

Proliferative and Migratory Responses of Astrocytes to In Vitro Injury

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An in vitro “scratch-wound” model was used to evoke and investigate some astroglial responses to mechanical injury. The changes in the morphology, locomotion, and proliferation of injured astrocytes were analysed under culture conditions devoid of blood-derived cells responsible for activating the inflammatory cascade. The rate of proliferation was determined by immunocytochemical detection of BrdU-incorporating cells located next to or far from the wound. The motility of individual cells and the mass-advancement of cell-assemblies were monitored by computer controlled video-microscopy both in injured monolayers and in preparations of single cells or aggregates of astrocytes. The large sets of digitalized data allowed a reliable statistical evaluation of changes in cell positions providing a *quantitative* approach for studies on dynamics of cell locomotion. The results indicated that cultivated astrocytes respond to injury (1) with enhanced nestin immunoreactivity at the expanding processes, (2) with increased mitotic activity exceeding the rate caused by the liberation from contact inhibition, but (3) without specific, injury-induced activation of cell locomotion. Some advantages and drawbacks of “scratch-wound” models of astrocytic responses to mechanical injury are presented and discussed. *J. Neurosci. Res.* 61: 421–429, 2000. © 2000 Wiley-Liss, Inc.

Key words: astrocytes; in vitro trauma; migration; proliferation; scratch-wound model; computer controlled video-microscopy

Repair processes following traumatic brain injury aim at the recovery of tissue integrity and at the isolation of intact areas from the outer world and from the scene of destruction (Kimelberg and Norenberg, 1994). Astrocytes, contributing to the scar-formation along the lesion site, acquire characteristic “reactive” morphology and display a variety of functional changes (see reviews of Eddlestone and Mucke, 1993; Kimelberg and Norenberg, 1994; McMillian et al., 1994; Ridet et al., 1997). The major source of astroglial cells repopulating the lesion-site, however, is still a matter of debate (Eclancher et al., 1996). It is not clear whether already differentiated, local astrocytes or some quiescent, precursor glial populations give rise to scar-forming cells. It is also uncertain how the most char-

acteristic responses of astrocytes to tissue damaging stimuli, i.e., alterations in morphology, activation of mitosis, and locomotion, co-operate in the course of cell replacement and scar formation.

To study the process of glial activation, several in vitro models have been proposed (see review of Wu and Schwartz, 1998). In a set of these model systems, the in vivo traumatic events were simulated by exposing cells to direct mechanical injury, namely by scratching confluent astrocytic monolayers by blades, needles, or pipette tips (Hou et al., 1995; Yu et al., 1993; Mukhin et al., 1998). As a response to “scratch-wound injury” some well-known signs of the in vivo reactivation of astroglial cells were recognized in the scratch-area, such as elongation of “hypertrophic” processes, hypertrophy of astrocytic nuclei, enhanced expression of extracellular matrix molecules, and increase in the glial fibrillary acidic protein (GFAP) content (e.g., Yu et al., 1993). The realignment and expansion of the intermediate filament network are the most characteristic responses of astrocytes to injury in vivo (Kimelberg and Norenberg, 1994). Recently, a novel intermediate filament molecule *nestin* (originally described for neural stem cells; Lendahl et al., 1990) was identified, and introduced as an earlier and more sensitive sign of glial activation than the increase of GFAP (Frisén et al., 1995). Based on the appearance of the above markers, the models were used to predict the effects of a variety of molecular factors on glial activation (Hou et al., 1995; Faber-Elman et al., 1995).

In our in vitro studies, scratch wound injury-induced changes in motility, proliferation, and cytochemical properties of astrocytes were analyzed. We intended to

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Abbreviations used: BrdU = 5-bromo-2'-deoxyuridine; CCVM = computer controlled videomicroscopy; DIV = days in vitro; GFAP = glial fibrillary acidic protein.

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make clear whether the observed alterations in the locomotory and mitotic activities were inherent and injury-related responses of astrocytes. Special attention was paid to the fact that many previous works have undervalued the mitotic and migratory activation caused by the liberation of cells from cell-to-cell contacts. In order to distinguish injury-specific responses from reactions caused by the release from contact inhibition, the motility and proliferation of the astrocytes were *quantitatively* analyzed in scratch-wounded monolayers and in non-injured, low cell density cultures.

