Antisense Approaches to the Function of Glial Cell Proteins[a]

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S100 refers to a protein fraction originally isolated from bovine brain over 25 years ago[1] and termed S100 to denote its partial solubility in 100% saturated ammonium sulfate. The bovine brain S100 fraction is composed primarily of two small (M_r = 10,000), acidic, calcium binding proteins called S100α and S100β.[2] These proteins share approximately 50% identity in amino acid sequence and can form homologous and heterologous dimers of the form αα ($S100a_o$), αβ (S100a), and ββ (S100b). The S100α and S100β polypeptides are also characterized by the presence of regions of amino acid sequence that could form calcium binding structures: a conventional helix-loop-helix (EF-hand) calcium binding site in their COOH-terminal region and an NH_2-terminal calcium binding site that resembles the site found in calbindin.[3,4] S100α and S100β are structurally conserved in different species. For example, the amino acid sequences of bovine, rat, and human S100β differ by only 3–4 residues (see ref. 5). Less information is available about the structural conservation of the S100α polypeptide, but the bovine and rat proteins show only four amino acid differences (see ref. 6).

In addition to S100α and S100β, several proteins or cDNA sequences have been discovered in recent years that have 30–50% sequence identity to the S100s. This family of S100-like proteins includes calcium binding proteins, proteins that are induced in cells after growth factor or serum stimulation, proteins increased on differentiation or transformation of cells, a protein subunit of a cytoskeletal protein complex, and serum proteins associated with diseases such as cystic fibrosis or rheumatoid arthritis (see ref. 7 for references). Even though these proteins share extensive sequence homology, little is known about their functional roles. In this chapter, we will concentrate on the S100β polypeptide, the principal member of the S100 family in brain.

In both developing and mature vertebrate nervous system, S100β is expressed primarily by glial cells. Although S100β was thought to be brain specific for many years, it is known now that the protein is not restricted to the brain or even to nervous tissue. S100β has been localized to a variety of peripheral tissues (see refs. 5, 7, and 8), and reports about the use of S100β localization in diagnostic pathology abound in

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the literature. In contrast to the extensive literature about the structural properties and localization of S100β, the biological roles of this protein have only recently begun to be elucidated. A number of intracellular functions for S100β in glial cells have been suggested by *in vitro* studies. For example, S100β has been reported to stimulate or inhibit phosphorylation of various proteins, to inhibit microtubule assembly, and to interact with various enzymes and cytoskeletal proteins (see ref. 7 for references).

In addition to these multiple intracellular activities, an increasing body of evidence indicates that S100β also has extracellular roles. S100β is known to be released from glial cells, being detected in brain extracellular fluid[9] and in conditioned media from glial cells.[10,11] Release of S100β from astrocytes can be stimulated by serotonin acting through the astrocyte 5-HT_{1A} receptor.[12] A disulfide-linked, dimeric form of S100β has shown at least two extracellular activities on cultured cells: neurotrophic activity on select neuronal populations and mitogenic activity on astrocytes. S100β stimulates enhanced survival of embryonic cortical neurons in culture[13] and neurite outgrowth from cortical neurons,[13–15] serotonergic neurons of the mesencephalic raphe,[16] cells of the optic tectum,[17] dorsal root ganglia,[18] and spinal cord,[19] and the neuro-2A neuroblastoma cell line.[20] Nanomolar concentrations of dimeric S100β stimulate proliferation and increase in the steady-state levels of c-*myc* and c-*fos* protooncogene mRNAs in glial cells.[21] S100β was recently shown to be a trophic factor *in vivo,* being able to prevent the late naturally occurring motoneuron cell death during embryonic chick development as well as the deafferentation-induced death of motoneurons of the embryonic chick spinal cord.[22,23]

