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Astrogliosis in Culture. IV. Effects of Basic Fibroblast Growth Factor

Y.-J. Hou, A.C.H. Yu, J.M.R.Z. Garcia, A. Aotaki-Keen, Y.-L. Lee, L.F. Eng, L.J. Hjelmeland, and V.K. Menon

Departments of Cell Biology and Human Anatomy (Y.-J.H., J.M.R.Z.G., V.K.M.), Biochemistry and Biophysics (A.A.-K., L.J.H.), University of California, Davis, and Department of Pathology, V.A. Medical Center, Palo Alto, and Stanford University School of Medicine, Stanford (A.C.H.Y., Y.-L.L., L.F.E.), California

Previous studies have shown that the mechanical wounding of 3-week-old cultured rat astrocytes results in cell proliferation and hypertrophy resembling astrocyte responses to a brain injury in vivo. We now report the effects of basic fibroblast growth factor (bFGF) and an anti-bFGF antibody on astrocyte morphology, proliferation, and migration following in vitro wounding of confluent secondary cultures. Addition of bFGF (20 ng/ml) to wounded cultures induced morphological changes characteristic of differentiation in wounded and nonwounded areas of the culture. Combined treatment with bFGF and an anti-bFGF antibody (100 μg/ml) prevented this effect. Astrocyte proliferation along the edges of a scratch wound was at maximum 24 hr after wounding in cells growing in Eagle's minimum essential medium (EMEM) containing 10% serum. Low serum concentration and treatment with dibutyryl cyclic adenosine monophosphate (dbc-AMP) reduced injury-associated astrocyte proliferation. Addition of bFGF to cultures in EMEM with serum increased astrocyte proliferation at 18 and 24 hr after wounding. This effect was reduced considerably by treatment of cultures with bFGF in combination with an anti-bFGF antibody. The combined treatment and the antibody alone reduced cell division to a level lower than in control cultures. Twenty-four hr following wounding, astrocytes along the edges of the wound exhibited extension of thick, flat processes into the wound area. At 3 and 5 days after wounding, a bodily migration of astrocytes into the wounded area was observed. Addition of bFGF significantly increased astrocyte migration 1 day after wounding, with maximum effect on day 3 and no subsequent increase on day 5. A combination of bFGF and anti-bFGF antibody as well as the antibody alone reduced astrocyte migration to a level lower than in controls. Immunohistochemical localization and isoform pattern of bFGF in astrocytes did not change with dbc-AMP treatment or wounding. We conclude that mechanically wounded confluent astrocytes respond to bFGF added to the culture medium by enhancing cell division, differentiation, and migration. In addition, the results of the antibody treatment also suggest a role for endogenous bFGF in astrocyte proliferation and migration elicited by wounding in vitro. These results support the notion that in vivo, both bFGF released by injury and endogenous bFGF synthesized by astrocytes, contribute to the cellular responses that lead to astrogliosis.

Key words: glia, trauma, culture, astrogliosis

INTRODUCTION

Several studies in the past have shown that the protein level and the message for basic fibroblast growth factor (bFGF) increase at the site of a traumatic injury to the rat brain (Finklestein et al., 1988; Frautschy et al., 1991; Logan et al., 1991, 1992). In vitro, bFGF induces both proliferation and differentiation of rat astrocytes (Hatten et al., 1988; Kniss and Burry, 1988; Perraud et al., 1988, 1990; Sensenbrenner et al., 1990; Loret et al., 1991; Petroski et al., 1991). Thus, it is reasonable to presume that bFGF is one of the many growth factors that mediate astrocyte responses to brain injury and the consequent astrogliosis in vivo. Neurons, astrocytes, and endothelial cells are known to contain bFGF (Ferrara et al., 1988; Emoto et al., 1989; Ernfors et al., 1990; Woodward et al., 1992; Gómez-Pinilla et al., 1992). Damage to these cells is likely to release bFGF into the extracellular space where it can interact with astrocytes to elicit injury responses. In addition, macrophages,

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Address reprint requests to Dr. V.K. Menon, Dept. of Cell Biology and Human Anatomy, School of Medicine, University of California, Davis, CA 95616.

have different affinities for the molecule (Matsuzaki et al., 1989). Type I antibody is believed to recognize the native form of bFGF whereas type II detects both the native and denatured forms of the molecule (Matzusaki et al., 1989). We have previously used both antibodies equally successfully for immunohistochemical localization of bFGF in cultured rat astrocytes (Vijayan et al., 1993). Since the crossreactivity of these antibodies with bFGF and other members of the FGF family has not yet been described, a dot-blot analysis was carried out (see below). In our initial testing of the antibodies in Western blot analysis and immunoneutralization, type II antibody proved to be far superior to type I. For this reason, type II antibody was consistently used for all procedures.

Treatment of Cultures

Immediately after wounding, cultures were treated in one of the following ways: 1) 20 ng/ml bFGF (human recombinant bFGF, Upstate Biotechnology, Lake Placid, NY); 2) 100 μg/ml type II anti-bFGF antibody; 3) 20 ng/ml bFGF and 100 μg/ml type II anti-bFGF antibody; 4) no test agent, as a control; and 5) 100 μg/ml mouse IgG_1 (Sigma) as a control for anti-bFGF antibody. Culture medium used for these treatments was EMEM with 10% FBS except in studies on astrocyte migration, where test agents were added to Ham's F-12 medium. This choice was based on our finding that astrocyte migration was low in Ham's F-12 medium and that the low baseline levels provided optimum detection of the effects of bFGF. The doses of bFGF and anti-bFGF antibody chosen were based on previous studies (Kniss and Burry, 1988; Perraud et al., 1988, 1990; Loret et al., 1991; Sato and Rifkin, 1988). Test agents were added at each medium change.