The proliferative response was quantified by assays on BrdU incorporation. Dynamic changes of glial cell locomotion were resolved by a computer-controlled videomicroscopic (CCVM) system, developed in our laboratories (Czirók et al., 1998). CCVM allowed the long-term observation of living cells, and the statistical analysis of data collected both on the level of individual cells and on multicellular arrangements. We found that while the migratory activation was not a specific, injury-related action of astrocytes, the rate of cell proliferation was significantly enhanced along the wound and proved to be a specific, injury-evoked response.

MATERIALS AND METHODS

Cell Cultures

Primary cultures. Cell suspensions of neonatal rat (Wistar) forebrains were prepared by enzymatic dissociation with 0.05% w/v trypsin (Sigma Aldrich Co., Budapest, Hungary) in PBS and were plated onto poly-L-lysine (Sigma Aldrich Co., Budapest, Hungary) coated plastic surfaces at a cell density of $3-4 \times 10^5$ cell/cm². The cultures were grown in Minimum Essential Medium (MEM; Sigma-Aldrich Co., Budapest, Hungary) supplemented with 10% fetal calf serum (FCS; Gibco-BRL-Life Technologies, UK), 4 mM glutamine, and 40 µg/ml gentamycin in humidified air atmosphere containing 5% CO₂, at 37°C. The culture medium was changed twice a week.

Secondary cultures. In the second week of in vitro maintenance, primary cultures were trypsinized and re-plated onto poly-L-lysine coated glass coverslips or into 35-mm petri dishes at 3×10^5 cells/cm² or 10^4 cell/cm² density, respectively. Astrocytes in secondary cultures were maintained for 2–3 weeks in MEM supplemented with 10% FCS.

Aggregates of astrocytes were prepared by bringing the cells in close contact by centrifugation (1,000 rpm, 5 min). Densely aggregated cells were seeded as droplets into 35-mm dishes. After an initial 15-min period of cell attachment, the dishes were loaded with 2 ml of culture media, and were transferred into the mini incubator of the videomicroscopic device (see Computer-Controlled Videomicroscopy (CCVM)). The aggregated cells were grown in the mini-incubator for 48 hr under conditions described for primary cultures.

Scratch-Wound Model

Confluent monolayers in secondary cultures were wounded by either scratching with a plastic pipette (yellow) tip along lines at right angles to each other, or by removing wide stripes of cells with rubber blades. Scratched cultures were

immediately transferred to the stage of the videomicroscope or processed for the assays on cell proliferation.

Determination of the Proliferation Rate

Cells were treated with 600 ng/ml bromo-deoxy-uridine (BrdU, Fluka) for 12 hr, starting at 0, 12, 24, and 48 hr after scratching. At the end of the incubation period, the cells were fixed and processed for anti-BrdU immunostaining. The rate of cell division was determined by relating the number of BrdU-positive nuclei to the total cell number found in individual microscopic fields at 400× magnification. Cells were counted on at least 15 microscopic fields (field size: 0.144 mm²) either from control cultures with low or high cell densities or from three different areas of the wounded preparations: inside the wound, next to the wound, and far from the wound-site. Data were collected from two to three sister cultures obtained from three independent experiments.