Evidence to date supports the idea that S100β is a multifunctional protein that plays important roles in nervous system development and maintenance. Therefore, the mechanisms involved in regulating the intracellular versus extracellular levels of S100β in the glial cell and the consequences of alterations in S100β levels need to be elucidated. S100β levels in the cell can be altered under certain conditions. For example, intracellular S100β levels increase as cells become confluent and at specific points in the cell cycle, in response to cAMP, and on treatment with various agents (see refs. 7 and 24 for references). In addition, S100β levels were reported to be increased in reactive glial cells of patients with Down syndrome and Alzheimer's disease,[25,26] a finding consistent with the localization of the gene for human S100β to the Down syndrome region of chromosome 21.[27,28] Recent studies with transgenic mice overexpressing S100β have demonstrated increased astroglial proliferation in specific areas of the brain,[29] suggesting that S100β may serve as a glial mitogen *in vivo.*

To directly address how perturbation of S100β levels in the glial cell correlates with changes in cell phenotype, we developed a biological system in which the S100β levels could be decreased in a specific and reproducible manner.[24] Specifically, we used antisense strategies to produce a selective decrease in S100β levels in rat C6 glioma cells. Two separate antisense approaches were used for inhibition of S100β production: (1) generation of clonal isolates of C6 cells stably transfected with an S100β antisense minigene under the control of a dexamethasone-inducible promoter, and (2) analysis of C6 cells treated with S100β antisense oligodeoxynucleotides. Both antisense procedures resulted in a decrease in intracellular S100β levels as measured by radioimmunoassay. This lowering of S100β levels correlated with three alterations in cellular phenotype: (1) a more flattened appearance, with a three- to fourfold increase in cellular area, (2) a more organized microfilament cytoskeletal network, and (3) a decrease in cellular proliferation rate. Similar results obtained with two distinct antisense methods provide strong evidence that S100β has important roles in glial cell morphology, cytoskeletal organization, and cell proliferation.

In this chapter, we briefly review the previous findings and report recent results examining in greater detail the effects of inhibition of glial S100β production on cell morphology.

MATERIALS AND METHODS

Cell Culture. Rat C6 glioma cells were used throughout these studies and were obtained and grown as previously described.[24] These cells contain high levels of S100β and have been used for many years in S100β studies as a biological model for glial cells.

Selection of Stable Clones Containing S100β Antisense Minigene. A plasmid (pS100AS) containing the S100β antisense minigene under the control of a dexamethasone-inducible promoter was constructed by using a synthetic S100β gene[15] as described.[24] C6 cells were transfected with pS100AS, and colonies were selected in G418, subcloned, and expanded. A number of stable clones containing the S100β antisense gene construct were developed, but the majority of experiments to date have used two clones, termed C6-AS1 and C6-AS2. Studies with these two clones yielded similar results and are used interchangeably in this chapter.

To induce expression of the S100β antisense gene, cells were treated for various times with 1 μM dexamethasone (10 μl of 1 mM dexamethasone in ethanol per 10 ml medium). Controls received diluent alone (10 μl of ethanol per 10 ml medium). We found that the endogenous S100β levels in the C6-AS1 and C6-AS2 clones are lower than those in the parental C6 cells. This may merely reflect selection of clones with lower amounts of S100β compared to the C6 cells, or the promoter may be somewhat "leaky," that is, there may be a low level of expression of the antisense gene in the absence of dexamethasone. In this regard, we found that it is important to use lots of fetal calf serum that do not contain high levels of steroid or to charcoal treat the serum before using in order to deplete the serum of steroids.

Treatment of C6 Cells with S100β Antisense Oligonucleotides. Oligodeoxynucleotides were synthesized by β-cyanoethyl phosphoramidite chemistry as described[24] and were used either directly from the synthesizer or after further purification using Sephadex G-25 spin columns. Cells were treated with oligonucleotides as described.[24] Briefly, before treatment, serum-containing medium was aspirated from the cells, and the cells were washed two times in serum-free medium. Cells were then incubated for 2 hours at 37°C in serum-free medium containing 30 μM oligonucleotide. After the 2-hour incubation, fetal calf serum was added to the wells (without changing the medium) to a final serum concentration of 2.5%. For our studies, cells received only a single addition of oligonucleotide at the beginning of the time course, and the medium was not changed during the experiment. In our experiments, we did not find it necessary to use modified oligonucleotides. An important factor, however, is the use of lots of fetal calf serum containing low levels of nuclease activity. Before embarking on an oligonucleotide experiment, we carried out experiments essentially as described[30] to test various lots of fetal calf serum for nuclease activity. We have had excellent results with fetal calf serum obtained from Hyclone Laboratories.

