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Quercetin Inhibits c-fos, Heat Shock Protein, and Glial Fibrillary Acidic Protein Expression in Injured Astrocytes

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Quercetin, a bioflavonoid, is found widely in many kinds of fruits and vegetables. It is known to engage in many bioactivities, such as interfering with of the progress of stress responses to injury. In the present study, we investigated the effects of quercetin on some injury responses in primary cultures of astrocytes. These injury responses included the elevation of c-fos protein, heat shock protein (HSP70), and glial fibrillary acidic protein (GFAP). After heat shock insult, the levels of c-fos protein and HSP70 in astrocytes increased. With quercetin treatment, these proteins were significantly reduced. The inhibition of these injury responses by quercetin in astrocytes indicated a dose dependency, with the highest effect at 100 μM. We have previously established a scratch injury model in a primary culture of astrocytes. In that model, astrocytes responded to the scratch injury by an elevation in their GFAP level and formation of hypertrophic cell processes, which extend into the scratch areas. Quercetin treatment reduced the number of hypertrophic cell processes being extended into the scratch areas. With 100 μM of quercetin, there was a complete inhibition of the formation of the hypertrophic cell process. Western blot analysis for GFAP indicated that quercetin significantly reduced the induction of GFAP in the scratch model. At 100 μM, the total GFAP content in the injured cultures was reduced to a level lower than that of the control. This implied that quercetin might possess an antigliotic property. J. Neurosci. Res. 62:730–736, 2000.

Key words: astrocytes; culture; injury; quercetin; GFAP

INTRODUCTION

Quercetin is a bioflavonoid found widely in plants, including many kinds of fruits and vegetables (Graefe et al., 1999), and has been shown to have over a dozen seemingly independent biological effects. Quercetin has been reported to inhibit cell growth (Duthie et al., 1997), glycolysis (Nass-Arden and Breitbart, 1990), lactate release (Volk et al., 1997), nitric oxide production (Soliman and Mazzio, 1998), to block metallothionein mRNA induction (Conklin et al., 1998) and macromolecule synthesis (Huang et al., 1997). It has also been found that quercetin can affect the progress of a stress response to injury by inhibiting the synthesis of heat shock proteins (HSPs; Koishi et al., 1992; Hansen et al., 1997). We investigated whether quercetin produces similar biological effects in injured cells in the central nervous system (CNS).

The astrocyte is a major cell type in the CNS. Astrocytes react to various types of injury and stress with rapid and vigorous astrogliosis (Fawcett and Asher, 1999). Prominent reactive astrogliosis is seen in AIDS dementia, a variety of other viral infections, prion-associated spongiform encephalopathies, inflammatory demyelinating diseases, acute traumatic brain injury, and neurodegenerative diseases such as Alzheimer's disease (for reviews see Forno et al., 1992; O'Callaghan and Miller, 1993; Eng and Ghirnikar, 1994; Unger, 1998). These reactive astrocytes are hyperplastic and possess hypertrophic nuclei, cell bodies, and cytoplasmic processes (Yu et al., 1991, 1993; Fawcett and Asher, 1999). Glial fibrillary acidic protein (GFAP), a major component of the intermediate filament in differentiated astrocytes, is extensively synthesized in astrocytes within and adjacent to the sites of injury (Eng and Ghirnikar, 1994; Wu and Schwartz, 1998). Astrogliosis can help to monitor rapid changes in the molecular and ionic content in the extracellular space (Fawcett and Asher, 1999). These beneficial effects, however, are nullified by the rapid formation of a glial scar that inhibits axonal growth. Therefore, it is essential to develop a means to delay or inhibit scar formation, so that neurons and oligodendrocytes might have an opportunity to reestablish a functional environment and to regenerate.

We have created a scratch wound model to study glial scar formation in a primary culture of astrocytes, in which injured astrocytes exhibited all the major properties

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of astrogliosis, including GFAP accumulation (Yu et al., 1991, 1993; Hou et al., 1995; Eng et al., 1995; Lau and Yu, 2000). Others have used heat shock treatment to study the injury response of astrocytes (Takuma et al., 1996; Xu and Giffard, 1997). In this study, we used heat shock treatment and the scratch wound models to elucidate whether quercetin exerts any effect on the induction of c-fos protein, HSP70, and GFAP in injured astrocytes and thereby to evaluate the potential of quercetin as an antiscar-formation agent for brain injury treatment.