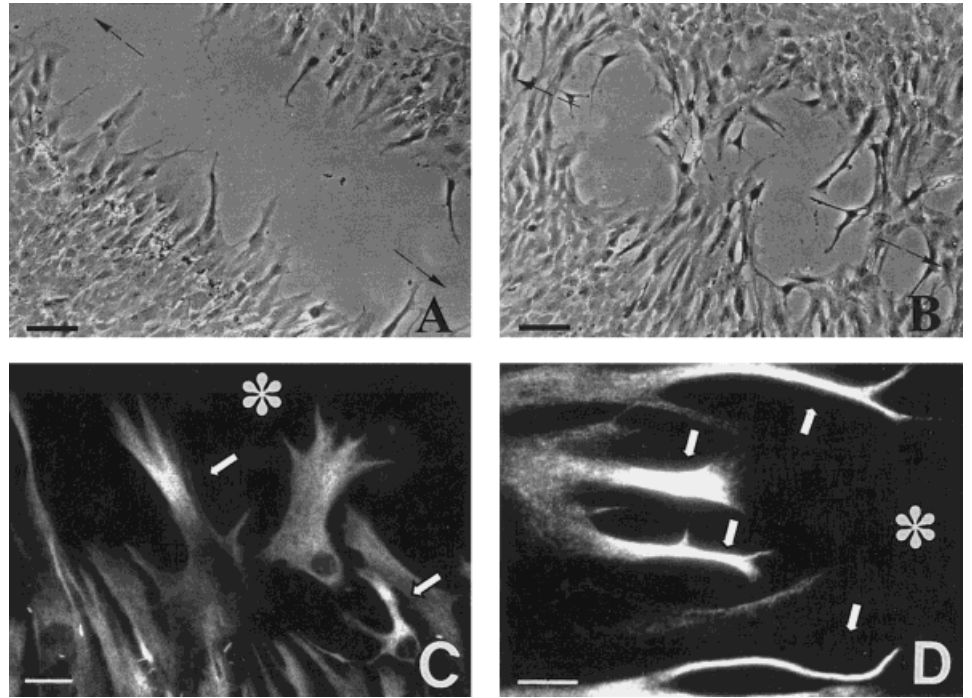
Immunocytochemistry

Cells grown on poly-L-lysine coated glass coverslips were fixed with 4% paraformaldehyde in PBS for 20 min, at room temperature. The membranes of fixed cells were permeabilized by treatment with Triton X-100 (5 min, 0.1% v/v in PBS). For the visualization of incorporated BrdU, nuclear proteins were detached from the DNA by treatment with 1 N HCl and 0.1% v/v Triton X-100 at 60°C, for 30 min, prior to the exposure to anti-BrdU antibody. Non-specific antibody binding was blocked by incubation with 5% FCS in PBS at room temperature, for 1 hr. Antibodies to BrdU (mouse IgG, Dako), GFAP (mouse, Sigma-Aldrich Co, Hungary), and nestin (mouse, Pharmingen) were diluted with MEM-FCS and were used at dilutions of 1 to 1,000–3,000, overnight, at +4°C. Secondary antibodies to anti-mouse IgGs conjugated with biotin (Sigma) were employed in a dilution of 1 to 1,000, for 2 hr at room temperature. The immunoreactions were visualized by standard streptavidin-peroxidase-diaminobenzidine (DAB) or the fluorescent avidin-TRITC reaction.

Computer-Controlled Videomicroscopy (CCVM)

Cell cultures were kept in a computer-controlled mini-incubator (Czirók, 1998), which provided stabilized temperature of $37 \pm 0.5^\circ\text{C}$, with 100% humidity and 5% CO₂, 95% air gas atmosphere and optical transparency for microscopic observations. The incubator was fastened to an inverted phase contrast microscope (Zeiss Telaval-3, Jena, Germany) equipped with a powered stage and with 10× or 20× long working distance objectives. Images were taken at every 5 min using a CCD camera (JVC KY-F30B, Japan) connected directly to the frame grabber card (Matrox Meteor, Matrox Electronic Systems LTD, Canada) of a computer running under Linux operating system. Cells living in the mini incubator were monitored by CCVM for at least 48 hr. The consecutive images of microscopic fields were stored as digitalized data. From the stored images, the average velocities of front advancements, the trajectories of migrating cells, and the distribution functions of cell velocities were determined. Statistically evaluated data were obtained from 3 different microscopic fields of two independent experiments.

Fig. 1. Phase contrast microscopic images and immunostained photomicrograms of scratch-wounded astroglial cultures. Images were taken on the 22nd–23rd days in vitro (DIV 22–23). **A:** Twelve hours after the injury the astrocytes extended tactile processes towards the denuded area. Note the cell-debris gathered on the surface of cells at the margin of the scratch. The arrows indicate the direction of the injury. **B:** Twenty-four hours after injury astrocytes migrated into the denuded area. Dividing cells with phase-bright spherical somata appeared within the gap. **C:** Twenty-four hours after injury some of the astrocytes displayed elevated GFAP-immunoreactivity in the growing processes extended towards the denuded area. **D:** Strong immunoreactivity of nestin intermediate filamentum protein was localized in the tips of elongating cell-processes 24 hr following scratching. Asterisk: Cell free gap created by scratching. Scale bars = (A,B) 50 μm , (C) 20 μm , (D) 10 μm .