Bromodeoxyuridine (BrdU) Uptake for Detecting Cell Division

Proliferative activity was assessed by the ability of astrocytes to incorporate BrdU, a thymidine analog, as described by Young and Kim (1987). Briefly, cells on coverslips and 35-mm petri dishes were incubated in medium containing 10 μM BrdU for 2.5 hr at 37°C. BrdU-containing medium was then removed and fresh medium without BrdU was added for 15 min. Cells were washed three times in phosphate-buffered saline (PBS), fixed, and used for BrdU/GFAP double immunofluorescence labeling.

Immunohistochemistry

Immunohistochemical stainings for GFAP, BrdU, and bFGF were carried out as previously described (Vijayan et al., 1993; Yu et al., 1993). For GFAP, a polyclonal antibody (Dako Corp., Santa Barbara, CA) was used at 1:100 dilution. A monoclonal anti-BrdU antibody (Dako) was used at 1:75 dilution, following denaturation of native DNA with 1 N HCl for 30 min at 37°C. Type II anti-bFGF antibody was used for bFGF staining at 1:50 dilution.

Controls for immunohistochemistry were prepared by incubating fixed cultures in the blocking buffer omitting the primary antibody, in normal rabbit serum in place of polyclonal antibodies, or mouse IgG_1 in place of monoclonal antibodies. The latter two were used at concentrations equivalent to that of the primary antibody. In addition, for bFGF, controls were also prepared by adsorbing the antibody with bFGF and removing the antigen-antibody complexes using heparin-sepharose beads as previously described (Vijayan et al., 1993).

Quantification of Cell Division and Cell Migration

Cell division was quantified by counting the number of BrdU-positive cells in 10 random fields along the edge of the wound at 18, 24, 48, and 72 hr after wounding. The data from each time period were averaged and divided by the number of BrdU-positive cells in control cultures.

Cell migration was measured according to the method of Sato and Rifkin (1988). Briefly, injured cultures with or without treatment were stained for GFAP and observed under a light microscope with an ocular grid fitted in the eyepiece. Migration was quantified by counting the number of cells in several (1–8) successive 100-μm × 400-μm zones away from the wound edge. This number was normalized by expressing it as a percentage of cells counted in an equivalent area of non-wounded sister cultures.

Western Blot Analysis of bFGF

Heparin-sepharose extracts of cultured astrocytes were prepared according to Ishigooka et al. (1992). Briefly, astrocytes were sonicated in an FGF extraction buffer (2 M NaCl, 20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% deoxycholic acid, 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A) at a dilution of 1 ml/10-cm petri dish. Sonicate was diluted 8-fold to reduce the salt and detergent concentrations. Previously washed heparin-sepharose (Pharmacia LKB Biotechnology, Piscataway, NJ) was added to the sonicate (approximately 3% v:v), and the mixture was agitated overnight at 4°C to absorb FGFs. After washing sequentially in 10 mM Tris, pH 7.6, and 1 M NaCl, and in Tris buffer only, beads were boiled in an equal volume of 2 × sample buffer to release bound FGFs. Beads were spun down, and the supernatant was used as the sample for Western blot analysis.

SDS-PAGE and Western blot analysis were carried out as described by Ishigooka et al. (1992), and blots

were stained with type II ant-bFGF antibody at 1:100 dilution (5 μg/ml). Heparin-Sepharose extracts were used at 15–20 μl volumes, with the volume of samples collected from wounded cultures adjusted so that all lanes contained an equivalent number of cells (5×10^4). Recombinant human bFGF (25–50 ng) was used as the standard. Low molecular weight markers (Bio-Rad Laboratories, Richmond, CA) were used at 6 μl per lane.

Dot-Blot Analysis

Dot-blot analysis was carried out to establish the crossreactivities of type I and type II anti-bFGF antibodies with bFGF, acidic FGF (aFGF), and fibroblast growth factor 5 (FGF-5). This was essential since bFGF shares approximately 55% and 43% structural homology with aFGF and FGF-5, respectively (Benharroch and Birnbaum, 1990). Recombinant human bFGF was obtained from R and D Systems (Minneapolis, MN) and from Upstate Biotechnology (Lake Placid, NY). Recombinant aFGF was from Upstate Biotechnology. Recombinant FGF-5 was expressed in *E. coli* and purified as previously described (Ishigooka et al., 1992). Samples of FGFs (1–100 ng) were dotted directly on nitrocellulose and the blots were processed in the same way as Western blots. Both type I and II anti-bFGF antibodies were used for staining at 1:500 dilution (1 μg/ml).

Statistical Analyses

One-way analysis of variance was done for establishing the significance of observed differences between means for various treatment and time points. Post hoc comparisons were carried out using the Fisher PLSD test. All analyses were done using the StatView 512 program on a Macintosh computer. Statistical significance was set at $P<0.05$. Data from a minimum of three separate cultures were used for all analyses.

RESULTS

Cultures

The purity of secondary astrocyte cultures was established by immunocytochemical staining for GFAP. GFAP-positive astrocytes constituted 90–95% of the total cells and appeared as flat and undifferentiated cells with few or no processes. Addition of dbc-AMP to the cultures induced striking morphological changes, converting the majority of flat protoplasmic-type astrocytes into process-bearing cells (not shown), as previously described by others (Shapiro, 1973; Sensenbrenner et al., 1980; Federoff et al., 1984; Shain et al., 1987).

Effects of Mechanical Injury

Astrocyte morphology. In 24-well cultures, astrocytes bordering the wound demonstrated an extension of cell processes into the wounded area at 24 hr after wounding (Fig. 1A). These processes appeared to be flat and spread out with no discernible changes in the morphology of the cell body. Wound area was devoid of cells at this time. By day 3, many astrocytes could be detected in the wound area, and these cells appeared to have migrated from the wound edge into the denuded zone (Fig. 1B). This bodily migration of cells was even more apparent by day 5, when migrated astrocytes covered a substantial portion of the wound area (Fig. 1C). Migrated astrocytes exhibited morphological changes, characterized by smaller cell bodies and thin, compact, and branched processes, and were randomly organized within the wound area.