Measurement of S100β Levels. To confirm that S100β production was being inhibited in our antisense experiments, S100β levels were measured in cell extracts by radioimmunoassay, as described.[24]

Analysis of Phenotypic Changes. Morphological examination of cells was performed as described[24] or by phase-contrast microscopy on a Leitz Diavert inverted microscope. The cytoskeletal proteins actin, tubulin, and vimentin were localized by fluorescence methods as described.[24] Cell counts were determined with a hemacytometer.

Time-lapse video microscopy was conducted on cells grown in a T-25 flask. The flask was sealed with a rubber stopper to maintain the 95% air, 5% CO_2 atmosphere. Extended through the stopper was a wire (sealed in the stopper with silicone rubber adhesive) connected to a small thermistor that served as a temperature sensor to regulate an infrared source (Opti-quip Red Beam Incubator) that maintained temperature in the flask at 37.0 ± 0.1°C. To allow injection of dexamethasone via a hypodermic needle and syringe, a small hole was melted in the flask top before culturing cells, using a 2-mm diameter metal rod that had been heated over a bunsen burner. The hole was immediately sealed with silicone rubber adhesive. The adhesive cured as cells were cultured in the flask. The flask was placed on the stage of a Leitz Diavert inverted microscope, and cells were imaged using a long working distance 40× phase contrast objective lens. A Dage-MTI newvicon video camera was used to obtain images, and the images were recorded by a Panasonic AG6750 time-lapse video recorder. For most studies, the recorder was set for a time compression of 240×.

RESULTS

As reported previously,[24] we developed two antisense methods for inhibiting the production of S100β in C6 glioma cells. One method used a eukaryotic expression vector constructed to produce RNA complementary to the coding sequence of an S100β gene. There are several key features in this S100β antisense vector. First, the S100β coding region was derived from a synthetic S100β gene[15] that contains 77% homology with the coding region of rat S100β cDNA,[31] and then inserted into the polylinker region of the pMSVneo eukaryotic expression vector.[32] Second, the pMSVneo vector allows the inducible expression of inserted genes, because of the presence of the dexamethasone-inducible promoter in the mouse mammary tumor virus-long terminal repeat (MMTV LTR). This was advantageous because antisense-containing clones could be selected before S100β inhibition occurred, thus avoiding potential cloning problems due to decreases in cell growth rate. Third, pMSVneo contains an ampicillin resistance gene and pBR322 origin of replication, allowing initial cloning in bacterial cells.

The second antisense method used S100β antisense oligodeoxynucleotides. An S100β antisense oligonucleotide was prepared whose sequence is the inverse complement of 15 bases of the rat S100β cDNA sequence,[31] beginning at the ATG that corresponds to the initiator methionine. The antisense oligonucleotide had the sequence (reading 5′ to 3′): CTCCAGCTCAGACAT. Controls included the sense oligonucleotide that is equivalent to the coding strand of the cDNA in this region (ATGTCTGAGCTGGAG), and a mismatch oligonucleotide that is equivalent to the antisense sequence in which two bases have been changed (CTCCACCTCAGAGAT). In designing the oligonucleotides, we searched sequence databases to confirm that no other known nucleotide sequence matched the sequence of our oligonucleotides. We also kept the base composition of the mismatch oligonucleotide the same as the antisense oligonucleotide, making a G to C change and a C to G change.