MATERIALS AND METHODS

Cell Cultures

Primary cultures of astrocytes were prepared from 1-day-old newborn ICR mouse brains (Animal Care Center, SHMU, Shanghai, China), following a process described previously (Yu et al., 1989, 1991, 1993; Lau and Yu, 2000; Yu and Lau, 2000). Briefly, the cerebral hemisphere was freed of the meninges and cut into small cubes (<1 mm^3) in DMEM. The tissue was dissociated by vortex mixing for 1.5 min, and the cell suspension was passed through 70 μm and 10 μm sterile mesh nylon filters (Spectrum Medical Industries, Inc., Houston, TX). A volume of cell suspension containing about 4.5×10^5 cells was seeded in a 35 mm Falcon tissue culture dish (Becton Dickinson and Co., San Jose, CA). Fresh DMEM supplemented with 10% fetal calf serum (FCS; Gibco BRL, Grand Island, NY) was added to yield a final volume of 2 ml. Cells were cultured at 37°C in a 5% CO_2 humidified atmosphere inside a CO_2 incubator. The medium was changed twice weekly. Cultures at least 4 weeks old were used for experiments.

Injury Models

The primary cultures of astrocytes were heat shocked by incubation in a water bath at 42°C for 30 min. Immediately after heat shock treatment, the cells were incubated in a 37°CCO_2 incubator for 2 hr. Control cultures were incubated at 37°C.

The scratch wound model was initiated by scratching confluent cultures of astrocytes with sterile plastic pipette tips according to a grid, a process that has been described previously (Yu et al., 1993; Lau and Yu, 2000). Cells along the wound were damaged to various degrees. A control culture without scratching contained 382.4 ± 12.3 μg ($n = 3$) protein. Cultures after scratching contained 221.8 ± 8.4 μg ($n = 3$) protein. Based on these protein values, a reproducible model of 42% scratch damage was established. Immediately following scratching, the culture medium was replaced with serum-free fresh DMEM containing quercetin. Morphological changes after scratching were observed under a Nikon phase-contrast microscope.

Drug Treatment

Prior to the experiment, quercetin (catalog No. 6151-25-3; Sigma, St. Louis, MO) was dissolved in dimethylsulfoxide (DMSO) and sterilized by filtration (0.2 μm; Whatman, Germany). The sterile solution was used for preparing quercetin stock solutions. In the experiments, 2 μl of the stock solutions was added to 2 ml of medium including 10% FCS, which was mixed by vortex before being applied to the culture. The concentrations of quercetin in the treatment medium were 1, 10, 50, and 100 μM. Immediately after scratching or heat shock treatment, the treatment medium was added to the cells and incubated in a CO_2 incubator for a designated time. Control cultures were treated with 2 μl DMSO only. The cell toxicity of quercetin was examined with a Live/Dead® Eukologht™ Viability/Cytotoxicity Kit (Molecular Probes Inc., Eugene, OR). TdT-mediated dUTP nick end-labeling (TUNEL) techniques (In Situ Cell Death Detection Kit Fluorescein from Boehringer Mannheim, Mannheim, Germany) were performed to detect apoptotic cells in cultures treated with quercetin.

Western Blotting

The primary cultures of astrocytes were harvested by dissolving the culture in 1 ml of 0.15% sodium dodecyl sulfate (SDS) in 50 mM phosphate buffer (pH 8.0; Yu et al., 1993). The cells on the dish were scraped off, and the cell suspension was sonicated at 4°C for 5 min. After centrifugation, the cellular extracts (5 μg of protein per lane) were separated by one-dimensional SDS-polyacrylamide gel electrophoresis using 10% polyacrylamide gels. The proteins were transferred to nitrocellulose membranes according to Towbin et al. (1979). The membranes were first incubated with antibodies of GFAP, HSP70, and c-fos for 1 hr at room temperature. After washing, horseradish peroxidase-conjugated anti-mouse IgG (1:2,000) was added, then imaged by the enhanced chemiluminescence (ECL) procedure (Amersham, Arlington Heights, IL). The films were quantified by an Is-1000 image system (Alpha Innotech Corp, CA), and protein levels were expressed as the relative values of the controls.

