring the specific properties of white matter astrocytes in vitro. We describe a procedure for obtaining cultures of white matter astrocytes from the rodent corpus callosum. In this procedure, we take advantage of our previous finding that white, but not gray matter astrocytes express the calcium-binding protein S100A4. S100A4 expressing astrocytes are abundant in the corpus callosum, and we show that cultures, highly enriched in S100A4 expressing white matter astrocytes, can be reproducibly generated from this area. Key factors for successful cultures are (i) meticulous dissection of the corpus callosum from 4-day-old rats, and (ii) Percoll density gradient centrifugation to purify astrocytes. As a means of exploring the possible role of S100A4 in white matter astrocytes, we describe the use of the siRNA technique to eliminate the expression of S100A4 in our in vitro system.

Theme: Development and regeneration
Topic: Glia and other non-neuronal cells

Keywords: Astrocyte; White matter; In vitro; siRNA

1. Type of research

Astrocytes are specialized to meet local functional requirements in different areas of the central nervous system (CNS). Whereas gray matter astrocytes mainly co-operate with neurons in synaptic networking [5,11], white matter astrocytes are primarily involved in the maintenance of secure impulse propagation of long distance projecting myelinated and non-myelinated axons. Furthermore, astrocytes in gray and white matter are likely to respond differently following injury and disease. Reactive white matter astrocytes are key players in the development of a non-permissive environment to neurite growth and in the development of a glial scar following injury [14]. Studies in vitro have provided valuable information on functional specializations of astrocytes from different brain regions (see e.g. [4]). Current protocols for astroglial cultures are likely to result in a mixture of gray and white matter astrocytes. Thus, these protocols are inadequate for analyzing the specific functions of white matter astrocytes. White, but not gray matter astrocytes in the rat express the calcium-binding protein S100A4 [1]. S100A4 has been implicated in tumor malignancy by promoting tumor cell motility [7], dysregulating the tumor suppressor gene p53 [3] and stimulating angiogenesis [15]. S100A4 is markedly up-

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regulated in white matter astrocytes following injury [8,9] and has neuritogenic effects on hippocampal neurons [12] in vitro and neuroprotective effects on hippocampal, dopaminergic, and cerebellar neurons in vitro [13]. Thus, S100A4 may be an important regulator of injury responses and plasticity in the central nervous system. The siRNA technique provides a tool for analyzing the role of S100A4 in white matter astrocytes. The presence of S100A4 exclusively in white matter astrocytes enables us to use this antigen as a marker for white matter astrocytes in vitro. Here, we describe a procedure for preparing cultures of white matter, S100A4 expressing astrocytes. In addition, we describe a protocol for silencing the expression of S100A4 in white matter astrocytes with the siRNA technique.

2. Time required

Step 1. Dissection of P4 rat brains (10–12): 15 min.
Step 2. Preparation of primary cultures: 2 h.
Step 3. Development of purified white matter astrocytic cultures: 1–3 weeks.
Step 4. siRNA transfection: 5 h.
Step 5. Post-transfection interval: 3 days.
Step 6. Immunocytochemistry: 2 days.
Step 7. Microscopic analysis: 2 h.
Total: 2–4 weeks.

3. Materials

3.1. Animals

Pregnant Sprague–Dawley rats (250–300 g; B & K Universal, Sollentuna, Sweden) to produce newborn pups of both genders.

3.2. Special equipment

- Curved, fine-tipped jeweller's forceps.
- Thin, sharp cutting blade with handle.
- Needles, BD Microlance 18-G, 20-G and 23-G (Becton Dickinson, Franklin Lakes, NJ)
- Cellstar 15 ml tubes (Greiner Bio-One Int. AG, Frickenhausen, Germany)
- Poly-L-lysine (Sigma-Aldrich, St. Louis, MO) coated coverslips 12 mm Ø (Histolab-Products AB, Gothenburg, Sweden)