Astrocyte proliferation. In confluent monolayers of astrocytes wounded with a pipette tip to remove a narrow zone of cells along the middle, or with a razor blade to remove half of the cells, injury-induced proliferative changes were demonstrated throughout the culture but were more dramatically evident in cells remaining along the borders of the wound. In control cultures grown in EMEM and serum, during the first 6 hr after wounding, no changes in the numbers of BrdU-positive cells were detected along the wound edges, compared to the time of wounding. At 12 hr following injury, more BrdU-positive cells appeared at the edges of the wound, and their number increased at 18 hr (24.54/mm^2 at wound edge vs. 13.18/mm^2 in nonwounded areas), with the maximum cell division occurring at 24 hr after wounding (72.72/mm^2 at wound edge vs. 26.13/mm^2 in nonwounded areas; Fig. 2A). At 48 hr, the number of dividing cells declined (28.02/mm^2 at wound edge vs. 11.17/mm^2 in nonwounded areas), and at 72 hr after wounding, the number had returned to that at 12 hr postinjury (Fig. 2A). Cultures grown in EMEM with 10% serum had the highest numbers of BrdU-labeled cells at all times after wounding. Cells grown in EMEM with 0.25 mM dbc-AMP or in Ham's F-12 medium also showed a temporal pattern of cell division along the wound edges similar to cells in EMEM, but the number of dividing cells was considerably lower at all times (Fig. 2A). The values for dbc-AMP-treated cultures and cultures grown in Ham's F-12 medium represented 14.79% and 4.68%, respectively, of the values obtained for cells in EMEM at 24 hr after wounding.

Astrocyte migration. Cell migration assay showed that with time, the number of cells migrating into the denuded area, and the distance moved by cells from the edge of the wound, progressively increased (Fig. 3A–C). Cells in EMEM exhibited the highest rate and distance of migration with maximum increase on day 5. Thus, on day 1, the mean numbers of cells detected in the first and second 100-μm × 400-μm zones were 12.75 and 2.0, respectively. On day 5, the corresponding num-

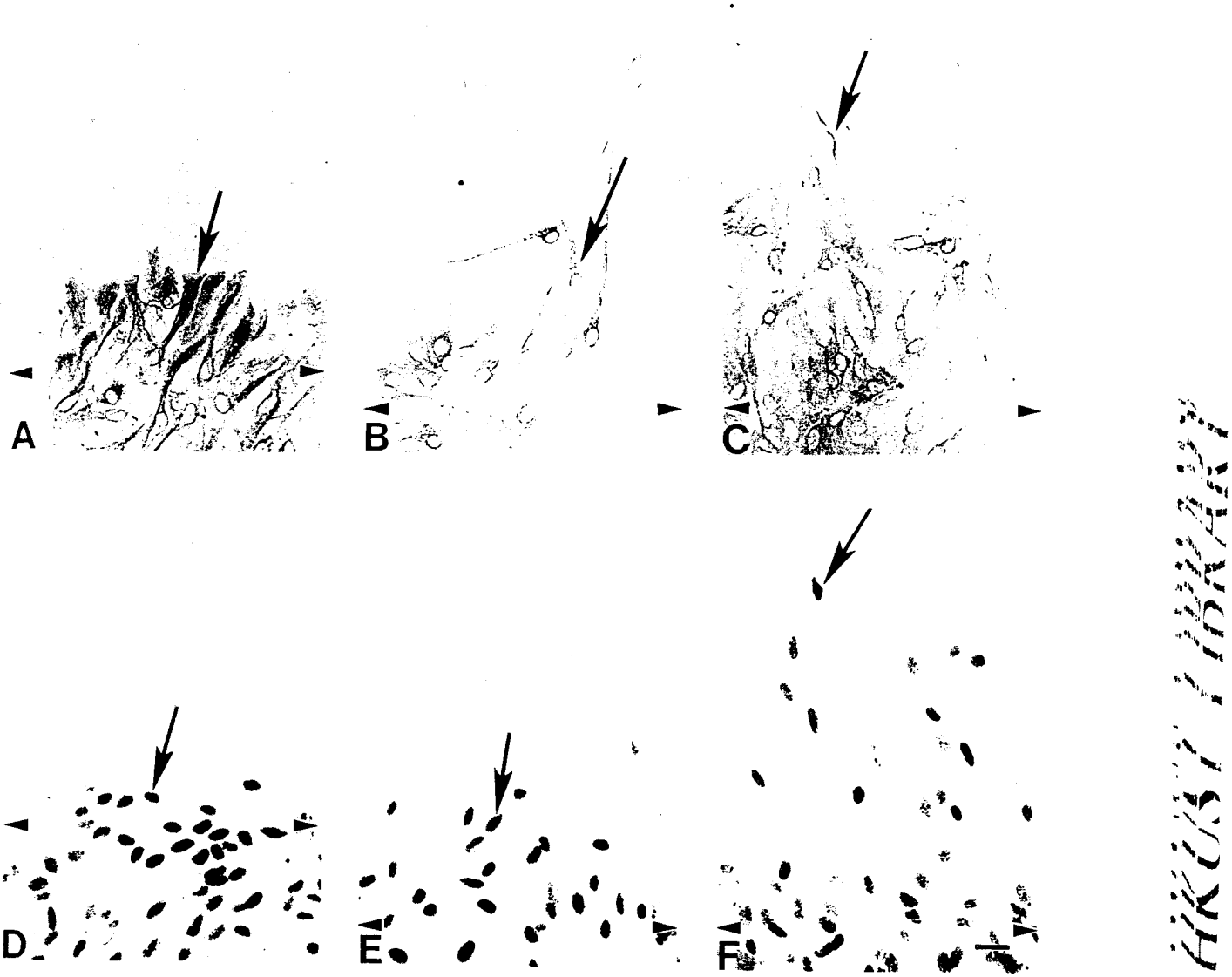

Fig. 1. GFAP (**top**) and bFGF (**bottom**) staining of mechanically wounded confluent cultures of astrocytes at 1 (**A,D**), 3 (**B,E**), and 5 (**C,F**) days after wounding. Arrowheads indicate the wound edge. On day 1 (A), astrocytes along the wound edge exhibited thick flat processes extending into the denuded area (arrow). On days 3 (B) and 5 (C), a bodily migration of cells into the wound area was apparent (arrows). Astrocyte nuclei stained for bFGF at all times (D–F). Bar, 20 μm.