Statistical Analysis

Data analysis determined that there were means ± SEM in at least four or five separate experiments with at least one culture each. The SPSS 10.0 software package allowed us to run one-way ANOVA and SNK for multiple comparisons among groups.

RESULTS

Cytotoxicity Test

Astrocytes were grown for 4 weeks, and the concentration-dependent cytotoxic effects of quercetin were examined. The results showed that quercetin did not induce any observable morphological changes in astrocytes (Fig. 1A), nor did it cause any death of astrocytes in the 7 days of treatment with 100 μM quercetin, as indicated by the lack of nuclei staining in the culture (Fig. 1B). TUNEL analysis did not indicate any positive staining, further indicating that quercetin did not cause apoptosis in these cells. However, direct addition of 2 μl stock solution, of which the final concentrations were 50 and 100 μM of quercetin, into the culture caused regional cell death after 3 days of incubation. Therefore, it is very important to mix the quercetin well with the incubating medium before adding it to the culture.

Heat Shock Treatment

After heat shock treatment, the levels of c-fos protein in the astrocytes were significantly increased by 2.3-fold

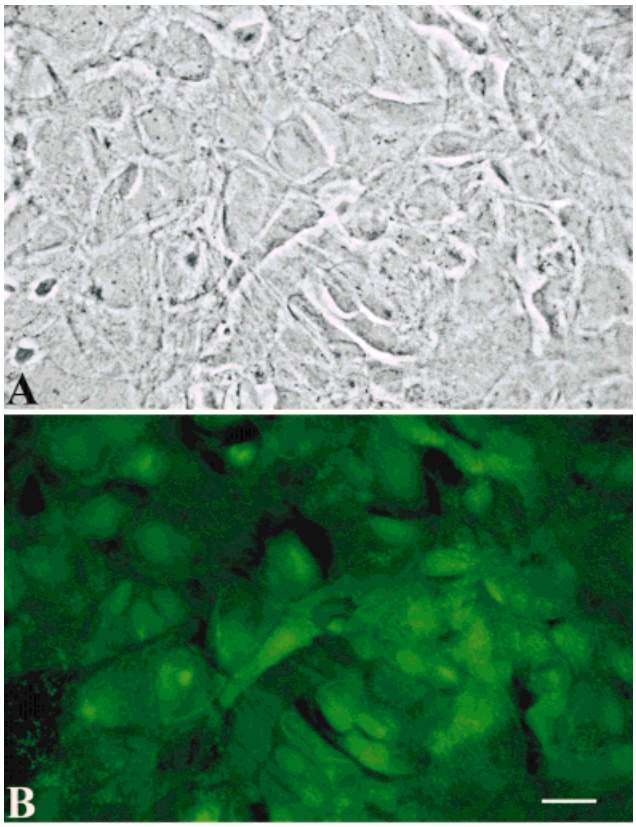

Fig. 1. Cyotoxicity study of primary astrocytes treated with 100 μM quercetin for 7 days. Quercetin was mixed well with the culture medium before being added to the culture. **A:** Phase-contrast micrograph. **B:** Fluorescent micrograph taken after staining with a Live/Dead Cytotoxicity Kit. Scale bar = 8 μm.