3.3. Chemicals and reagents for preparing white matter astroglial cultures

- Dulbecco's modified Eagle's medium containing 2% glucose and 3% glutamine (DMEM; Gibco), supplemented with fetal calf serum (Gibco), 100 units/ml penicillin (Sigma-Aldrich) and 100 μg/ml streptomycin (Sigma-Aldrich)
- DMEM (Gibco) containing 0.5 mg/ml DNase (Sigma-Aldrich)
- Phosphate-buffered saline (PBS; Merck, Darmstadt, Germany)
- PBS containing 2% glucose (Sigma-Aldrich) (PBS/glucose)
- PBS/glucose containing 10 mg/ml trypsin (Sigma-Aldrich) 1 mg/ml DNase (Sigma-Aldrich) and 5 mg/ml $MgSO_4$ (Sigma-Aldrich) (PBS/glucose/$MgSO_4$)
- Percoll/RediGrad (Amersham Biosciences, Uppsala, Sweden)
- Trypsin 0.05%/EDTA 0.53 mM (National Veterinary Institute, Uppsala, Sweden)

3.4. Chemicals and reagents for siRNA treatment

- BLOCK-iT Fluorescent Oligo (Invitrogen)
- Lipofectamine 2000 (Invitrogen)
- Opti-MEM (Invitrogen)
- Silencer Negative Control #1 siRNA (Ambion, Inc.)
- siRNA 21 nucleotide duplexes (Ambion, Inc., Austin, TX)

3.5. Chemicals and reagents for immunocytochemistry

- Bovine Serum Albumin, 3% (BSA; Sigma-Aldrich) in PBS
- FITC conjugated swine anti-rabbit IgG (DakoCytomation; 1:200)
- FITC conjugated goat anti-rabbit IgG (Sigma-Aldrich; 1:500)
- GFAP antibodies (rabbit polyclonal, DakoCytomation, Glostrup, Denmark; 1:200)
- GFAP antibodies (rat monoclonal, Zymed Labs., San Francisco, CA; 1:100)
- Glycerol 50% (Merck) in PBS containing 100 mM propyl-gallate (Sigma-Aldrich)
- Paraformaldehyde, 4% (Merck) in PBS
- S100A4 antibodies (rabbit polyclonal, 1:700, ([11])
- Texas Red conjugated donkey anti-rat IgG (Jackson ImmunoResearch, West Grove, PA; 1:100)
- Triton X-100, 1% (Sigma-Aldrich)

4. Detailed procedure

4.1. Astroglial cultures

The animal procedures were approved by the Uppsala county regional committee for research on animals. Pregnant Sprague–Dawley rats (250–300 g; B & K Universal, Sollentuna, Sweden) were housed individually in a temperature- and humidity-controlled animal facility with a 12-h light/12-h dark cycle. Animals were given laboratory chow

and tap water ad libitum. Brains from ten rat pups are used to prepare cell cultures as follows:

(i) Remove rat pups (n = 10) from the mother on postnatal day 4 (P4), anesthetize by cooling on ice, clean with 70% alcohol, and decapitate.
(ii) Remove the brain quickly from each pup and place it in a large Petri dish (PD) with cold PBS (Merck).
(iii) Transfer brains to a new, large PD with DMEM (Gibco, Invitrogen) containing 10% FCS (Gibco), supplemented with 100 units/ml penicillin (Sigma-Aldrich, St. Louis, MO) and 100 μg/ml streptomycin (Sigma-Aldrich).
(iv) Fix the rostral part of the brain gently with a pair of curved, fine-tipped jeweller's forceps. Make a 2 mm thick coronal slice at the level of rostral hippocampus with a sharp blade and place all slices in a new large PD with fresh medium (see above).
(v) Dissect the middle part of the corpus callosum area carefully under high magnification and collect the tissue blocks in a small PD.
(vi) Transfer tissue to a 15 ml test tube, rinse in PBS/2%glucose (Sigma-Aldrich), resuspend in PBS/glucose containing 10 mg/ml trypsin (Sigma-Aldrich), 1 mg/ml DNase (Sigma-Aldrich), and 5 mg/ml $MgSO_4$ (Sigma-Aldrich). Cap and put on 37 °C water bath for 3 min.
(vii) Remove trypsin/DNase solution and wash tissue 3 times with PBS/glucose.
(viii) Suspend the tissue in DMEM (Gibco) containing 0.5 mg/ml DNase (Sigma-Aldrich) and dissociate by drawing up and expelling the suspension with a 2 ml syringe (BD Biosciences) through needles of decreasing diameter (18-G, 20-G, and then 23-G). Repeat the procedure 15 times with every needle.
(ix) Centrifuge the tube at 1200 × g for 1 min at 4 °C. Resuspend the pellet in 1 ml of a 1:1 mixture of DNase (Sigma-Aldrich) solution and PBS/glucose/$MgSO_4$ (Sigma-Aldrich). If using more than one gradient, resuspend in as many milliliters as there are gradients. More than one gradient is required when using tissue from more than ten rat pups.
(x) Transfer the cell suspension with a Pasteur pipette onto a discontinuous Percoll gradient (Amersham Biosciences, Uppsala, Sweden) that is made in a 15 ml test tube (Greiner Bio-One Int. AG, Frickenhausen, Germany) by overlaying 2.5 ml of 30% Percoll on 2.5 ml of 60% Percoll in PBS/glucose. Do not disturb the gradient interfaces. The interfaces between the blue 60% Percoll and yellowish 30% Percoll should be clearly visible. Cold Percoll solutions give sharper interfaces.
(xi) Centrifuge at 2000 × g for 10 min at 4 °C. Use a Pasteur pipette with a bulb. Expire the air from the bulb before pipetting to avoid introducing bubbles into the gradient. Aspirate carefully the astrocyte enriched fraction that has migrated to the medium/30% Percoll interface.
(xii) Transfer the cells to a new 15 ml tube (Greiner Bio-One), suspend the cells in 15 ml of PBS/glucose, and centrifuge the cells at 2000 × g for 10 min at 4 °C.
(xiii) Suspend the pellet in DMEM (Gibco) containing 2% glucose and 3% glutamine (Gibco), supplemented with 20% FCS (Gibco), 100 units/ml penicillin (Sigma-Aldrich), and 100 μg/ml streptomycin (Sigma-Aldrich).
(xiv) Plate at a concentration of 1 × 10^5cells/ml in culture flasks (T25). Maintain in culture for 1 to 3 weeks. Change medium every 2nd or 3rd day. Reduce the concentration of FCS to 10% after the first few changes.
(xv) For subculturing for transfection, the cells are incubated with 0.05% trypsin/0.53 mM EDTA in PBS (National Veterinary Institute, Uppsala, Sweden) for 3 min at 37 °C. After adding an equal volume of DMEM/10% FCS/antibiotics, the cells are detached, centrifuged, and resuspended in DMEM/10%FCS/antibiotics in a concentration of 1 × 10^6cells/ml in a 12-well Microwell plate (Nunc, Nalge Nunc Corp., Rochester, NY) or plated on poly-L-lysine coated coverslips (Histolab Products, Gothenburg, Sweden).
(xvi) Cultures must be confluent during the next 1 to 2 days for transfection.

4.2. siRNA transfection

Use RNase-free sterile pipette tips and supplies to ensure that the stock solution does not become contaminated with RNase. Wear gloves when handling reagents and solutions. The stock solution (see below) can be frozen and thawed several times.

(i) siRNA mediated silencing of endogenous expression of S100A4 in astrocytes is performed using 21 nucleotide siRNA duplexes. The sequence of sense and antisense oligonucleotide was 5′-GGGUGACAA-GUUCAAGCUGtt-3′ and 5′-CAGCUUGAACUU-GUCACCCtc-3′, respectively.
(ii) S100A4 siRNA is transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. On the day of the experiment, siRNA–Lipofectamine 2000 complexes are prepared and transfection performed. Use Falcon 2058 tube to mix the reagents.
   a. Dilute 1 μl of siRNA (100 μM stock) in 100 μl of Opti-MEM (Invitrogen).
   b. Dilute 3 μl of Lipofectamine 2000 reagent in 100 μl of Opti-MEM and leave for 5 min at room temperature.
   c. Combine the diluted siRNA solution with the diluted Lipofectamine 2000 solution. Mix gently and incubate for 20 min at room temperature.
(iii) Change the medium for astrocytic cultures to 0.8 ml of Opti-MEM 20 min before transfection.

