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Astrogliosis in Culture: I. The Model and the Effect of Antisense Oligonucleotides on Glial Fibrillary Acidic Protein Synthesis

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Astrogliosis is a predictable response of astrocytes to various types of injury caused by physical, chemical, and pathological trauma. It is characterized by hyperplasia, hypertrophy, and an increase in immunodetectable glial fibrillary acidic protein (GFAP). As GFAP accumulation is one of the prominent features of astrogliosis, inhibition or delay in GFAP synthesis in damaged and reactive astrocytes might affect astrogliosis and delay scar formation. The aim of this study is to investigate the possibility of utilizing antisense oligonucleotides in controlling the response of astrocytes after mechanically induced injury. We scratched primary astrocyte cultures prepared from newborn rat cerebral cortex with a plastic pipette tip as an injury model and studied the astrogliotic responses in culture. Injured astrocytes became hyperplastic, hypertrophic, and had an increased GFAP content. These observations demonstrate that injured astrocytes in culture are capable of becoming reactive and exhibit gliotic behaviors in culture without neurons. The increase in GFAP content in injured astrocytes could be inhibited by incubating the scratched culture with commercially available liposome complexed with 3′ or 5′ antisense oligonucleotides (20 nt) in the coding region of mouse GFAP. The scratch model provides a simple system to examine in more detail the mechanisms involved in triggering glial reactivity and many of the cellular dynamics associated with scar formation. Antisense oligonucleotide treatment could inhibit the GFAP synthesis in injured astrocytes, hence it may be applicable in modifying scar formation in CNS injury in vivo.

Key words: astrocytes, transfection, injury, scratch-wound model

INTRODUCTION

Astrocytes react to various types of injury by rapid and vigorous astrogliosis (Reier, 1986; Eng, 1988a,b; Eng et al., 1992). Astrogliosis is characterized by hyperplasia and extensive hypertrophy of the cell body, nucleus as well as cytoplasmic processes (Eng, 1988a), and an increase in immunodetectable glial fibrillary acidic protein (GFAP) (Eng, 1988a; Condorelli et al., 1990; Hozumi et al., 1990; Vijayan et al., 1990; Eng et al., 1992). Such a response may be beneficial in the healing phase of injured CNS by actively monitoring and controlling the extracellular molecular and ionic contents, and walling off areas of the CNS from non-CNS tissue environments following trauma. However, the process is rapid and may result in interference with the function of residual neuronal circuits, remyelination, or axonal regeneration (Eng et al., 1987; Reier and Houle, 1988). If astrogliosis could be delayed or inhibited, neurons and oligodendrocytes might have an opportunity to reestablish a functional environment and to regenerate.

We had previously reported that antisense GFAP mRNA was capable of inhibiting GFAP synthesis induced by dibutyryl cyclic AMP (dBcAMP) in primary cultures of astrocytes (Yu et al., 1991). In this report, we described the establishment of a mechanical injury model by scratching a confluent culture of rat cerebral cortical astrocytes with a plastic pipette tip. Injured astrocytes in this culture model exhibited the major properties of astrogliosis. This system allows the observation of astrocytic response to an injury in a culture environment lacking interactions with neurons. As mentioned, GFAP accumulation is one of the prominent features of astrogliosis; inhibition or delay in GFAP synthesis in damaged and reactive astrocytes might affect astrogliosis and delay scar formation. Therefore, we also extended our investigation on the effect of antisense oligonucleotides on GFAP synthesis in these injured astrocytes.

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MATERIALS AND METHODS

Cultures

The preparation of primary cultures of rat cerebral cortical astrocytes from newborn Sprague-Dawley rats (VAMC Animal Facility, Palo Alto, CA) has been described previously (Yu et al., 1989, 1991). Briefly, the neopallia freed of meninges were cut into small cubes (<1 mm^3) in a modified Dulbecco's Modified Eagle Medium (DMEM) (Applied Scientific, San Francisco, CA). The tissue was disrupted by vortex mixing for 1 min and the suspension was passed through two sterile nylon Nitex® sieves (L. and S.H. Thompson, Ontario, Canada) with pore sizes of 80 and 10 μm. A volume of cell suspension equivalent to approximately one-40th of cerebral hemispheres from one brain was placed in a 35 mm Falcon tissue culture dish (Becton Dickinson and Co., Lincoln Park, NJ). Fresh DMEM supplemented with 20% fetal calf serum (FCS) (Hyclone Laboratories, Inc., Logan, UT) was added to yield a final volume of 2 ml. All cultures were incubated at 37°C in a 95%:5% (vol:vol) mixture of atmospheric air and CO_2 with 95% humidity. The culture medium was changed after 3 days of seeding and subsequently 2 times per week with DMEM containing 10% FCS. Cultures more than 4 weeks old were used for experiments.