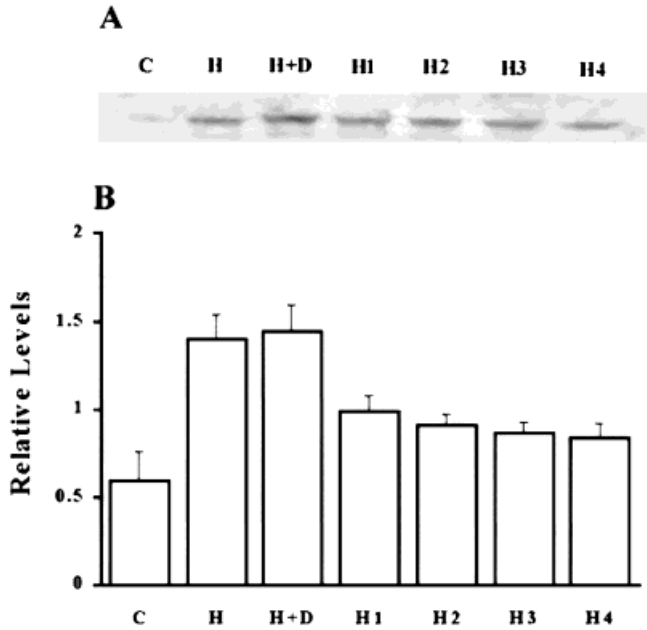

Fig. 2. Inhibition of c-fos expression by quercetin in primary astrocytes under heat shock treatment. **A:** Sample Western blot analysis. **B:** Statistical analysis of densitometric readings from four separate Western blots. C, control without heat shock treatment; H, control with heat shock treatment; H+D, with heat shock treatment and the addition of DMSO; H1, H2, H3, H4, with heat shock treatment and the addition of 1, 10, 50, and 100 μM quercetin, respectively.

over the experimental controls both with and without DMSO (Fig. 2). Quercetin significantly inhibited the heat shock-induced c-fos protein expression at all the concentrations studied. The inhibition ranged from 29% with 1 μM, to 35% with 10 μM, to 37% with 50 μM, and to 45% with 100 μM of quercetin. The levels of c-fos protein in the treated cultures were always higher than in the controls without heat shock treatment, indicating that c-fos protein induction by heat shock treatment could not be completely inhibited at the concentrations studied.

The Western blot analysis (Fig. 3A) showed that heat shock treatment significantly induced an increase in heat shock protein content. In the control with DMSO, the amount of heat shock protein was elevated but not to as high a level as in the heat shock control. Quercetin treatment reduced the band intensity (Fig. 3A). Densitometric measurement of the bands showed that the HSP70 content in heat-shocked astrocytes was 1.4 times that in astrocytes that had not undergone heat shock treatment (Fig. 3B). In all the quercetin treatments, the HSP70 content was not elevated by the heat shock treatment. Quercetin at 1, 10, 50, and 100 μM was completely able to prevent heat shock induction of HSP70. The HSP70 content in cultures treated with quercetin was statistically significantly inhibited compared to that in the experimental controls. Quercetin at 100 μM was very effective in inhibiting the induction, and the reduction was 60% of the level in the control with no heat shock treatment and 45% of the level in the control with heat shock treatment.

Scratch Wound Model

The morphology of cells along the wound 4 days after scratching with DMSO treatment (Fig. 4A) or without DMSO treatment (Fig. 4B) did not show any observable differences. Many hypertrophic cell processes migrated into the scratch area. In cultures treated with 1 (Fig. 4C), 10 (Fig. 4D), and 50 μM (Fig. 4E) of quercetin, the hypertrophic processes migrated into the denuded area, as in the control cultures (Fig. 4A,B). There was an obvious difference in the duration and the area covered in the quercetin-treated cultures, with shorter processes covering smaller areas in cultures treated with higher concentrations of quercetin. The most distinct inhibition of the hypertrophic process formation was observed in cultures treated with 100 μM of quercetin (Fig. 4F). There was practically complete inhibition of the hypertrophic process formation in astrocytes along the scratch.

The Western blot analysis demonstrated that the scratch significantly induced a slight but significant increase of 20% in the GFAP content in scratch cultures