(iv) Add the 200 μl siRNA–Lipofectamine 2000 complexes dropwise to each well while gently rocking the plate and incubate cells for at least 5 h at 37 °C.
(v) Add 1 ml fresh Opti-MEM containing 20% FCS and incubate cells overnight.
(vi) Change medium to DMEM supplemented with 10% FCS and antibiotics (see above) and incubate for 2–3 days. The amounts of siRNA, Lipofectamine 2000, and siRNA transfection medium are proportionally scaled up to the surface area of cell culture.
(vii) As a control for S100A4 siRNA, BLOCK-iT Fluorescent Oligo (Invitrogen) or Silencer Negative Control #1 siRNA (Ambion, Inc.) is used.

4.3. Immunocytochemistry

(i) White matter astrocytes on glass coverslips are washed three times with PBS and fixed for 30 min with 4% formaldehyde in PBS.
(ii) After washing, permeabilize the cells with 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO), incubate with 3% BSA (Sigma-Aldrich) in PBS to block non-specific binding, and then incubate with rabbit polyclonal anti-GFAP antibodies (DakoCytomation, Glostrup, Denmark, 1:200) for 1 h.
(iii) After extensive washing with PBS, the primary antibody is localized with the FITC-swine anti-rabbit IgG (DakoCytomation, 1:200).
(iv) After rinsing, mount the coverslips in 50% glycerol (Merck) in PBS containing 100 mM propyl-gallate (Sigma-Aldrich) to inhibit photobleaching.
(v) To verify the efficiency of transfection, coverslips with fixed white matter astrocytes are also double labeled with rat monoclonal anti-GFAP antibodies (Zymed Labs, San Francisco, CA, 1:100) and rabbit polyclonal anti-S100A4 antibodies (1:700, [16]). Secondary antibodies are Texas Red conjugated donkey anti-rat IgG (Jackson Immunoresearch, West Grove, PA, 1:100), and FITC conjugated goat anti-rabbit IgG (Sigma-Aldrich, 1:500), respectively.
(vi) The immunostainings are viewed and photographed in a Nikon Eclipse fluorescence microscope equipped with filter for simultaneous examination of FITC and TRITC fluorescence.

5. Results

Percoll purified astrocytes from the corpus callosum of P4 rats developed to cultures of GFAP positive cells with about 95% of them expressing S100A4. Already after 3 days, when cells were dispersed, the young astrocytes were S100A4 positive. After 1 week in culture, most of the astrocytes expressed S100A4, even if their morphology was immature with few and short processes (Fig. 1A). One to two weeks later, cultured astrocytes displayed strong S100A4 expression and had developed long processes (Fig. 1B). As in vivo, S100A4 immunoreactivity was located in the cell bodies of astrocytes, whereas their processes were only positive for GFAP [8,9].

Following transfection with S100A4 siRNA, the expression of S100A4 protein gradually declined until it was completely eliminated on day 3 after transfection (Fig. 2A). Astrocytes transfected with control siRNA maintained their expression of S100A4 (Fig. 2B). Concomitantly with the S100A4 siRNA mediated down-regulation of S100A4 expression, the immunoreactivity for GFAP increased (cf. Figs. 2C and D).