Scratch-Wound Model

Cultures were scratched with a sterile plastic pipette tip (Cat. 3507; E&K Scientific Products, Inc., Saratoga, CA) according to a grid which resulted in an average of 37% injury. The degree of injury was estimated by comparing the protein content in injured cultures to the uninjured cultures. Protein content was measured by the Lowry procedure (Lowry et al., 1951). Immediately following the scratch-wound, the culture medium was changed in order to remove most of the detached cells and debris. This was done to prevent conditioning the medium by cell debris and factors released from the detached cells. Injured cultures were incubated in fresh DMEM containing 10% FCS or immediately transfected with liposome-antisense oligomer complex (see below).

Morphology Studies

Cultures after scratching were observed under a Nikon phase contrast microscope. The morphologic response of astrocytes along the margin of the wound was followed by taking phase contrast micrographs from the same field at different time intervals following the scratching. Sister cultures were fixed in 0.3 M NaCl in 70% ethanol and immunostained with anti-GFAP serum (see below).

Cell Proliferation

The effect of scratch injury on astrocyte proliferation was estimated by both ^{3}H-thymidine and 5-bromo-2-deoxyuridine (BrdU) uptake. Two hour uptake of [6-^{3}H]-thymidine (0.5 μCi per culture; 20.1 Ci/mmol; NEN® Research Products, Boston, MA) was performed at various times after the injury. At the end of incubation, cultures were quickly washed twice with cold fresh DMEM and dissolved in 1 M NaOH. The radioactivity in the cell solution was determined in a Packard Tri-carb 460C Liquid Scintillation Counter.

For BrdU uptake, cultures were incubated for 8 hr in 10 μM BrdU (Becton Dickinson and Co.). At the end of incubation, the cultures were washed twice with 0.9% NaCl before fixing with 0.3 M NaCl in 70% ethanol for 30 min. The cultures were stored in 70% ethanol at 4°C until processed for immunostaining.

Immunocytochemistry

For immunostaining of GFAP in astrocyte culture, polyclonal rabbit antibodies to multiple sclerosis plaque GFAP prepared in our laboratory (Eng and DeArmond, 1983) were used with peroxidase-antiperoxidase (PAP) (Sternberger et al., 1970; Eng and DeArmond, 1983; Yu et al., 1991). Rabbit and mouse PAP and rabbit IgG antibody to mouse IgG were purchased from Sternberger-Monoclonals Incorporated (Baltimore, MD).

Cultures treated with a single pulse of BrdU were immunostained with monoclonal mouse antibody to BrdU (Nagashima et al., 1985). All cultures were counterstained with hematoxylin.

GFAP Measurement

Both control and injured culture (transfected and non-transfected) were harvested by addition of 1 ml of 0.15% sodium dodecyl sulfate (SDS) in 50 mM phosphate buffer (pH 8.0) (Morrison et al., 1985) 1, 2, 3, 5, or 7 days after scratch-wound. GFAP content was measured by a solid phase enzyme-linked immunosorbent assay (ELISA) method, using p-nitophenyl phosphate as the substrate chromogen (Eng et al., 1986; Yu et al., 1991). The change in GFAP content was further visualized (Yu et al., 1991) by one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% acrylamide gels (Laemmli, 1970), followed by transfer to Immobilon-P membrane (Millipore Products, Bedford, MA) according to Towbin et al. (1979) and immunoperoxidase staining with antibody to GFAP (Sternberger et al., 1970; Eng and DeArmond, 1983).

Antisense Nucleotides and Transfection

Antisense constructs were 5′ and 3′ oligonucleotides (20 nt) in the coding region of mouse GFAP (Op-

eron Technologies, Inc., Alameda, CA). The sequence for the 5′ oligomer is 5′-GCT CAA TGC TGG CTT CAA GG-3′ and was selected from the initiation site of the mouse GFAP cDNA described in Lewis and Cowan (1985). The 3′ sequence was 5′-GGA GGA GCT CTG CGT TGC GG-3′ from the same coding region of mouse cDNA. Some of the oligomers were conjugated to biotin in order to investigate whether the modification of the oligomers would affect their efficacy. Antisense oligomers were transfected into astrocytes with Lipofectin® (LF) (BRL Life Technologies, Inc., Gaithersburg, MD) (Eng et al., 1990; Yu et al., 1991). To prepare the complex, the nucleotide and liposome (48 μg of antisense oligomers to 11 μg of LF per culture) were mixed in a polystyrene tube for 15 min at ambient temperature. Transfection was performed on astrocytes immediately after scratching by adding the complex to the culture in serum-free DMEM (Yu et al., 1991). After 3 hr of incubation, FCS was added directly to the incubation medium to acquire a final serum concentration of 10%. Cultures were fed with normal DMEM containing 10% FCS thereafter twice a week until experiments were done.