bers for the first, second, fifth, and eighth zones were 27.6, 19.75, 8.25, and 1.25, respectively. Cells grown in EMEM containing dbc-AMP as well as cells grown in Ham's F-12 medium exhibited lower rates and shorter distances of migration, compared to cells grown in EMEM. For example, on day 1, only cells in EMEM reached the second 100-μm zone from the wound edge. On day 5, only cells in EMEM could be detected beyond the first 300-μm distance away from the wound edge. BrdU labeling of migrating cells failed to demonstrate significant nuclear uptake of the label beyond day 2 (not shown).

Effect of Treatments on Wounded Cultures

Astrocyte morphology. Addition of bFGF to the medium resulted in changes in the morphology of astrocytes starting at 2–3 days following wounding. These changes were evident in both the injured and noninjured

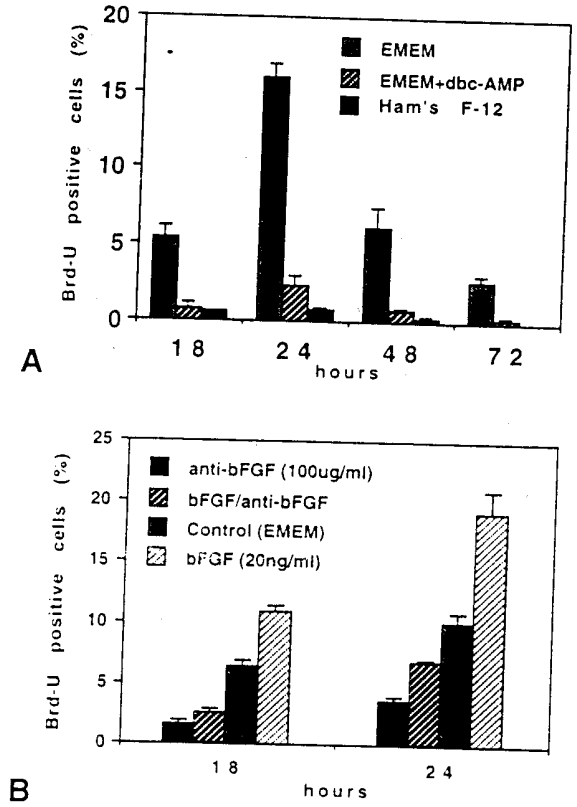

Fig. 2. Uptake of bromodeoxyuridine (BrdU) per unit length of the wound edge by mechanically wounded confluent cultures of astrocytes. BrdU-labeled cells were counted and the number expressed as a percentage of labeled cells in non-wounded areas of the culture. **A:** Untreated wounded cultures grown in EMEM containing 10% serum showed a significantly greater magnitude of cell division compared to cells grown in the presence of dbc-AMP in the same medium or in Ham's F-12 medium containing 0.5% serum ($P<0.001$). The values for dbc-AMP and F-12 were not significantly different from each other. **B:** Effects of bFGF, anti-bFGF, and combined treatment with bFGF and anti-bFGF on BrdU uptake at 18 and 24 hr after mechanical wounding of confluent astrocyte cultures grown in EMEM and 10% serum. BrdU uptake was expressed as in A. All values were significantly different from each other ($P<0.001$) except at 18 hr, when no significant difference was detected between anti-bFGF treatment and combined treatment with bFGF and anti-bFGF.

areas of the culture (Fig. 4A,B), but were more easily detected along the wound edge. Astrocytes in treated cultures appeared to be more compact, with smaller cell bodies and the cytoplasm condensed to the perinuclear area, often along one side of the nucleus. Cell processes detected by GFAP staining were less flat, more compact, narrower, and longer with many branches, compared to untreated controls. The presence of anti-bFGF antibody along with bFGF in the medium reduced cellular differentiation induced by bFGF (Fig. 4C,D). Anti-bFGF antibody alone did not significantly alter the morphological changes resulting from wounding.

Astrocyte proliferation. Addition of bFGF, anti-bFGF antibody, and bFGF along with the anti-bFGF antibody altered the magnitude of cell division following injury in cultures grown in EMEM containing 10% fetal serum (Fig. 2B). The presence of bFGF in the culture medium resulted in a sharp increase in the number of dividing cells at 18 hr, and more so at 24 hr after wounding. When these values were expressed as percentages of control values, this represented an approximately 90.9% increase in cell division at 24 hr following injury. In the presence of anti-bFGF antibody alone in the medium, cell division was 63.2% lower, compared to control values at 24 hr after wounding. In cultures which received bFGF along with anti-bFGF antibody, cell division was 32% less than in control and 64.2% less than in cultures which received bFGF. Substitution of anti-bFGF antibody with an equivalent concentration of mouse IgG_1 did not have any effect on astrocyte proliferation (not shown).