6. Discussion

6.1. Overall assessment of protocol

The results show that cultures of white matter astrocytes can be reproducibly generated from the corpus callosum of P4 rats. This offers new possibilities to explore the func-

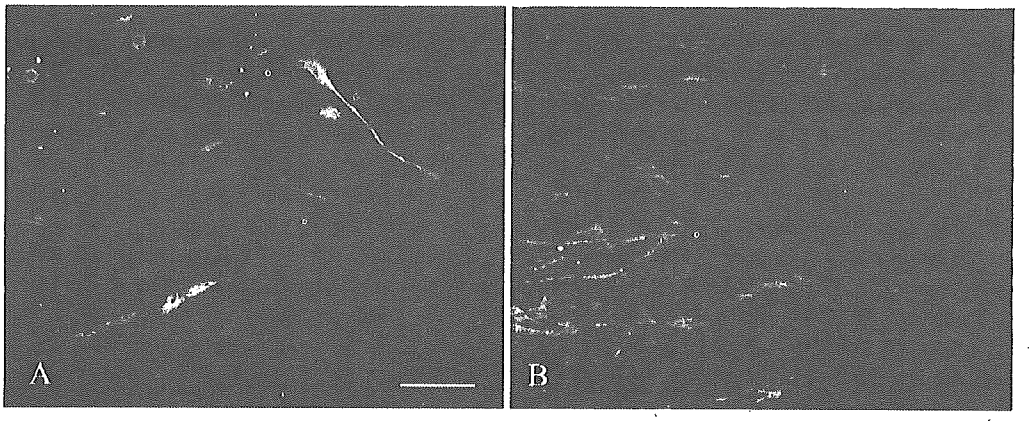

Fig. 1. Astroglial cultures from the corpus callosum of P4 rats. Cultures from 1 (A) and 3 (B) weeks, double labeled with antibodies to GFAP (red) and S100A4 (green). Scale bar = 100 μm.