RESULTS

Scratch-Wound Model

Scratching a confluent culture of astrocytes created denuded areas by removing cells from the substratum. Cells along the wound were damaged to varying degree. A control culture without scratching contained 427.3 ± 4.6 (n = 9) μg of protein. Cultures after scratching with a plastic pipette tip according to a grid contained an average of 281 ± 1.6 (n = 6) μg protein. Based on these protein values, a reproducible model of 37% scratch damage was established.

Morphology

Figure 1 shows a culture after injury under phase contrast microscopy. The edge of the scratch was lined with irregularly shaped cells (Fig. 1A). Some cells had swollen cytoplasmic processes while some had a dark and dense cytoplasm. Six hours after injury, astrocytes began to send pale and flat cytoplasmic processes toward the denuded area (Fig. 1B). Cells near the wound showed loose contacts with each other. The cytoplasmic processes elongated further at 12 and 24 hr after injury (Fig. 1C,D). Some small cells, both phase bright and dark, were occasionally seen in the denuded area immediately after the damage (Fig. 1A). These round cells differentiated and sent out processes within the first day of injury (Fig. 1B–D). Some cells acquired morphological appearances of microglia and oligodendrocytes.

GFAP Staining

Cultures injured under identical conditions were immunostained for GFAP and viewed under light microscopy. Figure 2 shows the morphology of cells along the scratch-wound 0, 1, 2, and 3 days after injury, where all astrocytes were positively stained for GFAP. Figure 2A shows that the cells along the wound appeared to be damaged. Cells adjacent to the wound did not show any abnormality in their morphology. One day after the injury (Fig. 2B), the cells along the edge lose their integrity and tight contact with each other. Their cytoplasm was extremely positive to GFAP staining. Many astrocytes were multinucleated and some nuclei were lobulated. Many active nuclei could be located. A few appeared to be hypertrophic. Some astrocytes started to send out cytoplasmic processes into the scratched area. Some tiny cells could be seen which were GFAP negative. These cells might be microglia and need future characterization for confirmation. Two days after injury, numerous long cytoplasmic processes grew into the wound (Fig. 2C). Many small branches radiated in different directions at the end of their growth cones. Figure 2D shows a culture 3 days after the injury. The denuded areas became almost completely covered with cytoplasmic processes. Most astrocytes responded to the injury by sending cytoplasmic processes toward the site of injury without migration of their cell bodies and nuclei.

Hypertrophy

Hypertrophy of nuclei, cell bodies, and cytoplasmic processes is another characteristic of astrogliosis observed along the scratch-wound in astrocyte cultures. A variety of insults may lead to size and shape changes of astrocytic nuclei. Nuclear hypertrophy in astrocytes along the wound was observed (Fig. 2B). In many cases the nuclei became highly lobulated. Hypertrophy of astrocytic cytoplasmic processes was observed within a few days after injury (Figs. 1, 2). They show an unusually prominent positive reaction for GFAP (Fig. 2). Hypertrophic astrocytes are easily distinguished and have a clear starfish-like pattern and expansion of their processes.

Hyperplasia

Figure 3 shows a BrdU-treated culture stained with anti-BrdU 1 day after injury. Areas distant from the injury are shown in Figure 3A. Areas adjacent to the wound are shown in Figure 3B. The number of nuclei stained with anti-BrdU were higher in Figure 3B than in Figure 3A. This indicates that cells closer to the injury site were more mitotic than cells farther away. Scratch-wound induction of cell proliferation in astrocytes was further demonstrated by ^{3}H-thymidine uptake. Figure 4