We confirmed by radioimmunoassay that both of these antisense methods resulted in an inhibition of S100β production in the glial cell. FIGURE 1 summarizes

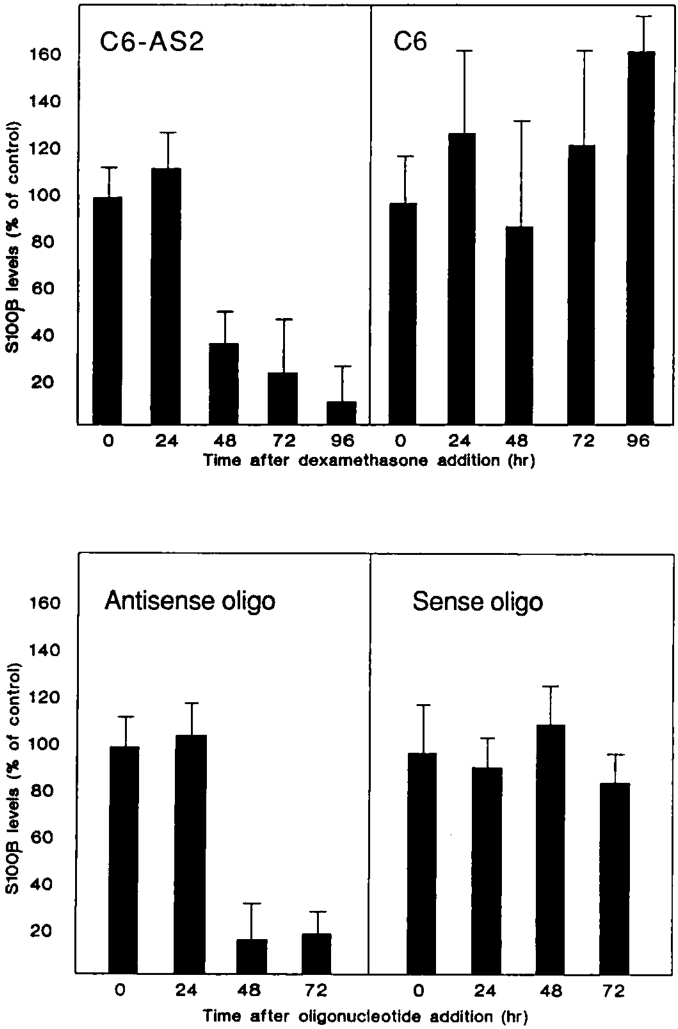


FIGURE 1. Inhibition of S100β production in a C6 clone containing the S100β antisense gene or in C6 cells treated with antisense oligonucleotides. In the top panel, the C6-AS2 clone (*left*) or untransfected C6 cells (*right*) were grown in the presence of dexamethasone for various lengths of time. S100β levels in cell extracts were determined by radioimmunoassay, and calculated as ng S100β/μg total protein. S100β levels are expressed as a percentage of the control levels at the 0-hour time point (i.e., in the absence of dexamethasone). Bars represent the mean ± SEM of four determinations (two separate experiments analyzing two wells per time point). In the bottom panel, C6 cells were treated with 32 μM antisense oligonucleotide (left) or sense oligonucleotide (right) for various lengths of time. S100β levels were determined as above, and expressed as a percentage of the control levels (levels in untreated C6 cells at each time point). Bars represent the mean ± range of duplicate cultures from a representative experiment. (Figure adapted from Selinfreund.[24])

these data, expressed as a percentage of the control S100β levels. It should be noted that by both antisense methods, S100β levels were not significantly lower than the controls until between 24 and 48 hours after antisense gene induction or the addition of antisense oligonucleotide. This is not an unexpected finding, as S100β is a relatively long-lived protein. We also could not completely inhibit S100β production by either method; however, S100β levels were only 15–20% of control values after 72–96 hours of treatment with dexamethasone or antisense oligonucleotide. Even though some S100β could be detected by radioimmunoassay at the 72- and 96-hour time points, we still obtained dramatic phenotypic effects of S100β inhibition, as will be discussed in more detail.