Calculation of Front Advancement Velocities

As a first step we identified the areas covered by cells on the images. Briefly, after correcting the unevenness of the background illumination, a Gaussian filter was applied for each image to reduce noise. The pixels belonging to the cell-free area were then detected on the basis of the lack of local brightness variation. Since we compared front displacements in experiments with different initial configurations (i.e., the quasi linear arrangements at the wounds or the irregular shapes of the aggregates), simple area measurements were not sufficient to characterize the front advancement speeds. Instead, we replaced each pixel on a certain image i by a circle of radius n pixels, in this way isotropically expanding the region at the edge of the image corresponding to cells (A_i). This process resulted in an enlarged cell covered area $A_i'(n)$. The enlarged area $A_i'(n)$ was compared to the area covered by cells (A_j) on a subsequent image j . The displacement of the front (d_{ij}) during a time interval $t_j - t_i$ (where t_i denotes the moment when image i was taken) was determined by the following way: we compared the areas $A_i'(n)$ and A_j for various values of n . Then d_{ij} was set equal to the value of n , which minimizes the difference between $A_i'(n)$ and A_j . Applying this procedure to growing cell assemblies, we found a linear relationship between d_{ij} and $t_j - t_i$ holding for a wide range of time intervals separating the images i and j .

Determination of Individual Cell Positions and Velocities

The position of selected cells was tracked manually on each image (i.e., in every 5 minutes in real time, denoted by $t_0, t_1, t_2, \dots, t_m, \dots$ from the beginning of the recordings) with a precision of approximately 10 μm , comparable to the average cell diameter (20–25 μm). In the following, the position of the

i th cell at time t_m is denoted by $x_i(t_m)$. Between two consecutive frames, the difference in the location of a given cell i , i.e., $x_i(t_m + 1) - x_i(t_m)$ was considered as a migratory segment. Trajectories $x_i(t)$ were constructed from these segments. Cellular velocities $v_i(t)$ were defined as the soma displacement in one hour ($\Delta t = 1$ h), as $v_i(t) = |x_i(t + \Delta t) - x_i(t)|$.

RESULTS

Scratch-Wound Model

Monolayer cultures of astroglial cells derived from neonatal rat forebrains were scratched with plastic pipette tips according to simple geometrical patterns (Fig. 1). The tips removed most of the debris from the “wound-site.” A one- or two-cell wide edge of the scratched astroglial sheet detached from the substrate, and curled into a freely floating cylinder that was still bound to the rest of the monolayer (see Fig. 3A). The cells did not release each other as a consequence of wounding, at least not during the first few hours after injury. A total cell loss of 5 to 10% was calculated by comparing the denuded areas to the cell-covered surfaces. Astrocytes along the wound-site, directly exposed to mechanical injury, represented no more than 2% of the entire population. After an initial cell decay indicated by the cell debris gathering on the surface of intact astrocytes (Fig. 1A), no signs of further cell death were observed at the margin of the wound, in a period of 24 to 60 hr after injury.

Based on phase-contrast microscopic observation of living cells and on the evaluation of immunostained preparations, three steps of astrocytic reactions to scratching were distinguished. (1) Cells surviving the acute damage at the wound-edge, re-attached to the substrate in 0–30 min

following injury. (2) Astrocytes extended flat, cytoplasmic processes to the denuded area, without considerable dislocation of the nuclei, in a 1–12-hr period after scratching (Fig. 1A). (3) Migration and enhanced proliferation within and next to the wound-site started at 12–24 hr postlesion, and lasted until cell density approached confluency (Fig. 1B).

All cells lining the scratch were GFAP-positive (Fig. 1C), and many of them displayed enhanced immunoreactivity for nestin. Nestin-positivity was strictly localized in elongating cell-processes, emerging from a 1 to 3 cell-row distance from the wound (Fig. 1D). Though astrocytes with characteristic process-bearing morphology (Seniuk et al., 1994) were also detected among the migrating population, the majority of cells filling the wound were identified as flat, type I astroglia.