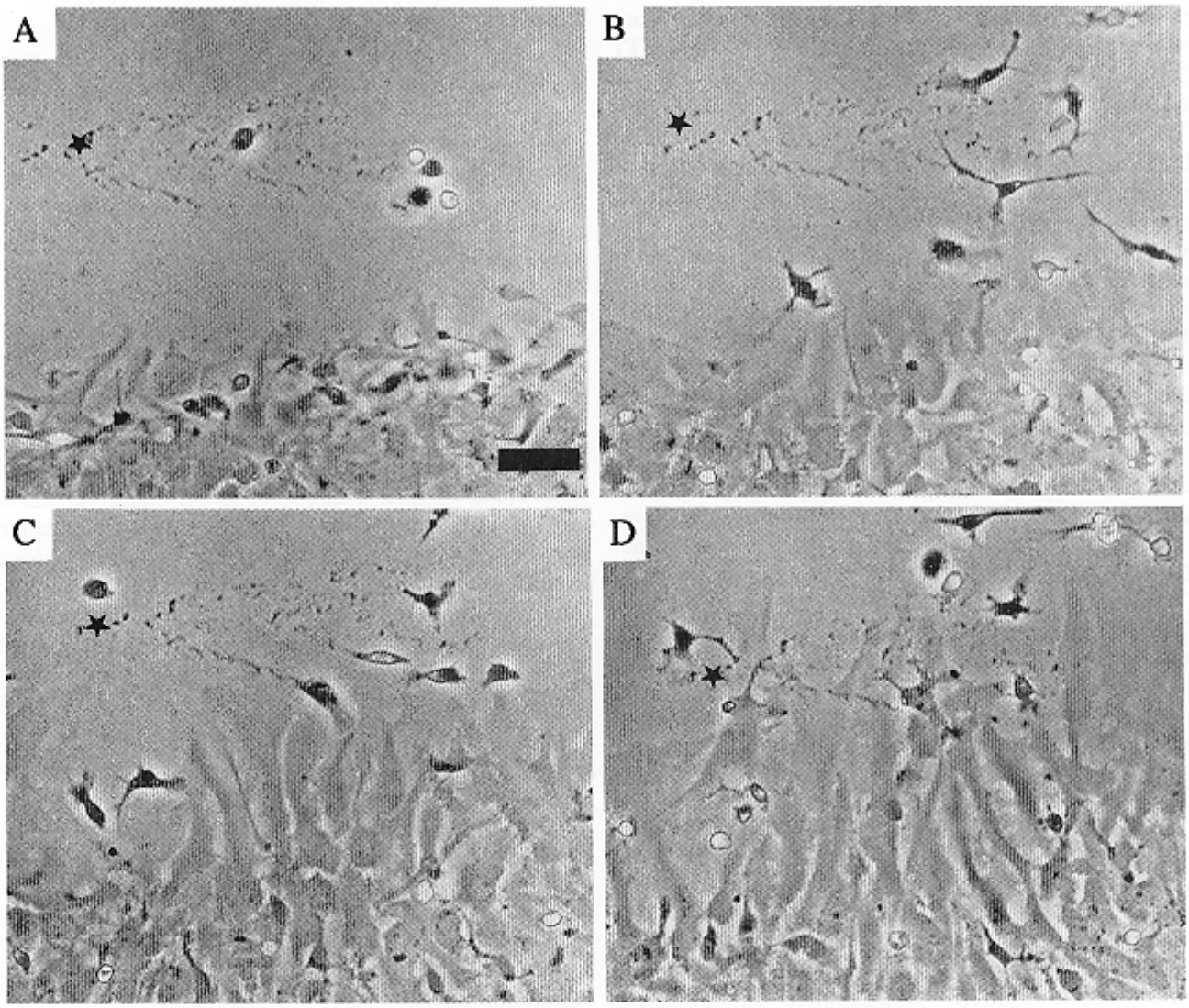

Fig. 1. Phase contrast micrographs of primary culture of astrocytes after scratch-wound. All pictures were taken from the same field. **(A)** 0 hr, **(B)** 6 hr, **(C)** 12 hr, and **(D)** 24 hr after injury. A scratch mark (star) was in the denuded area for reference. In A, there is a swollen cytoplasmic process on the left side of the scratch. All pictures are the same magnification. Bar = 5 μm.

shows that injured astrocytes began to take up more ^{3}H-thymidine 12 hr after injury. The uptake peaked at 1 day and remained high 2 days after injury.

GFAP Content and Effects of Antisense Nucleotides

Cultures injured by scratching had a higher GFAP content, when measured by ELISA, than control cultures without injury throughout the 7 day experimental period (Table I). Injured cultures treated with antisense nucleotides had lower GFAP: total protein ratios than injured cultures with no antisense treatment. All antisense nucleotides tested in this experiment were effective in inhibition of GFAP synthesis in injured astrocytes. The suppressive effect on GFAP content was most obvious on the third day after injury and treatment. The biotin-conjugated oligomers did not show a stronger or longer inhibitory effect in all the conditions studied.

Western blots of similar experiments confirmed the ELISA result. In Figure 5, 3′ oligomers and 3′ oligomer conjugated with biotin (3′-biotin) exerted a stronger and longer inhibitory effect than 5′ oligomers. According to these blots, biotinylation on 3′ oligomer but not 5′ oligomer might improve the consistency and duration of the antisense effect.