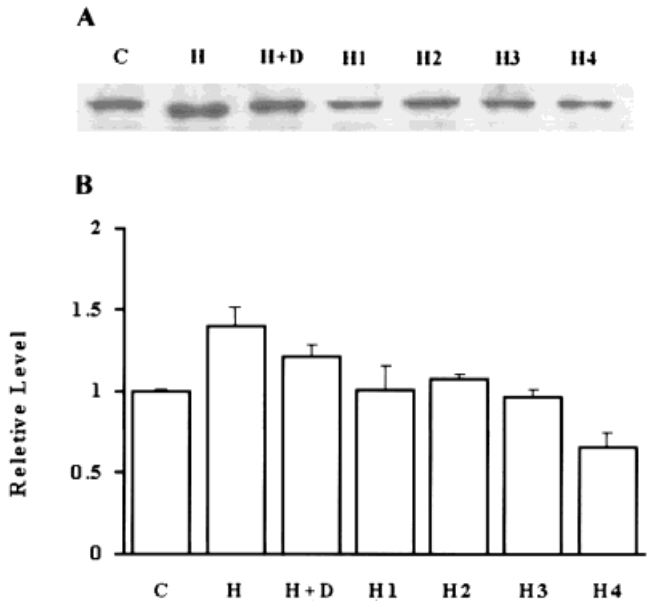

Fig. 3. Inhibition of HSP70 synthesis by quercetin in primary astrocytes under heat shock treatment. **A:** Sample Western blot analysis. **B:** Statistical analysis of densitometric readings from four separate Western blots. C, control without heat shock treatment; H, control with heat shock treatment; H+D, with heat shock treatment and the addition of DMSO; H1, H2, H3, H4, with heat shock treatment and the addition of 1, 10, 50, and 100 μM quercetin, respectively.

both with and without the presence of DMSO (Fig. 5). Treatment with 1 and 10 μM of quercetin did not show any detectable inhibition of GFAP induction. Quercetin at a concentration of 50 μM completely inhibited the GFAP induction by the scratch, holding the GFAP to the same level as in the cultures without a scratch. At 100 μM of quercetin, the amount of GFAP was drastically and significantly reduced by 42% compared to the control scratch cultures. This amount of GFAP was 30% lower than the amount in the control culture without a scratch.

DISCUSSION

Studies have shown that quercetin induces cytotoxicity in tumor cells (Shi et al., 1995) and the induction of apoptosis in leukemic cells (Larocca et al., 1997), HL-60 cells (Xiao et al., 1997), as well as liver cells after partial hepatotectomy (Iwao and Tsukamoto, 1999). In this study, there was no detectable astrocyte death at up to 7 days of exposure in cultures with the highest concentration of quercetin studied. The TUNEL study did not show the induction of apoptosis in the astrocytes. Therefore, the observed inhibitory effect of quercetin was a functional event and was not due to cytotoxicity.

Immediate early gene (IEGs) products such as c-fos protein and HSPs are known to be reliable stress indicators in cells after injury (Lowenstein, 1995; Herrera and Robertson, 1996; Sharp et al., 1999). There have not been many reports on the effect of quercetin on c-fos expression. Only recently, Iwao and Tsukamoto (1999) showed that quercetin induced an increase in c-fos mRNA levels in liver after partial hepatotectomy. The observed inhibition of c-fos protein induction in this study was contradictory to the finding of Iwao and Tsukamoto (1999). If quercetin produces a similar effect in astrocytes and liver cells in c-fos expression, quercetin must enhance the c-fos mRNA expression but inhibit the translation of the c-fos mRNA into the protein. Another explanation for the difference in c-fos expression between astrocytes and liver cells is due to the variation in the action of quercetin among cell types, as was suggested by Hansen et al. (1997).

HSP70 protects cells from various injurious stimuli and is important in cell growth and differentiation (Sharp et al., 1999). Quercetin inhibits the heat-induced synthesis of HSPs in a variety of cell lines (Hansen et al., 1997; Morino et al., 1997; Kudo et al., 1999) by inhibiting the heat shock factor (HSF; Nagai et al., 1995). Quercetin has been known to hinder the thermotolerance and ischemic tolerance of the CNS (Koishi et al., 1992; Nakata et al., 1993). Quercetin similarly inhibited HSP70 in astrocytes. This inhibition of HSP70 might lower the tolerance of astrocytes to injury (Takuma et al., 1996; Xu and Giffard, 1997). Whether the inhibition of c-fos protein and HSP70 by quercetin was harmful or beneficial to astrocytes and the CNS requires further clarification.

We have previously shown that scratching upregulates the expression of the mRNAs of IEGs, HSPs, and cytoskeletal proteins (Eng et al., 1995) in astrocytes. A similar observation has been made in astrocytes under ischemic insult (Yu et al., 1995). Among all the changes in astrocytes under injury, GFAP should be the most specific and significant marker. We have shown that astrocytes after scratch injury increase their GFAP content (Yu et al., 1993). We have also shown that GFAP synthesis could be inhibited by antisense oligos treatment (Yu et al., 1991, 1993; Ghirnikar et al., 1994). The GFAP induction by the scratch was inhibited by the presence of 50 μM of quercetin. Quercetin at 100 μM not only abolished the scratch-induced GFAP but also decreased the GFAP to a level lower than that of the control. This indicated that quercetin can attenuate GFAP synthesis and even disassemble GFAP. It is known that quercetin inhibits many kinases (Agullo et al., 1997). We suspect that quercetin might inhibit some kinases that are necessary for GFAP synthesis and result in the alteration of the phosphorylation of specific proteins, thus affecting the structure and/or function of intermediate filament proteins.