Proliferative Response of Astroglial Cells to In Vitro Injury

In a set of experiments, sister-cultures of astroglial monolayers were scratched and incubated with BrdU for 12 hr, starting at 0, 12, 24, and 48 hr following injury. At the end of the incubation periods, the cultures were fixed and subjected to immunostaining. The proportion of the BrdU-positive S-phase cells was calculated as a percentage of the total cell number in a given microscopic field. Data were collected from fields of equivalent size in three different areas of scratched monolayers: within the denuded area, next to the wound, and far from the wound-site. In fields next to the wound, the average rate of cell-division was elevated 1.5–2-fold compared to non-injured control cultures, during the first 24–36 hr after scratching, (Fig. 2A). From the 12th hour, the repopulation of the denuded area was accelerated by enhanced cell-proliferation and the percentage of S-phase cells increased 3–4-fold between 24 and 36 hr, compared to control cultures (Fig. 2A). When cell density approached semi-confluency in the wound (60 hr after trauma), the rate of cell proliferation decreased. Cells in culture areas far from the scratch did not show enhanced proliferation in comparison to non-wounded control cultures. The pattern of mitotic activation was similar in wounded cultures grown in chemically defined medium (data not shown).

Migration of Astrocytes in Injured Cultures

Displacement of astroglial cells at the edge of the wound site was followed by computer controlled videomicroscopy for a 48-hr postlesion period. After scratching, a 2–3-hr period elapsed before well-detectable cell displacement took place. During the first 24 hr only a minute fraction of the cells departed from their neighbours (Fig. 1B). The majority of astrocytes retained their cell-to-cell connections and the injured edges of the monolayer expanded with a well defined front line. The interface between the cell-covered and cell-free areas roughened with time, reflecting the fluctuations in cell migration activity (illustrated by Fig. 3A–D) and the asynchronous cell proliferation. The mean advancement of the interface was proportional to the time elapsed, thus an average front

advancement speed of $10 \pm 1 \mu\text{m/hr}$ has been determined (Fig. 4A).

In order to get more detailed data on the occupation of cell-free areas by astrocytes, individual cell positions were followed by tracking the cell bodies on consecutive frames of the time-lapse recordings. The trajectories of individual cells showed a remarkably ordered, outward directed migration superimposed with the inherent randomness of cell locomotion (Czirók et al., 1998, Dunn and Brown, 1987, Wichterle et al., 1997) (Fig. 4C). Most cells facing the empty areas moved with a velocity of 10–30 $\mu\text{m/hr}$ (Fig. 4B) and the average velocity of the tracked cells was 12 $\mu\text{m/hr}$.

Migratory and Proliferative Activity of Astrocytes in Non-Injured Cultures

In order to test whether the observed responses were specific to the in vitro injury, migration of astrocytes were studied in non-injured cultures, as well. As an origin for outmigration, astrocytes were let to aggregate and the aggregates were seeded onto poly-L-lysine coated plastic surfaces. Images of expanding aggregates were taken in a period of 48 hr (Fig. 3E–G). Despite previous trypsinization, continuous spread of the outgrowth area was observed during the entire recording period, excluding the first 2–3 hr when the aggregates became attached to the substrate (Figs. 3H, 4A). The migrating cells followed similar, outward-oriented routes both in the outgrowth zones of the intact aggregates and at the wounded edges of the monolayers, in contrast to the random movement of individual cells plated with low cell density (Fig. 4C–E). The values of the front advancement velocities indicated no significant differences between the extension of the wounded monolayer and the expansion of the aggregates (Fig. 4A). The distribution of individual cell velocities (Fig. 4B) also indicated that scratch-wound did not cause any specific elevation in the migratory activity above the locomotory activity of astrocytes in populating empty culture surfaces.

In order to reveal whether the trauma-induced proliferative responses were not entirely induced by a release from contact inhibition, the distribution of cells with BrdU label was analysed in dense and sparse populations of cells, as a function of cell density (Fig. 2C–F). In confluent monolayers of astrocytes (50–200 cell/field), the proportion of BrdU positive cells did not change with cell density and never exceeded 40% (Fig. 2C). In areas next to the wound, where cell-density varied between 35 to 175 cells/field, however, an increase in the proportion of mitotically active cells was detected (Fig. 2D). Among the cells repopulating the scratch-area, a characteristic distribution of BrdU-labelling was found (Fig. 2E). The high BrdU-index and the apparent lack of mitotically silent cells indicated that the proliferation activity was higher in these areas than in non-injured cell populations with similar low cell densities (Fig. 2E,F). The significant difference in BrdU-index between the scratch-wounded and low density control cultures was confirmed by Wilcoxon-test ($P \leq 0.05$) (Fig. 2B).