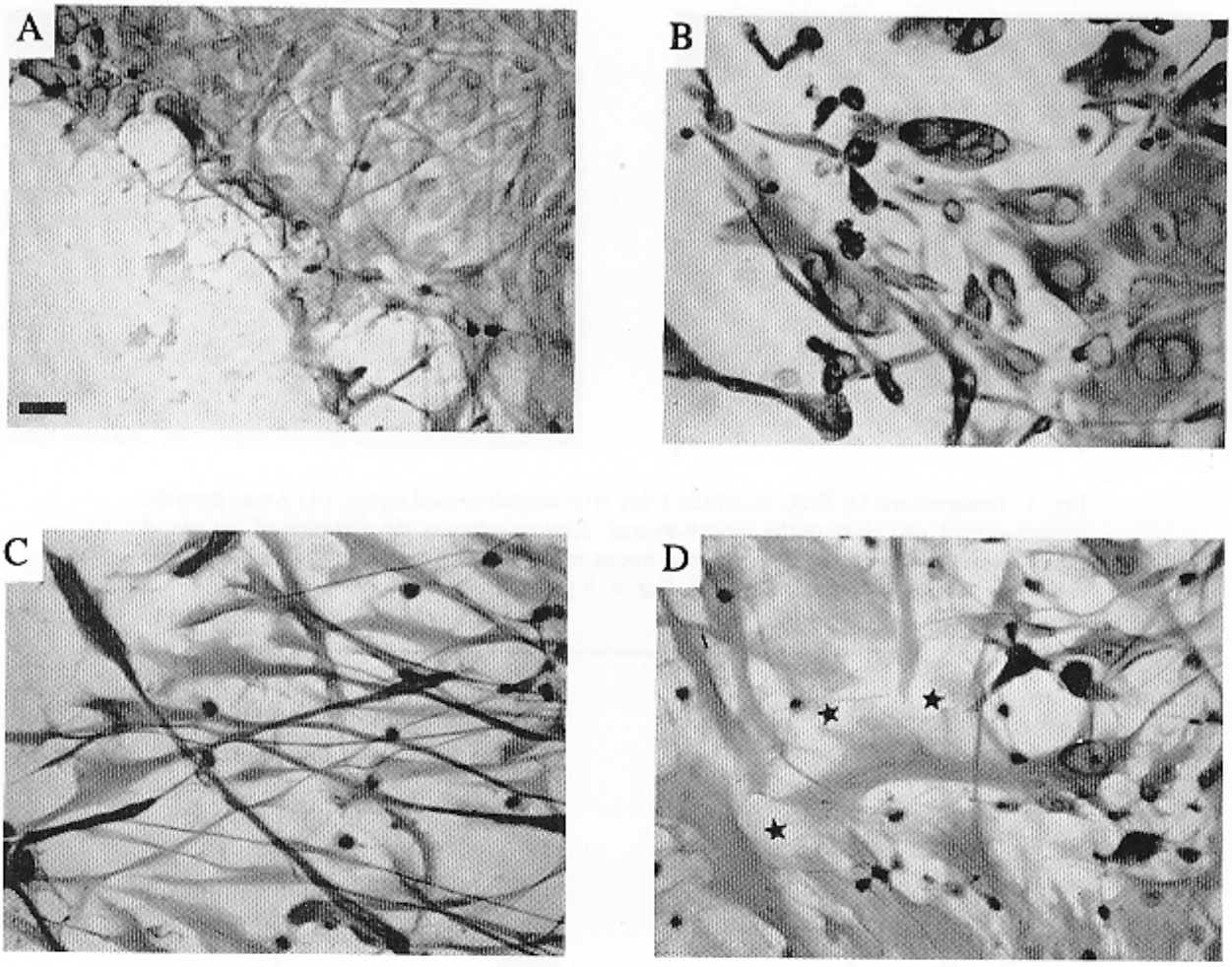

Fig. 2. Immunostain for GFAP in cultures. **(A)** 0 hr, **(B)** 1 day, **(C)** 2 days, and **(D)** 3 days after scratch-wound was performed. Note the multinucleated astrocytes in B. The nuclei in B and D were hypertrophic compared to A. In C, long cytoplasmic processes grew toward the wound and were stained strongly with anti-GFAP. In D, the site of scratch-wound (stars) was filled with hypertrophic cytoplasmic processes of astrocytes. All photographs are the same magnification. Bar = 5 μm.

DISCUSSION

Astrogliosis is a stereotypic response of astrocytes to various types of injury. While gliosis has received considerable attention in terms of its proposed inhibitory effect on CNS repair, there is still very little specific information available concerning what triggers glial reactivity, and many of the cellular dynamics associated with scar formation.

Astrocytes can be activated by numerous factors in response to injury. Some of these factors are anoxia, dilution of inhibitory "chalones" around the site of edema, blood-borne substances, substances derived from infiltrating cells, and mitogenic and non-mitogenic substances from degenerating neurons and fibers. None of these factors can be easily controlled experimentally in intact animals. This makes the evaluation of astrocytic response to injury in intact CNS often difficult as additive and complex interactions are likely to occur. The scratch-wound model established in this study provides a culture system free of neurons for addressing these questions. It is a simple model accurately mimicking the wounds or injuries likely to be experienced by astrocytes in vivo after mechanical trauma. Astrocytes are either irreversibly or transiently disrupted along the scratch. The results clearly demonstrate that astrocytes in scratched cultures are activated. In these cultures, we observed the major characteristics of astrogliosis—hyperplasia, hypertrophy, and increase in immunostainability for and content of GFAP.