Astrocyte migration. Addition of bFGF to Ham's F-12 medium containing 0.5% FBS induced promotion of cell movement from day 1 following wounding, with maximum effects detected on day 3 (Fig. 5). When the values obtained for bFGF treatment were expressed as percentages of control values, on day 1, bFGF-treated cells demonstrated 186.5% greater migration into the first 100-μm zone, compared to controls. On day 3, cells treated with bFGF had moved into the the third 100-μm zone, whereas control cells in Ham's F-12 medium moved into the second zone only. In bFGF-treated cultures, at 3 and 5 days after wounding, cell migration within the first 100-μm zone was 142.7% and 73.7% greater than the control values, respectively. It was also apparent that bFGF-induced increase in astrocyte migration was considerably lower than that observed in serum-containing medium. Presence of anti-bFGF antibody in the culture medium reduced the migration of cells into the denuded area. In the presence of the antibody, on day 1, no astrocyte migration was detected in the denuded area. On day 3, some cells had moved into the first 100-μm zone, but this represented only 17.3% of the control values for medium alone. On day 5, an increase in the number of cells in the first 100-μm zone was evident over day 1, but this still represented only 32.3% of the control value. No cell migration into the second and third zones occurred at this time. Use of mouse IgG_1 in place of the anti-bFGF antibody did not have any effect on cell migration (not shown).

Immunohistochemistry and Western blot analysis of bFGF. Astrocytes at the wound edge, and cells migrating into the denuded area, as well as cells in the

nonwounded area of cultures, exhibited strong bFGF staining in their nuclei with very little cytoplasmic or process staining (Fig. 1D–F). Western blot analyses (Fig. 6) revealed three bands, one at 18 kD and the other two in the 20–22 kD range in astrocyte extracts. The 18-kD band corresponded in location to the bFGF activity present in the human recombinant bFGF standard (Fig. 6C). Identical results were obtained with the polyclonal anti-bFGF antibody directed against the aminoterminal of the molecule (not shown). Wounding of cells grown in EMEM (Fig. 6A) and in EMEM containing dbc-AMP (Fig. 6B) did not alter the isoform pattern at any survival time.

Dot-blot analysis. Dot-blot analysis (Fig. 6D) revealed that both type I and II anti-bFGF antibodies detected human recombinant bFGF (1–100 ng) at 1:500 dilution (1 μg/ml). Both antibodies failed to recognize aFGF or FGF-5 (Fig. 6D).

DISCUSSION

Proliferation or cell division and hypertrophy are reactive responses of astrocytes in vivo to a variety of brain injuries (Murabe et al., 1982; Janeczko, 1989; Topp et al., 1989; Vijayan et al., 1990). Yu et al. (1993) have recently shown that mechanical wounding of a monolayer of cultured rat astrocytes results in proliferation and process-hypertrophy comparable to the known in vivo responses of these cells to trauma (Miyake et al., 1988; Topp et al., 1989; Janeczko, 1989; Vijayan et al., 1990). The results of the present study confirm many of the previous observations (Yu et al., 1993) and further show that this in vitro model is suitable for exploring the effects of putative agents suspected to play a role in inducing reactive changes in rat astrocytes.

Our observations on astrocyte cell division in response to scratch wounding are comparable to previous observations of Yu et al. (1993). In addition, we show that cell division in response to wounding can be modulated by serum concentration and treatment with dbc-AMP and bFGF. Thus, low serum concentration and pretreatment with dbc-AMP significantly reduced astrocyte proliferation in response to wounding. In contrast, addition of bFGF increased cell division in response to wounding, a predictable effect since bFGF has been shown to induce mitosis in cultured rat astrocytes (Hatten et al., 1988; Kniss and Burry, 1988; Perraud et al., 1988, 1990; Loret et al., 1991; Petroski et al., 1991). This

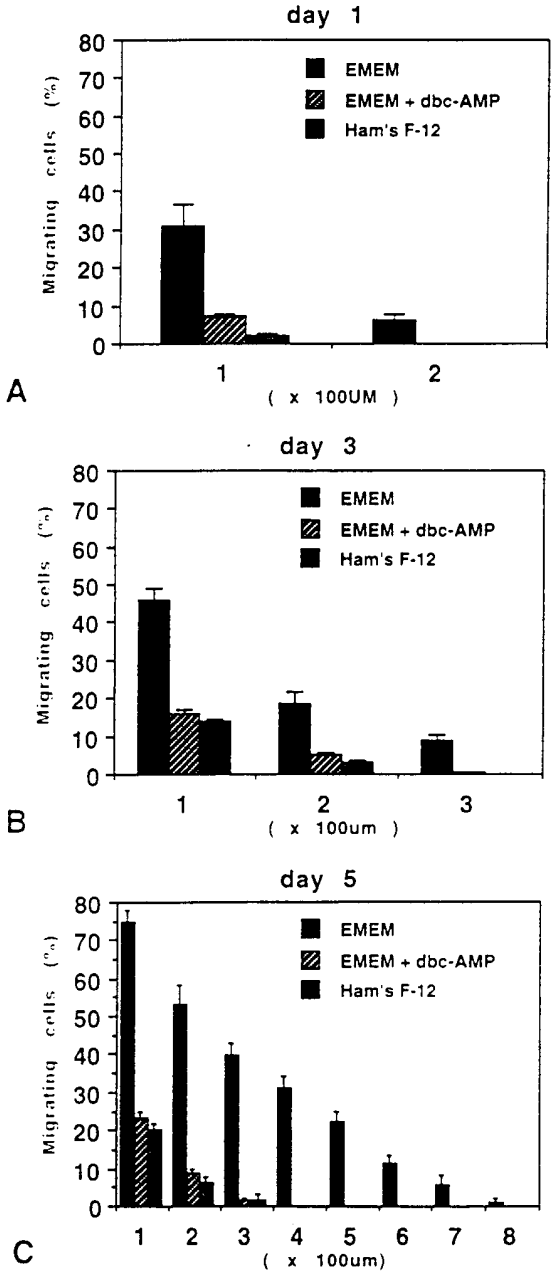

Fig. 3. Astrocyte migration 1 (**A**), 3 (**B**), and 5 (**C**) days following mechanical wounding of confluent cultures. Number of GFAP-stained cells within successive 100-μm zones from the wound edge was expressed as a percentage of cells within an equivalent area of the nonwounded parts of the culture. Values for the first 100-μm zone for cells grown in EMEM and 10% serum at all time periods were significantly different from each other ($P<0.001$). Values for the first 100-μm zone for cells grown in EMEM and 10% serum were significantly greater than those in the presence of dbc-AMP or Ham's F-12 medium on day 1 ($P<0.001$), day 3, and day 5 ($P<0.0001$). Values for dbc-AMP-treated cells and cells in F-12 medium were not significantly different from each other on any day. In the third 100-μm zone, values for cells in EMEM and 10% serum were greater than for the other groups on day 3 ($P<0.0006$) and day 5 ($P<0.0001$).