A readily observable effect of selective inhibition of S100β production in C6 cells was a change in cellular morphology. An example of this morphological change is shown in FIGURE 2 for one of the C6 clones treated with diluent alone (**a**) or treated with dexamethasone for 48 hours (**b**). The C6 clone in the absence of dexamethasone (**a**) show a bipolar stellate shape typical of untransfected C6 cells, with a rounded and refractile appearance as the cells become confluent. After treatment of cells with antisense oligonucleotide (data not shown) or induction of the S100β antisense gene with dexamethasone (**b**), the majority of cells exhibit a flattened, enlarged, and less refractile appearance. This change in cellular morphology was also accompanied by alterations in cytoskeletal organization and a decrease in cellular growth rate.[24] These phenotypic changes were not observed in untransfected C6 cells treated with dexamethasone or in C6 cells treated with the sense or mismatch oligonucleotides.[24]

To further analyze the time course of the morphological changes that occur on S100β inhibition, we examined S100β antisense-containing clones by time-lapse video microscopy at various times after the addition of dexamethasone (FIG. 3). Before dexamethasone treatment (panels **a–c**), the cells exhibit rapid undulations or "boiling" of the cell surfaces, along with numerous areas of membrane ruffling at the cell edges. Cells appear small and rounded, and each cell appears to have only one nucleolus. Mitoses are frequent, with an average cell-cycle time of 12–14 hours calculated from the video images.

After 1 day of dexamethasone exposure (panels **d–f**), the cell surface becomes more quiescent with slower undulations and fewer regions of membrane ruffling. The cells still undergo frequent mitoses. After 2–3 days in the presence of dexamethasone (panels **g–i**), the cells exhibit much reduced membrane activity, with very few surface movements or membrane ruffling. The cells are more flattened and spread, with larger nuclei often containing many nucleoli. Mitoses are much less frequent. From examination of the video images, it appears that cells may enter mitosis, but often do not complete cytokinesis. The resultant cells exhibit an enlarged cytoplasm and large nuclei containing multiple nucleoli. Estimates of cell cycle time from the few cells that underwent mitotis gave a value much greater than 30 hours. This lengthening of cell cycle time is consistent with our previous observations[24] of a reduced rate of proliferation after inhibition of S100β production.

DISCUSSION

Our studies demonstrate that selective inhibition of S100β production in glial cells produced by using either an S100β antisense minigene construct or S100β antisense oligonucleotides is correlated with at least three phenotypic changes: (1) a more flattened cellular morphology, with enlarged cytoplasmic area and reduced membrane ruffling; (2) a more organized cytoskeleton at the level of the microfilament network; and (3) a decrease in the cellular proliferation rate. These data