Astrocytes are known to become mitotic after in-

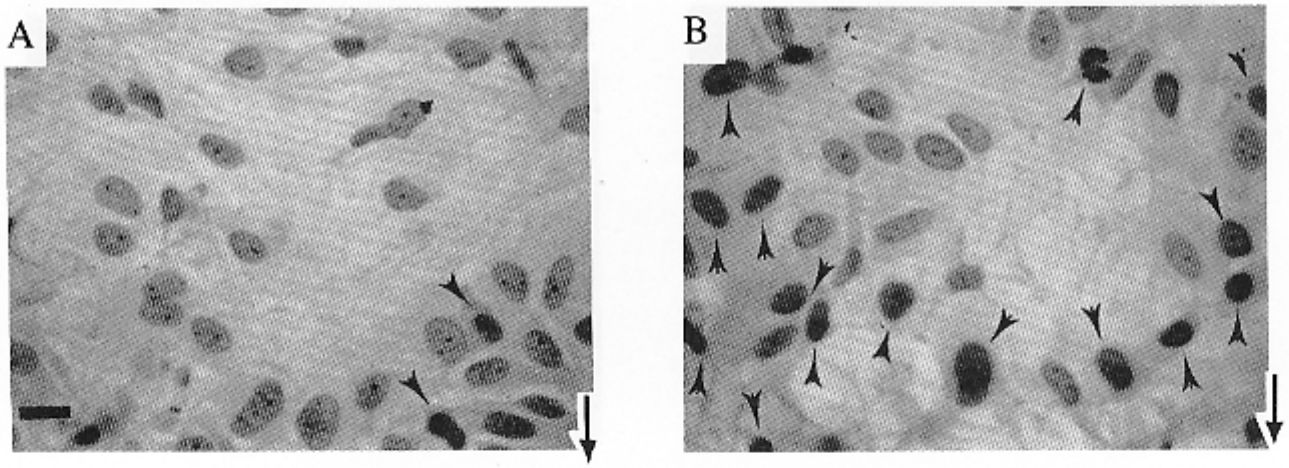

Fig. 3. Immunostain for BrdU in culture 1 day after scratch-wound injury. **(A)** Away from the scratch-wound; **(B)** close to the scratch-wound. Arrows indicates the direction of the site of injury. Note the number of BrdU-stained nuclei in B (arrowheads), indicating more mitotic cells in the area adjacent to the wound. Bar = 5 μm.

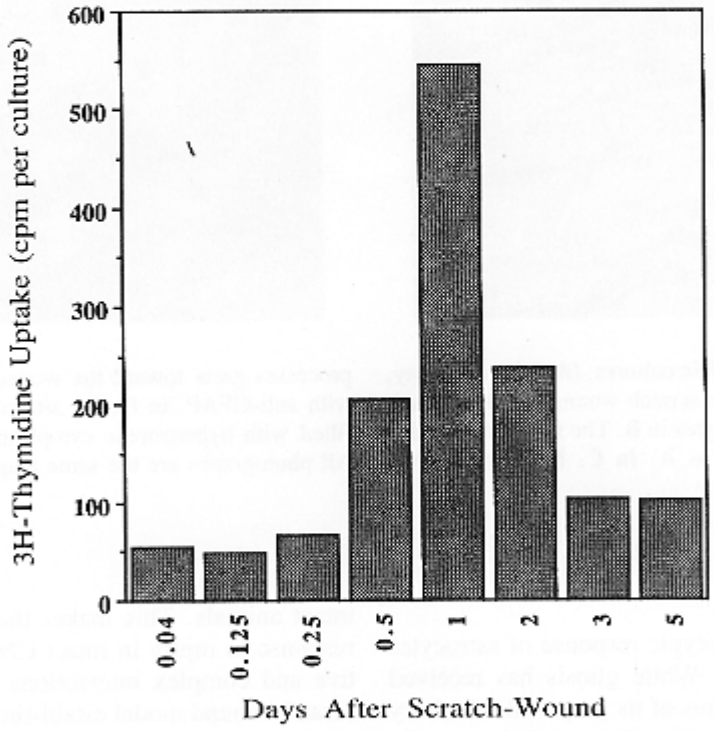

Fig. 4. ^{3}H-thymidine uptake days after scratch-wound in astrocytes.

jury. This has been shown in rats subjected to needle wounds and anoxic encephalopathy (Cavanagh, 1970; Diemer and Klinken, 1976; Latov et al., 1979; Jeneczko, 1988; Miyake et al., 1988; Takamiya et al., 1988; Topp et al., 1989). The large number of mitotic astrocytes next to the wound indicated that damage to the culture stimulated mitotic activity in the astrocytes. The mitotic activity detected in undamaged astrocytes adjacent to the wound indicated that the stimulant was transmitted from the wound to areas near by. The mitotic activity diminished in areas farther away from the wound indicating the stimulation was focal (Latov et al., 1979).