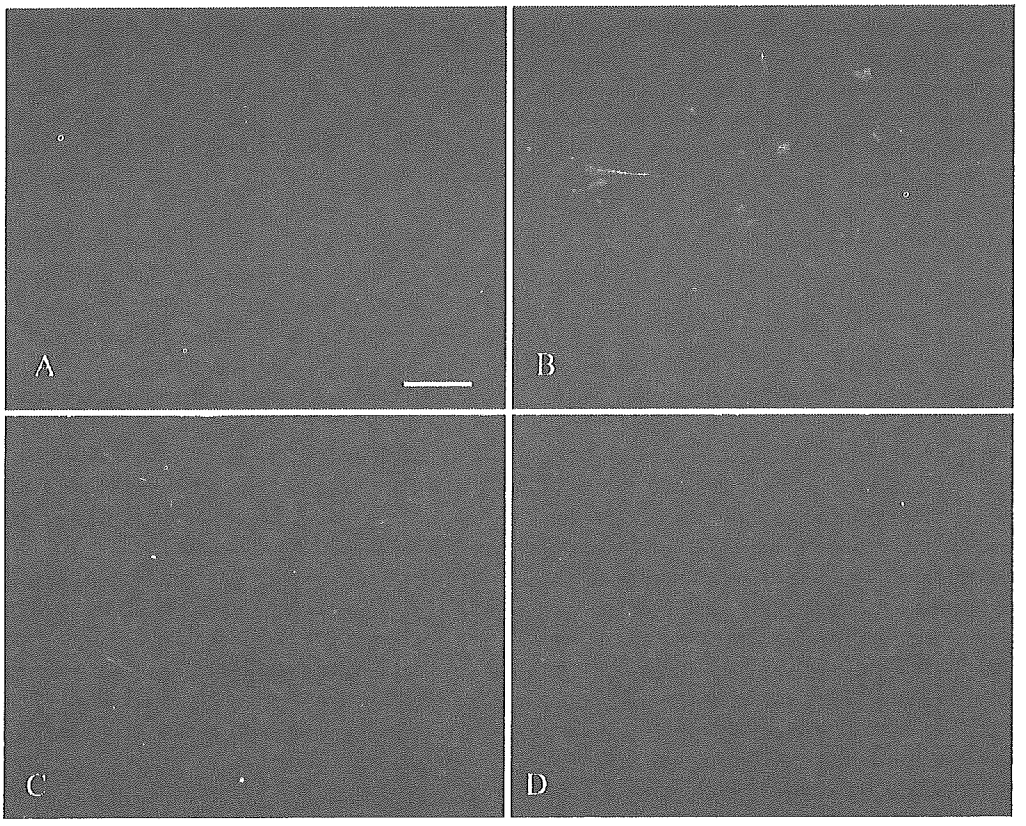

Fig. 2. White matter astrocytes from the corpus callosum of P4 rats transfected with S100A4 siRNA (A, C) or control siRNA (B, D). Cultures were examined 3 days following transfection. Cultures were stained with antibodies to GFAP (red) and S100A4 (green). Note the elimination of immunoreactivity for S100A4 after treatment with S100A4 siRNA (A). Immunoreactivity for GFAP appears to increase following silencing of S100A4 (cf. C and D). Scale bar = 100 μm.

tional properties of white matter astrocytes, and we show that the siRNA technique can be effectively applied to these cultures for this purpose. The aim of the siRNA silencing was to eliminate the expression of the S100A4 protein, which is expressed postnatally in white, but not gray matter astrocytes of the rat [1]. S100A4 is markedly up-regulated in reactive white matter astrocytes after injury and following demyelination [8,9], indicating that the protein is involved in the emergence of the non-permissive environment to neurite growth in degenerating white matter, as well as in the subsequent development of a glial scar [14].

Astroglial cultures are typically generated from brains of newborn rats or mice. At that stage, the corpus callosum cannot be unambiguously identified, and it is therefore not feasible to obtain cultures enriched in white matter astrocytes from this stage. We find that this is possible from P4 in the rat, which is the earliest time point when the corpus callosum can be clearly distinguished. However, the primary cultures made from the corpus callosum of P4 rats are extensively contaminated with other cells, such as fibroblasts, microglia, and oligodendroglial precursors. We find, in line with previous experience, that white matter astrocytes in these cultures can be effectively purified by density gradient centrifugation in Percoll. This procedure resulted in a well defined collection of astrocytes, which were subsequently plated and maintained in appropriate culture medium. Since the corpus callosum is easier to separate at later postnatal stages, we produced cultures from this area up to the age of P8, using Percoll density gradient centrifugation for purification. Although it was possible to prepare such cultures, the survival of astrocytes was poor compared to cultures produced from P4 rats (unpublished observations).

For the purpose of comparing white and gray matter astrocytes from the same stage, we tried to produce also primary cultures of gray matter astrocytes from the cerebral cortex overlying the corpus callosum of P4 rats. However, because of the difficulties to effectively remove connective tissue components in the cerebral cortex at this stage, these cultures were always overgrown by fibroblasts and only scattered astrocytes survived. Thus, to obtain gray matter astroglial cultures, we use the conventional approach to take tissue from the brains of newborn rats. These cultures develop fine but are always contaminated with S100A4 positive astrocytes (unpublished observations). The presence of these astrocytes most likely reflects the difficulty in completely avoiding parts of future white matter areas, including the corpus callosum, when preparing astroglial cultures from newborn cerebral cortex.

In theory, astroglial cultures can be generated from stem cells by directing their differentiation along the astroglial lineage [6]. However, at present, we do not know what factors are needed to induce differentiation of stem cells towards the astroglial phenotype characteristic of white matter. With increasing knowledge in this respect, embryonic or neural stem cells may be a useful alternative to cultures made from early postnatal white matter.

6.2. Trouble-shooting

A key factor for successful culture of white matter astrocytes is rapid and gentle handling of the tissue. Postnatal day 4 neural cells are likely to be more sensitive than those from newborn rats. Therefore, dissection of the brains has to be carried out quickly since the extent of astroglial survival will depend on the time that this procedure takes. At the same time, care has to be taken to restrict the dissection to the corpus callosum, since the inclusion of parts of the overlying gray matter will reduce the proportion of white matter astrocytes. We find that the dissection is facilitated by using a curved, fine-tipped jeweller's forceps to stabilize the rostral end of the brain, while the coronal slice is made with a sharp blade. The slice can then be kept flat by pressing gently on its basal part, while the corpus callosum is carefully separated from the ventricles and adjacent basal tissue. Finally, from the remaining corpus callosum-cerebral cortex piece, small blocks of the midportion of the corpus callosum are cut out. Large blood vessels are removed from the blocks to minimize contamination from non-neural cells.