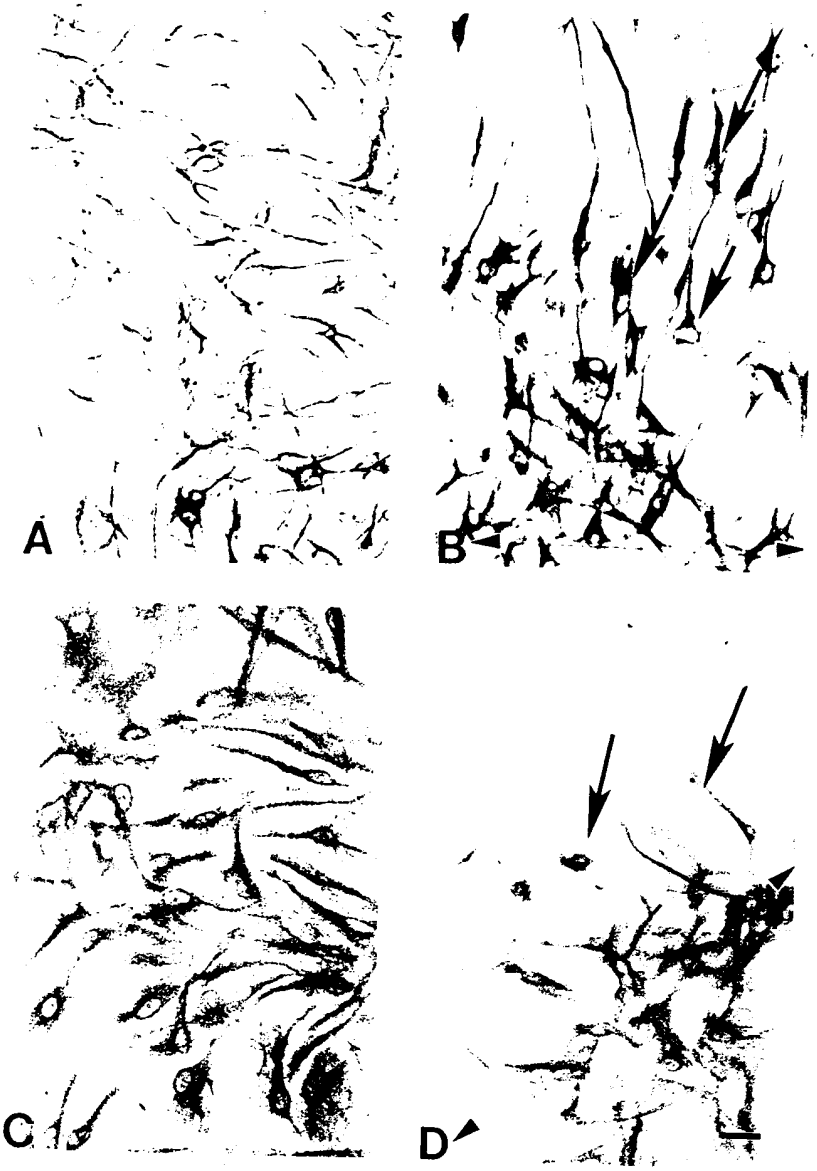

Fig. 4. Effects of bFGF, type II anti-bFGF antibody, and combined treatment with bFGF and type II anti-bFGF antibody on astrocyte morphology. GFAP staining at 3 days following mechanical wounding of confluent cultures is shown. **A,C:** Non-wounded area of the culture. **B,D:** Wounded area of the culture. Wound edge is demarcated by arrowheads. A,B show cultures treated with 20 ng/ml bFGF. C,D show cultures treated with bFGF and anti-bFGF (100 μg/ml). Arrows (B,D) indicate astrocytes migrating into the denuded area. bFGF-treated cultures (A,B) exhibited morphological features of differentiation and greater migration than cultures treated with bFGF in combination with anti-bFGF (C,D), which resembled control (untreated) cultures. Bar, 20 μm.

mitogenic effect could be blocked by a neutralizing anti-bFGF antibody which is believed to recognize the receptor binding domain of bFGF (Matsuzaki et al., 1989). The anti-bFGF antibodies used in our studies have been shown to reduce cell division of 3T3 cells in a dose-dependent manner in the presence and absence of exogenous bFGF (Matzusaki et al., 1989). Specificity of the antibodies is further supported by our dot-blot analysis, which showed that the antibodies recognized human recombinant bFGF but did not crossreact with human recombinant aFGF or FGF-5. In Western blots, type II antibody recognized three isoforms of bFGF in rat astrocyte extracts, comparable to what was previously demonstrated in the rat brain by other investigators (Woodward et al., 1992). For these reasons, we conclude that the observed effects of antibody treatment resulted from