Hypertrophy of nuclei, cell bodies, and cytoplasmic processes is another characteristic of astrogliosis observed next to the scratch damage. It is interesting that all the hypertrophic cytoplasmic processes were directed to-

TABLE I. Effects of Antisense Constructs on GFAP Content in Primary Cultures of Astrocytes After Scratch-Wound Injury*

Conditions	Days after injury				
	0	1	3	5	7
Control	2.57 ± 0.13	1.79 ± 0.39	1.52 ± 0.05	1.61 ± 0.09	1.84 ± 0.16
Injured	3.53 ± 0.23	3.32 ± 0.42	2.86 ± 0.31	2.90 ± 0.15	2.75 ± 0.21
Primers					
3′-Biotin		3.79 ± 0.30	1.45 ± 0.04	1.84 ± 0.38	0.79 ± 0.01
3′ Oligonucleotides		2.70 ± 0.06	1.82 ± 0.35	1.48 ± 0.19	1.30 ± 0.26
5′-Biotin		4.24 ± 0.87	1.94 ± 0.25	2.33 ± 0.31	2.16 ± 0.22
5′ Oligonucleotides		2.58 ± 0.62	1.38 ± 0.06	3.12 ± 0.23	1.08 ± 0.12

*GFAP contents (ng/μg protein ± SD) were measured by ELISA. Data were averaged from two experiments. Antisense constructs were 3′ and 5′ oligonucleotides (20 nt) from the coding region of mouse GFAP. The 3′- and 5′-biotin were nucleotides conjugated to biotin. The sequence for the 5′ oligomers was 5′-GCT CAA TGC TGG CTT CAA GG-3′ and was selected from the initiation site of the mouse GFAP cDNA described in Lewis and Cowan (1985). The 3′ sequence was 5′-GGA GGA GCT CTG CGT TGC GG-3′ from the same cDNA.

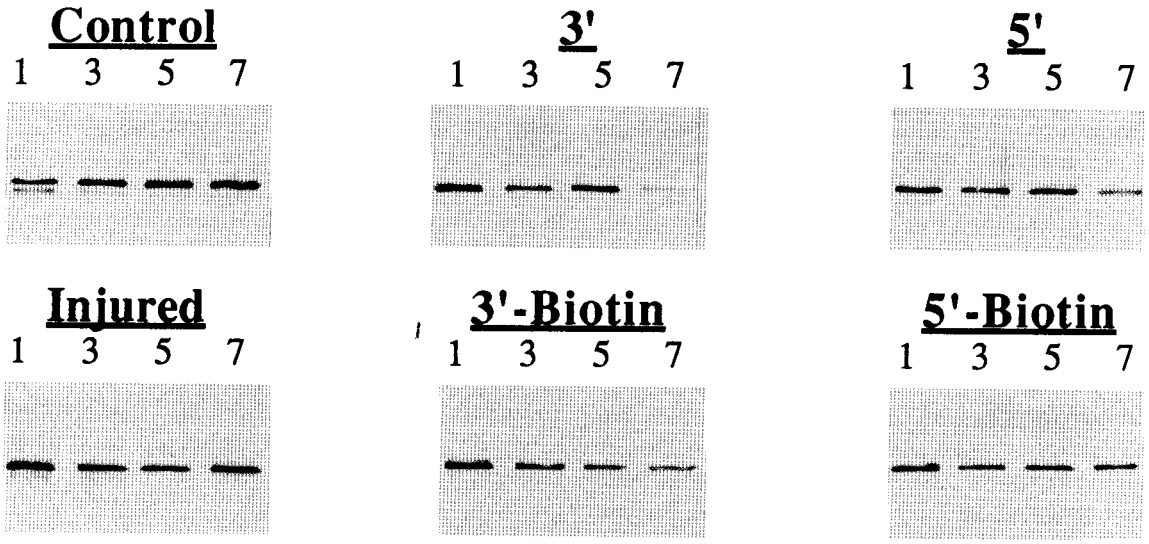

Fig. 5. Western blots of GFAP extracts from astrocyte cultures after scratch-wound. Control: culture without injury. Injured: culture injured by scratch-wound. 3′: injured culture treated with 3′ oligonucleotides (20 nt). 3′-Biotin: injured culture treated with 3′ oligonucleotides (20 nt) conjugated to biotin. 5′: injured culture treated with 5′ oligonucleotides (20 nt). 5′-Biotin: injured culture treated with 5′ oligonucleotides (20 nt) conjugated to biotin. All antisense oligonucleotides were from the coding region of mouse GFAP. **Lanes 1, 3, 5,** and **7** correspond to the number of days after injury. Transfection was done immediately with 48 μg of antisense constructs and 11 μg of Lipofectin® in serum-free DMEM for 3 hr. After transfection, cultures were incubated in medium with 10% FCS throughout the experiment.

ward the scratch-induced denuded area. There was no migration of cell bodies or nuclei into the wound observed in the 7 day experimental period. The directional growth of the processes may be a response of astrocytes to the release from contact inhibition after their neighboring cells were removed.