6.3. Alternative and support protocols

The dissociation and purification steps in Section 4.1 (ix–xiii) can be replaced with

- mechanical dissociation as described in (ix),
- filtering the cell suspension through a 70 μm nylon filter (Falcon, BD Biosciences)
- filtering through a 40 μm nylon filter (Falcon, BD Biosciences) followed by placing cells in culture.

Cultures prepared in this way from the corpus callosum will be contaminated with e.g. microglia and fibroblasts, and have to be purified by shaking and re-seeding according to conventional protocols (see e.g. [2,10]). Finally, these cultures will contain numerous S100A4 expressing astrocytes and can be used for studies of white matter astrocytes, including transfection with siRNA.

7. Quick procedure

(i) Remove brains from P4 rat pups, make a 2 mm thick coronal slice at the level of the rostral part of the hippocampus, and identify the corpus callosum.
(ii) Prepare small blocks from the corpus callosum and place them in DMEM containing 10% FCS.
(iii) Rinse, place tissue in dissociation solution, and dissociate cells mechanically using needles of decreasing diameter.
(iv) Centrifuge the cell suspension and resuspend the pellet in a mixture of DNase solution and PBS/glucose/$MgSO_4$.
(v) Transfer the cell suspension onto a discontinuous Percoll gradient and centrifuge.
(vi) Aspirate the astrocyte enriched fraction carefully with a Pasteur pipette and suspend the cells in 10 ml of PBS/glucose.
(vii) Centrifuge and resuspend pellet in DMEM/10% FCS/3% glucose, plate in culture flasks, and maintain in culture for 1 to 3 weeks.
(viii) For subculturing for transfection, incubate cells with 0.05% trypsin/0.53 mM EDTA and add an equal volume of DMEM/10% FCS. Detach the cells from the culture flask, centrifuge, and resuspend in DMEM/FCS/glucose in a concentration of 1×10^6 cells/ml in 12-well Microwell plates or plate on poly-L-lysine coated coverslips.

7.1. siRNA treatment

(i) siRNA mediated silencing of endogenous S100A4 protein expression is performed using 21 nucleotide S100A4 siRNA duplexes.
(ii) S100A4 siRNA is transfected with Lipofectamine 2000 according to the manufacturer's instructions.
(iii) Change medium for astrocytic cultures to Opti-MEM before transfection and transfect cells with S100A4 siRNA for at least 5 h at 37 °C. Switch to fresh Opti-MEM containing 20% FCS and incubate overnight.
(iv) Change the medium to DMEM supplemented with 3% glutamine, antibiotics, and 10% FCS. The amounts of siRNA, Lipofectamine 2000, and siRNA transfection medium are proportionally scaled up to the surface area of cell culture.
(v) As a control, BLOCK-iT Fluorescent Oligo or Silencer Negative Control #1 siRNA is used.

7.2. Immunocytochemistry

(i) Astrocytes on glass coverslips are washed and fixed.
(ii) After washing, permeabilize the cells with 1% Triton X-100 and incubate with 3% BSA/PBS.
(iii) Incubate cells with primary antibodies for 1 h: rabbit polyclonal anti-GFAP (1:200) for single cell labeling, or rat monoclonal anti-GFAP (1:100) and rabbit polyclonal anti-S100A4 (1:700) for double labeling.
(iv) After washing with PBS, the primary antibody is localized with the FITC-swine anti-rabbit IgG (1: 200)

for single cell labeling or Texas Red conjugated donkey anti-rat IgG (1:100) and FITC conjugated goat anti-rabbit IgG (1:500), respectively, for double labeling.

8. Essential literature references

[1,8,9]

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Mammalian Polycomb-mediated repression of *Hox* genes requires the essential spliceosomal protein Sf3b1

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