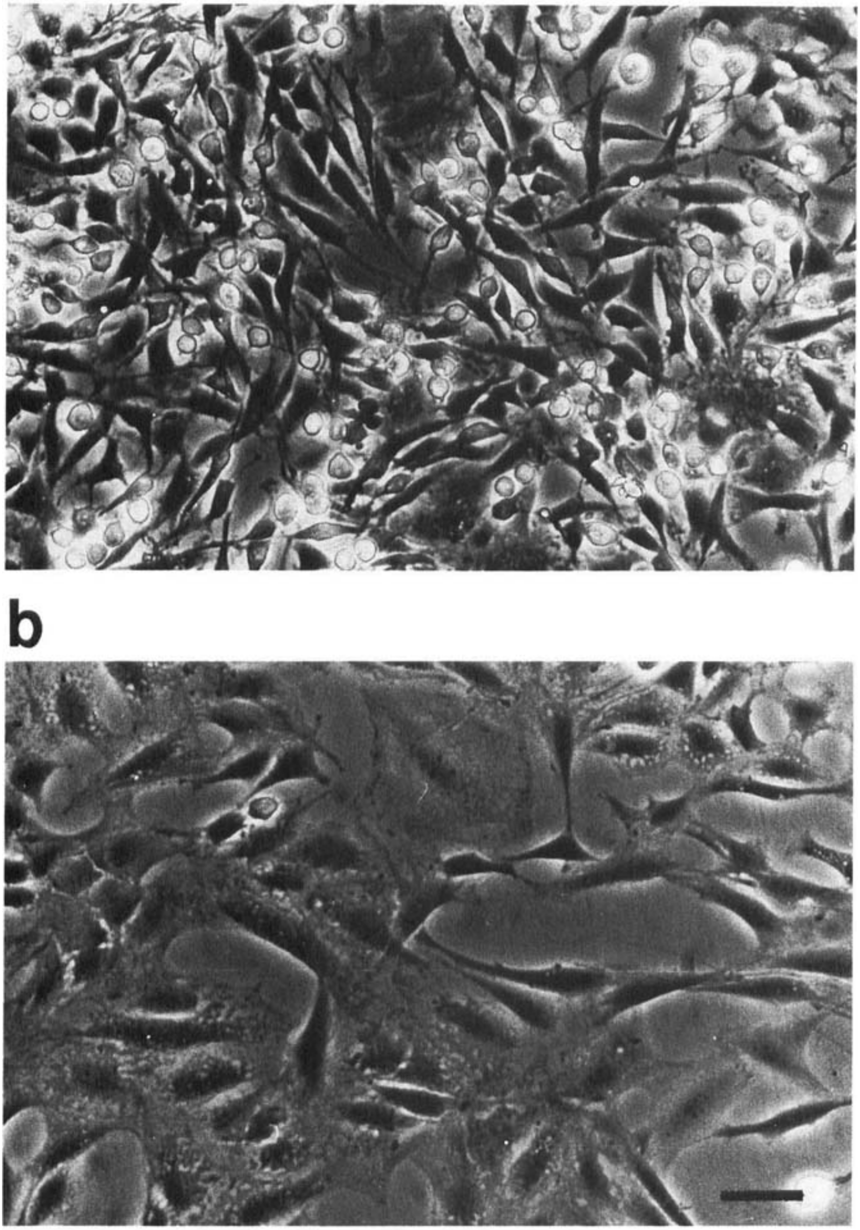
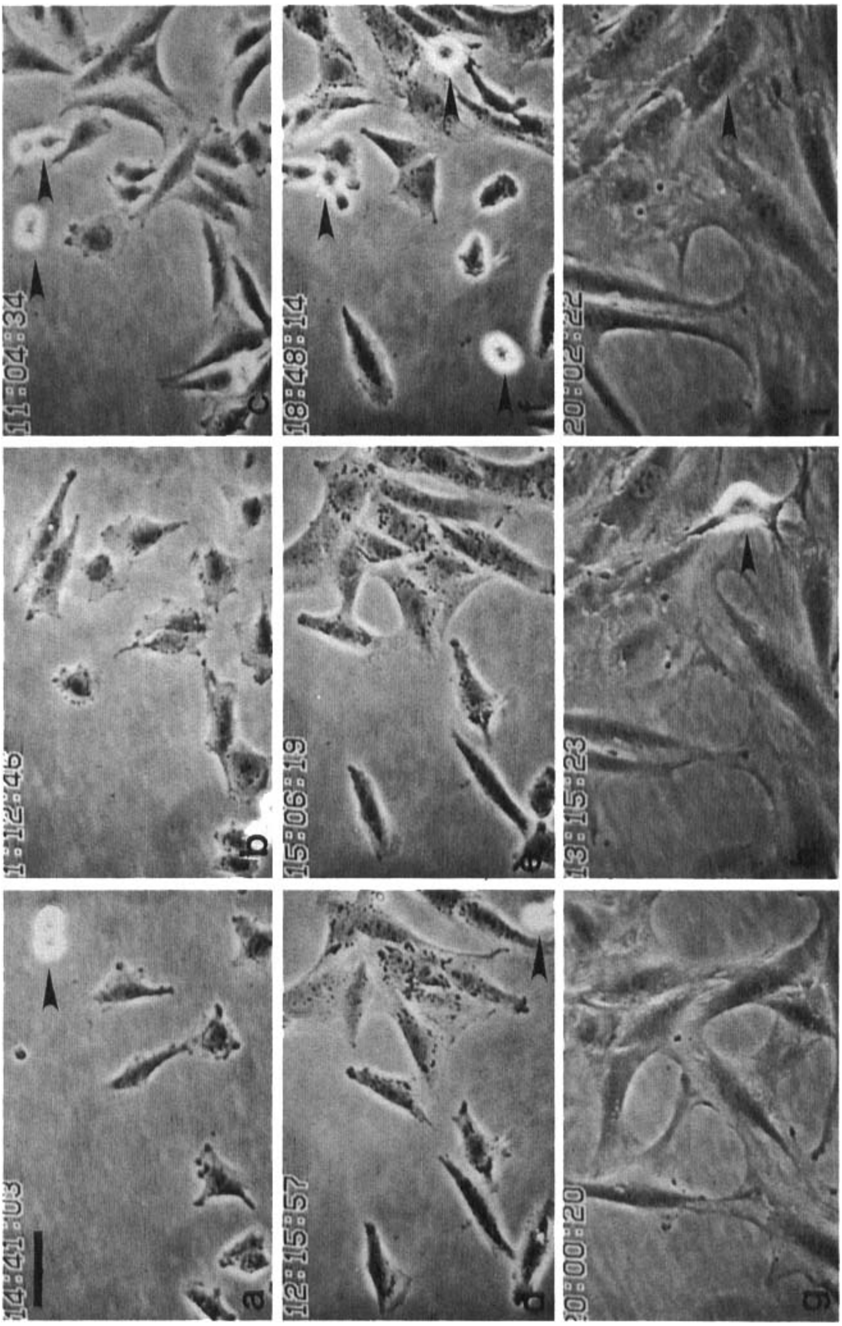