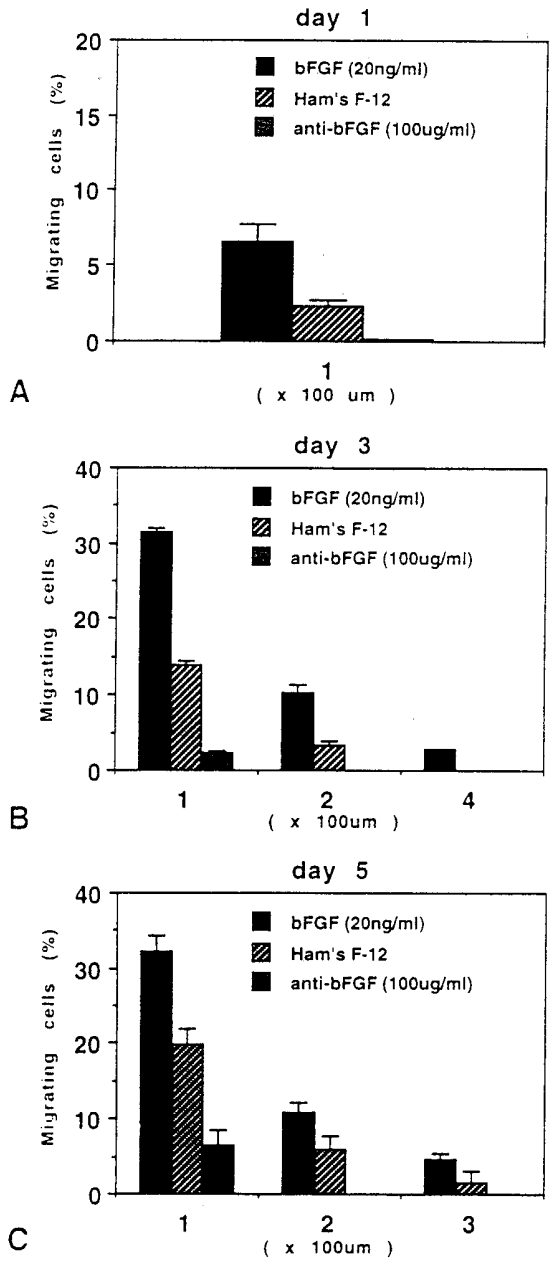

Fig. 5. The effects of bFGF and type II anti-bFGF antibody on astrocyte migration 1 (**A**), 3 (**B**), and 5 (**C**) days following mechanical wounding of confluent cultures grown in Ham's F-12 medium. Within the first 100-μm zone, values for bFGF-treated cultures were significantly greater than those for anti-bFGF-treated cultures and cultures grown in F-12 medium on days 1 ($P<0.001$), 3 ($P<0.0001$), and 5 ($P<0.0002$). In the third 100-μm zone, values for bFGF treatment were significantly greater than for the other two treatments on day 3 ($P<0.0001$) and day 5 ($P<0.02$). Values for anti-bFGF-treated cultures were lower than for control cultures in F-12 medium on day 3 ($P<0.0001$ for both the first and second 100-μm zones) and day 5 ($P<0.0002$ for the first 100-μm zone and $P<0.001$ for the second 100-μm zone).

a selective binding of antibody to the functional high-affinity receptor-binding or heparin-binding domains of the bFGF molecule and from the consequent inactivation of the growth factor. Whether the observed antibody effects may have alternatively resulted from steric hindrance conferred by the antibody cannot be ruled out by our studies. It is interesting that the antibody alone reduced cell division to a level lower than what was observed in the presence of serum, suggesting that astrocytes utilize endogenous bFGF to undergo cell division, and that the antibody was capable of blocking this effect as well. These results confirm our hypothesis that astrocytes are capable of utilizing endogenous bFGF, presumably synthesized by the cells, as well as bFGF made available in their external milieu, to initiate and sustain cell division in response to wounding. This is further supported by findings that astrocytes synthesize bFGF (Hatten et al., 1988; Ferrara et al., 1988; Flott-Rahmel et al., 1992) and that bFGF antisense oligodeoxynucleotides significantly reduce DNA synthesis of cultured rat astrocytes (Gerdes et al., 1992).

One of the major responses of confluent astrocytes subjected to wounding was a bodily migration of the cells from the wound edge into the denuded area. Cells grown in EMEM in the presence of serum showed maximal migratory capacity in terms of the distance moved and the number of cells entering the denuded area. Dbc-AMP treatment, as well as growing cells in low-serum medium, diminished astrocyte migration. Since dbc-AMP-treated cells are known to undergo stellation, whereas untreated cells will be expected to retain an immature morphology, this suggests that stellation or induced maturation may diminish astrocyte migration. A recent in vivo study supports our findings by demonstrating that dbc-AMP-treated astrocytes implanted into neonatal rat cerebrum failed to migrate into the parenchyma until the effect of the treatment diminished (Hatton and Sang, 1993). Our results show that bFGF treatment enhances astrocyte migration in a low-serum medium and that an anti-bFGF antibody reduces the effect of bFGF. The observation that treatment with anti-bFGF antibody alone reduced astrocyte migration to a level lower than in controls suggests that astrocytes not only utilize exogenous bFGF in the culture medium, but are also capable of using endogenous bFGF for cell migration. Similar results have been obtained with bovine aortic and capillary endothelial cells (Sato and Rifkin, 1988). Although we cannot unequivocally rule out a role for cell division in the migration of astrocytes that we observed, the time course of the two events does not support this possibility. Thus, the mitogenic response of astrocytes in EMEM containing 10% serum was maximal as early as day 1, and had subsided by day 3. In contrast, under identical conditions, cell migration progressively increased from