The reduction in GFAP content might also be explained by the inhibition of the GFAP decaying pathway by quercetin. Chiu and Goldman (1984) showed biphasic decay kinetics in the GFAP pool. One is a fast-decaying pool with a half-life of 12–18 hr, and the other is a stable pool with a half-life of 8 days. In this study, the longest treatment using quercetin was 4 days. If quercetin has an affect on this pathway, it ought to be on the fast-decaying pool. Shi et al. (1995) found that quercetin inhibits tubulin polymerization in some cancer cells. Kudo et al. (1999) showed that quercetin delayed the reorganization of fila-

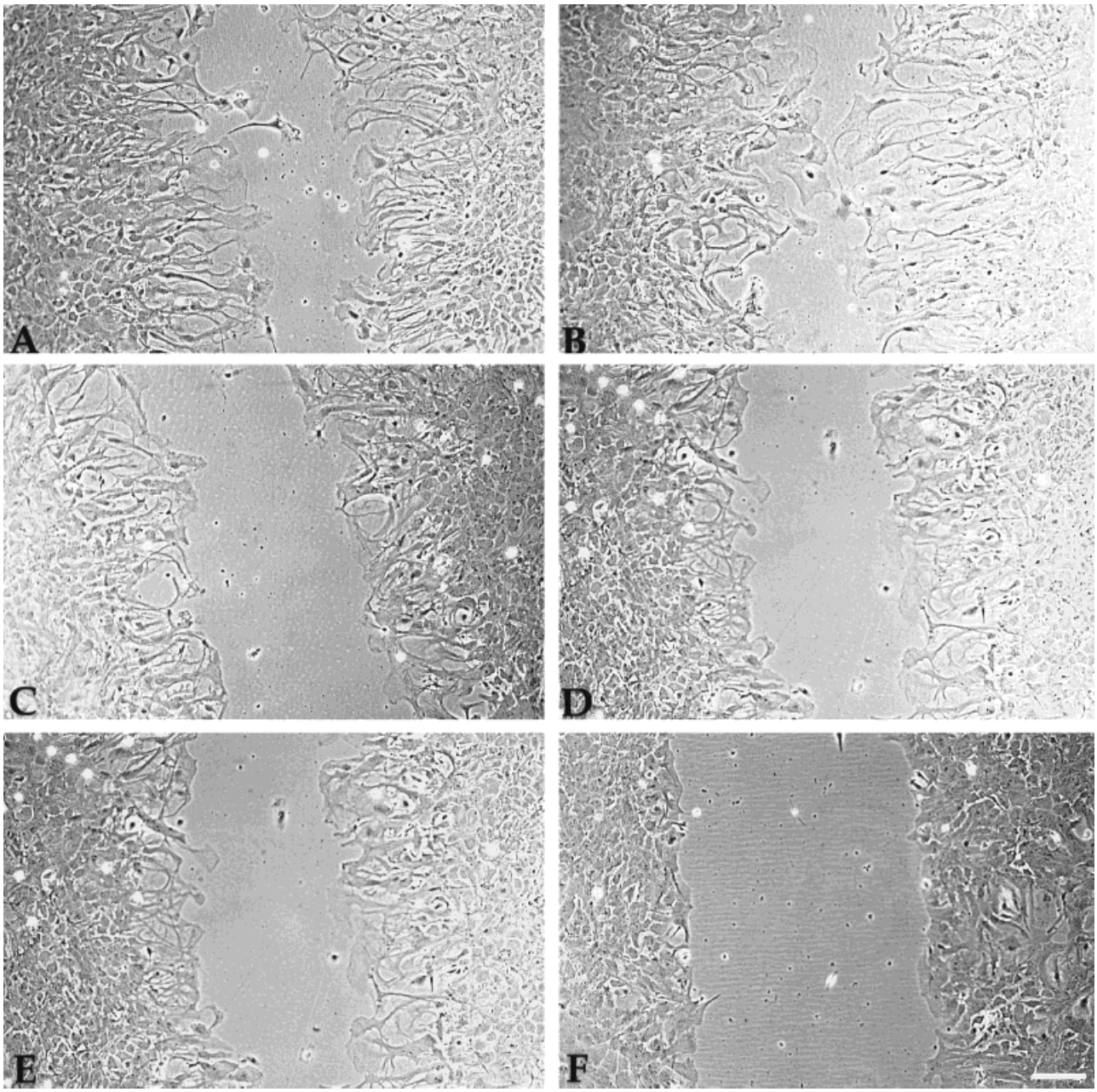

Fig. 4. Morphological changes of astrocytes in a primary culture of astrocytes after scratch wound and quercetin treatment. Quercetin was added to the culture immediately after scratching and the treatment lasted 4 days. **A:** Control with a scratch wound. **B:** Scratch wound and the addition of DMSO. **C–F:** Cultures with a scratch wound and the addition of 1, 10, 50, and 100 μM of quercetin, respectively. Scale bar = 10 μm.

mentous actin during the recovery period after heat shock. Therefore, it is not surprising that quercetin was able to affect GFAP metabolism. These effects of quercetin on cytoskeletal proteins would explain the lack of cytoplasmic processes being sent into the scratch area observed in this study.