Whether the growth-promoting activity induced by the scratch was a response to the physical disturbance induced by the scratch or to a local release of growth or mitogenic factors from wounded or disturbed astrocytes requires further investigation. McNeil et al. (1989) has shown that mechanical disruption of plasma membrane of endothelial cells released basic fibroblast growth factor (bFGF). Astrocytes are known to secrete many factors (Hatten et al., 1991; Martin, 1992). Dead, dying, or surviving cells along the wound all rapidly release growth factors such as bFGF at the site of traumatic injury. Therefore, it is very likely that release of growth factors may be through membrane disruption in astrocytes, rather than via the typical, membrane-bound secretory route. Such a mechanism would clearly insure that release is precisely localized to sites requiring the repair processes initiated by the growth factor. Although the culture medium was changed immediately following

the scratch-wound in order to prevent conditioning of medium by debris and factors released from the detached cells, this would not jeopardize the local response described above.

The increase in GFAP content and stainability after scratching may be explained by an increased synthesis of GFAP intermediate filaments (IFs). The functional significance for this increase in IFs is not known. Evidence from studies with rat optic nerve astrocyte cultures suggested that the content and subcellular distribution of IFs are important for cytoplasmic process formation and for structural stability of astrocytes. The relatively slow metabolic turnover rate for GFAP is consistent with such a structural role (Smith et al., 1984; DeArmond et al., 1986). This is further confirmed by Weinstein et al. (1991) who transfected AS2 and AS3 glial cell lines with plasmid DNA which constitutively transcribed an antisense mRNA of the GFAP gene. These cell lines expressed some glial properties but no longer expressed GFAP or extended glial processes in coculture with neurons.

The scratch-wound model, as a method for rapid, reproducible, synchronous, and efficient injury of astrocytes in vitro, appears to closely mimic the mechanical trauma observed in vivo and in vitro. The injury induced by the scratch may be different from injury or cell death induced by metabolic poisoning or hypoxia. Regardless of the method for inducing injury, all astrocytes along the scratch were activated and exerted gliotic behavior. The results have proven that this scratch-damage model in primary culture of astrocytes provides a simple system to examine in more detail the mechanisms involved in triggering glial reactivity and many of the cellular dynamics associated with scar formation. Another important observation from this study is that astrocytes were injured in an environment free of neuronal components, debris, and other systemic interactions. Under such conditions, astrocytes are still capable of exerting many of the gliotic responses observed in vivo. Astrogliosis, therefore, may be a primary astrocytic phenomenon.

We have reported previously that dBcAMP treatment induced an increase in GFAP in cultured astrocytes. Such an increase could be inhibited by antisense GFAP mRNA-liposome complex (Yu et al., 1991). In this study, we were able to demonstrate that a single dose of antisense nucleotide could suppress the increase in GFAP induced by mechanical injury. The effect of inhibition might be prolonged if multiple doses were applied to the injured culture. Antisense nucleotide inhibition of gene expression offers a method for neutralizing normal and harmful genes (Melton et al., 1984; Strickland et al., 1988; Yu et al., 1991; Eng, 1992). The delivery of the oligonucleotides into astrocytes utilized LF (Felgner and Ringold, 1989; Eng et al., 1990; Yu et al., 1991), which we have previously reported to be efficient and non-toxic to astrocytes at the concentration used in this study (Eng et al., 1990). The degree and length of the inhibitory effect of antisense oligomers varied according to the design of the oligomers. The sequence of the oligomers is important for the antisense action and also determines its specificity. In our case, 3′ oligomers seem to be more effective than the 5′ oligomers. The modification of the 3′ oligomers with biotin might slightly enhance its antisense activity. Whether the enhancement was due to improved resistance to nucleases or other mechanisms requires future clarification. The long-lasting but transient effect of the single dose treatment of the antisense oligomers might be due to two phases of action (Yu et al., 1991): a pure antisense effect followed by a slow replenishment of new sense RNA by transcription.

Our working hypothesis is that control of astrocyte proliferation, differentiation, and astrogliosis may be linked to GFAP synthesis. For example, inhibition of GFAP synthesis immediately following injury might delay astrogliosis, thereby giving neurons the opportunity to regenerate and allowing oligodendrocytes to proliferate and remyelinate. The inhibition of GFAP synthesis in injured astrocytes by a single dose treatment of 3′ and 5′ nucleotides and their biotin conjugates was efficient. These findings form an important basis for the ongoing investigation in our laboratory on whether antisense treatment, in addition to inhibiting GFAP synthesis, will modulate other astrogliotic responses such as hypertrophy and hyperplasia.

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