FIGURE 2. Effects of S100β antisense gene induction on cellular morphology. A C6 clone (C6-AS2) containing the S100β antisense gene under the control of a dexamethasone-inducible promoter was grown for 48 hours in the absence (**a**) or presence (**b**) of 1 μM dexamethasone. Cells were examined by phase contrast microscopy.


14:41:03
a
11:12:45
b
11:04:34
c
12:15:57
d
15:06:19
e
18:48:14
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g
13:15:23
20:02:22

FIGURE 3.

support the idea that S100β has important *in vivo* roles in regulation of glial cell morphology, cytoskeletal organization, and cell proliferation.

Antisense strategies are becoming increasingly important tools in attempting to understand the function of specific gene products by inhibiting the production of the protein and determining the functional consequences. It should be noted that the S100β gene[15] used in our studies for construction of the antisense minigene has only a 77% homology with the coding region of rat S100β cDNA. Even without complete homology, the antisense gene construct was able to be used successfully to inhibit S100β production in rat C6 cells, as confirmed by direct demonstration of decreased S100β levels by radioimmunoassay. This finding provides a precedent for potential utilization of DNA sequences from one species to inhibit protein production in cells from another species. These data are also consistent with previous studies[33] showing that complete DNA sequence homology is not required for inhibition of gene function by antisense constructs.

The use of antisense approaches has allowed us to begin to address the *in vivo* roles of S100β in glial cells. Our findings suggest that S100β plays a role in regulation of glial cell morphology, cytoskeletal structure, and cell proliferation. The molecular mechanisms by which alterations in S100β levels lead to these phenotypic changes are just beginning to be explored. Our initial findings[24] that a decrease in intracellular S100β levels resulted in a decrease in C6 cell growth rate, along with the detection of S100β in conditioned media of rapidly proliferating C6 cultures,[11] suggested that S100β may be secreted from C6 cells to act as an autocrine growth factor. To test this possibility, we examined the effects of exogenous S100β on C6 cell growth rate. We found[21] that exogenous S100β could stimulate C6 cell proliferation, as measured by both increases in (^{3}H)-thymidine incorporation and cell number. More recent studies[34] have shown that S100β evokes a transient rise in intracellular calcium and stimulates phosphoinositide turnover, which is consistent with the signal transduction of other extracellular mitogenic factors. The hypothesis that S100β antisense procedures slow the growth rate by decreasing production of an autocrine growth factor should be further tested by measuring the amount of S100β released from C6