In the adult CNS, one of the most remarkable characteristics of astrocytes is their vigorous response to diverse neurologic insults. The hallmark of this response, often called *reactive gliosis*, is the enhancement of GFAP content. Successful inhibition of GFAP using antisense oligonucleotides has also been reported by Yu et al. (1991, 1993), Ghirnikar et al. (1994), and Lefrancois et al. (1997); however, there are many limitations in the clinical use of antisense RNA treatment resulting from the difficulty of delivery to the site of injury in the CNS. Previous studies

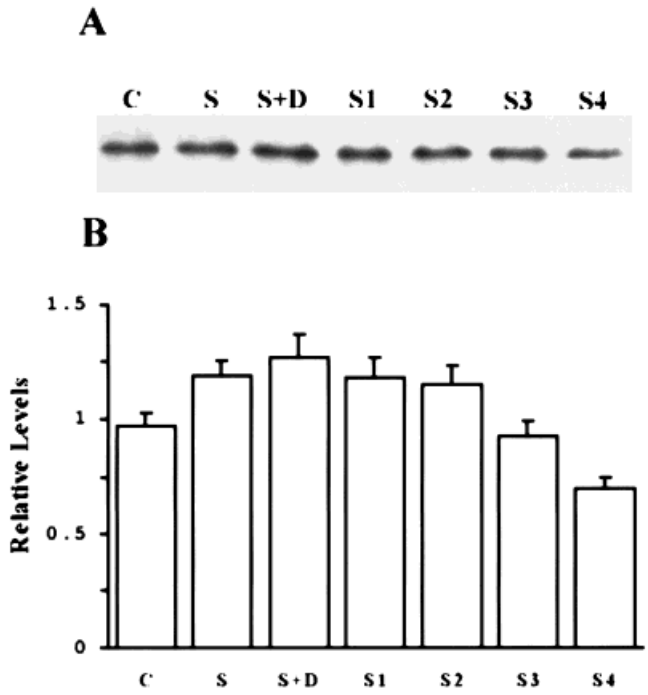

Fig. 5. Inhibition of GFAP synthesis by quercetin in primary astrocytes after scratch injury. **A:** Sample of the Western blot analysis. **B:** Statistical analysis of densitometric readings from five separate Western blots. C, control culture; S, culture with scratch wound only. S+D, with scratch wound and the addition of DMSO. S1, S2, S3, S4, with scratch wound and the addition of 1, 10, 50, and 100 μM quercetin, respectively.

showed that the inhibition of GFAP synthesis with antisense GFAP mRNA not only inhibited astrogliosis but also relieved the blockade of neuritic outgrowth (Lefrancois et al., 1997). The inhibitory effect of quercetin on GFAP synthesis might make quercetin a potential drug in preventing the formation of an astrocytic scar after brain injury. Therefore, quercetin might be able to be used as an astrogliosis inhibitor. This is a first report on the quercetin effect on astrogliosis- and injury-induced c-fos protein, HSP70, and GFAP expression.

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