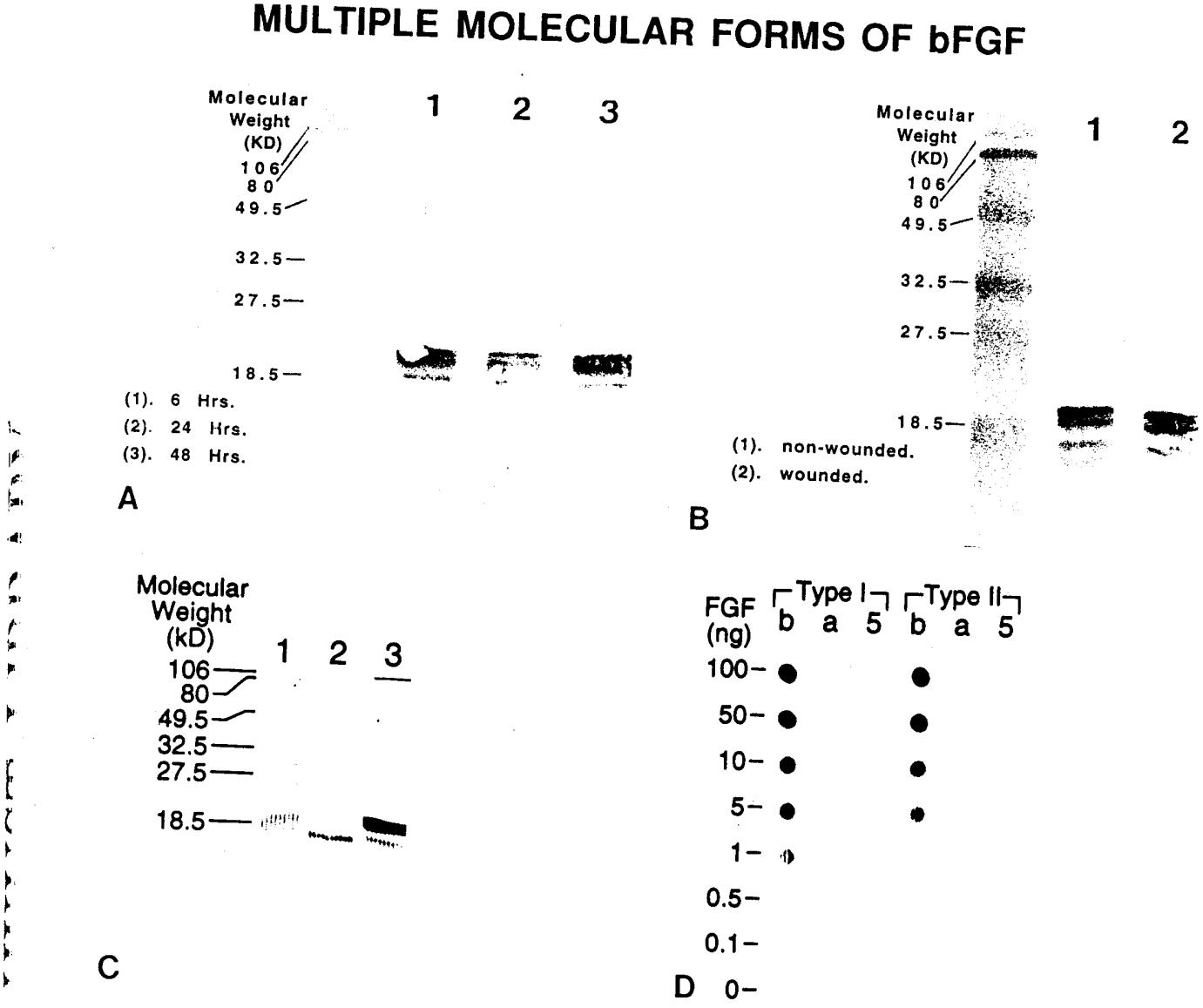

Fig. 6. A–C: Western blot analysis of bFGF in heparin-sepharose extracts of confluent astrocyte cultures. Blots were stained with type II anti-bFGF antibody. **A: Lanes 1, 2,** and **3** represent samples prepared at 6, 24, and 48 hr, respectively, after wounding. Molecular weight markers are shown on the left. **B:** Cultures treated with 0.25 mM dbc-AMP 5 days before and after wounding. **Lanes 1** and **2** represent samples of non-wounded cultures and cultures at 48 hr after wounding, respectively. Molecular weight markers are shown on the left. In A and B, astrocyte extracts were diluted appropriately so that all lanes contained approximately 5×10^4 cells. **C:** bFGF isoforms detected in a heparin-sepharose extract of the rat brain (**3**) and in 20 ng recombinant human bFGF (**2**). Molecular weight markers are also shown (**1**). **D:** Dot-blot analysis. Recombinant bFGF (**b**), aFGF (**a**), and FGF-5 (**5**) were dotted on nitrocellulose paper at 0–100 ng and exposed to anti-bFGF type I and II antibodies at 1:500 dilution (1 μg/ml).

day 1 to day 5, implying a lack of correlation between cell division and migration.

In contrast to endothelial cells which increase their cytoplasmic bFGF staining during migration (Muthukrishnan et al., 1992), in our study, astrocytes failed to alter their nuclear staining pattern for bFGF under conditions of cell division, differentiation, or migration. Whether this is a true cell-specific difference between astrocytes and endothelial cells, or whether it is methodological, remains to be determined. Western blot analysis showed that astrocyte extracts contained one isoform of bFGF with an approximate molecular weight of 18 kD

and a doublet in the area of 21–22 kD. Other studies have shown the existence of the three isoforms of bFGF in the rat brain and in cultured astrocytes (Woodward et al., 1992). We show that the isoform pattern for bFGF remains unaltered with wounding or dbc-AMP treatment. It remains to be determined whether wounding increases bFGF mRNA in astrocytes, leading to an increase in the level of the endogenous growth factor and its subsequent release into the culture medium.

In conclusion, our study demonstrates that cultured astrocytes subjected to mechanical wounding respond to exogenous bFGF by increasing their proliferation and migration. It also suggests a role for endogenous bFGF, presumably synthesized by astrocytes, in these injury responses. However, more direct evidence is needed to substantiate the presumed increase in bFGF level in astrocyte cultures as a result of wounding. Our results support the view that bFGF may play a role in the induction of astrocyte responses to brain injury in vivo where the cells are likely to synthesize bFGF and become exposed to bFGF in their immediate vicinity. Further, since the actions of exogenous and endogenous bFGF could be blocked in vitro by an anti-bFGF antibody, it is likely that similar interventions may reduce trauma-induced astrogliosis in vivo.

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