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FIGURE 3. Time-lapse video analysis of cellular morphology in a C6 clone containing the S100β antisense gene. Cells were cultured for video recording as described in Methods, and grown in the absence (**a–c**) or presence (**d–i**) of dexamethasone for various lengths of time. In panels **a–c**, the same region of the culture substrate is shown for cells grown in the absence of dexamethasone for ~3, 10, and 20 hours, respectively. In panels **d–f**, the same group of cells are shown after treatment with dexamethasone for ~24, 27, and 31 hours, respectively. In panels **g–i**, cells have been treated with dexamethasone for ~56, 73, and 80 hours, respectively. In the absence of dexamethasone, cells undergo frequent mitoses (*arrows* in panels **a–c**), with a cell cycle time of about 13 hours. Cells treated with dexamethasone for 1 day (panels **d–f**) appear more spread and somewhat larger than untreated cells. Mitosis is still evident in occasional cells (*arrows* in **f**); however, the cell cycle time is significantly longer than that of untreated cells. After 2–3 days of dexamethasone treatment (**g–h**), cells are much larger and more spread than untreated cells; mitosis is very infrequent (*arrow* in **h**). On those rare occasions when a cell does undergo mitosis, it may not go through cytokinesis. The cell indicated by the *arrow* in **h** is the single cell indicated by the *arrow* in **i**. After this cell went through mitosis, it did not undergo cytokinesis but became a daughter cell with two nuclei initially. The two nuclei then fused to form one larger nucleus. The infrequency of mitosis in cells treated with dexamethasone for 2 or more days indicates that the cell cycle time of these cells increases to much longer than 30 hours. Assuming that the cells are still growing, the lack of mitosis may account for the large size of the cells and their nuclei as time in culture in the presence of dexamethasone increases. Bar = 50 μm.

cells before and after inhibition of intracellular S100β production, and by determining if the addition of exogenous S100β can reverse or delay the phenotypic effects of antisense inhibition of S100β production.

The precise role of S100β in cell proliferation is not known, and it is not possible to exclude the involvement of intracellular functions of the protein. One of the most striking morphological changes resulting from a decrease in S100β levels was the establishment of stress fibers, indicating a change in microfilament organization.[24] These effects may be related to the ability of S100β to alter interactions of actin with caldesmon *in vitro.*[35] Effects of S100β on cytoskeletal elements presumably could alter cell division rates. In this regard, we observed in video images of S100β-antisense clones treated with dexamethasone for 2–3 days (i.e., at times when S100β levels are decreased significantly) that some of the cells appeared to enter the mitotic cycle, but then did not complete cytokinesis (FIG. 3). This apparent arrest of cell division resulted in enlarged cells containing large nuclei with multiple nucleoli, and would help explain the reduced proliferation rate observed after S100β inhibition. We have not yet pursued these observations further, but deficiencies in cytokinesis are very likely to result from alterations of cytoskeletal kinetics. A previous report[36] has also implicated S100β in regulation of progression through the cell cycle. Thus, it is possible that S100β plays regulatory roles during specific points in the cell cycle, but eludication of the mechanism of S100β regulation will require further study.

In summary, the available data suggest that homeostasis of S100β levels in the glial cell may be critical to normal brain development and function. The ability to manipulate glial cells by antisense strategies to produce selective decreases in S100β levels provides a useful biological system for directly testing the contribution of S100β to biological processes in the brain. Our studies using antisense approaches have provided new insights into possible *in vivo* roles for S100β in glial cell morphology, cytoskeletal organization, and growth regulation. In addition, these data provide the framework for our continuing studies aimed at defining the molecular mechanisms of S100β action in nervous system development and maintenance.

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