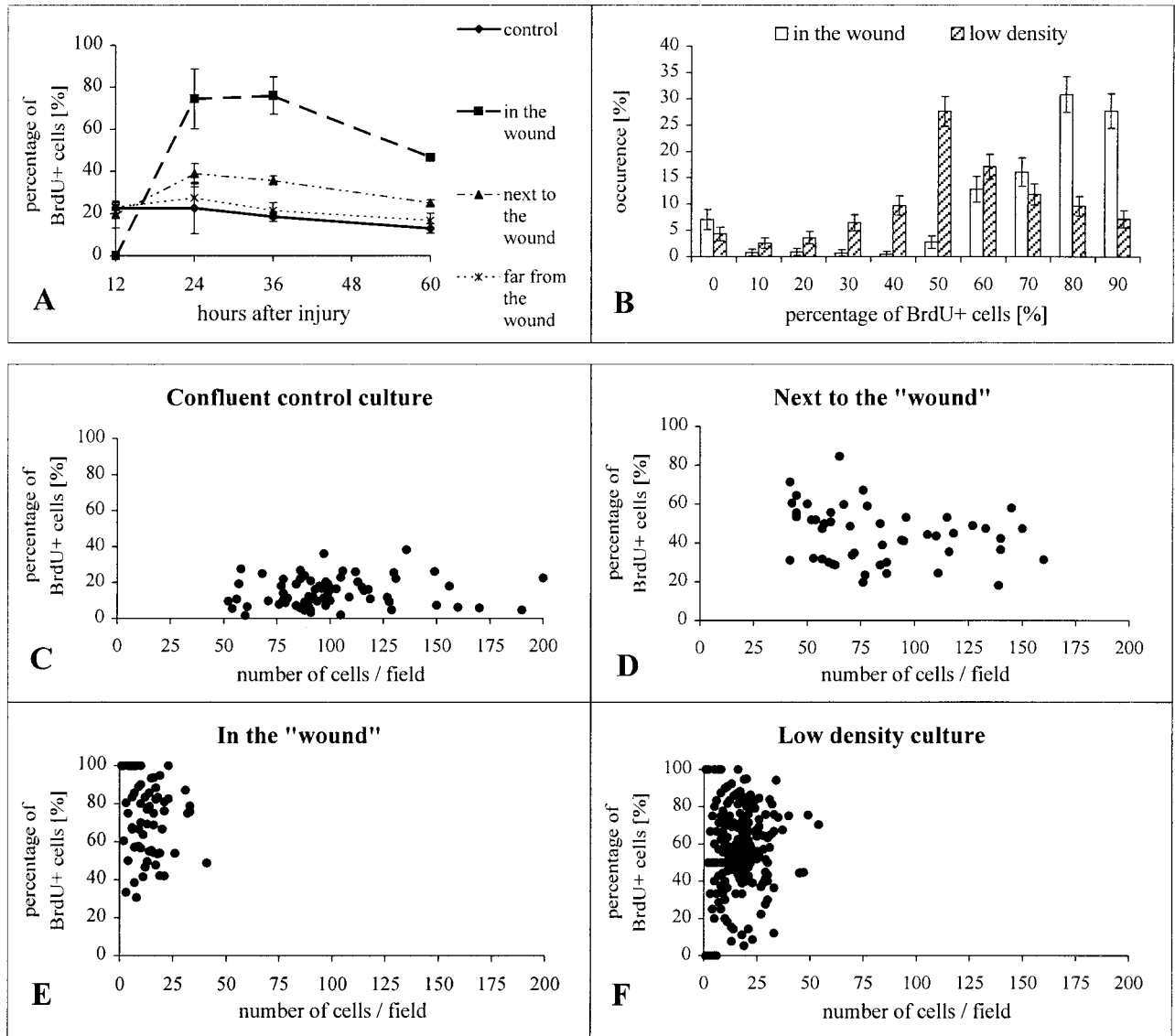


Fig. 2. Changes in the rate of cell proliferation in different regions of scratch-wounded monolayers of astrocytes. Cell number and BrdU index was determined inside the wound, close to the wound, far from the wound and in non-injured control cultures. **A**: Percentage of astroglial cells carrying BrdU label at different points of time after scratching. Averages and standard deviations of data obtained from 3 independent series of experiments are presented. **B**: Occurrence of microscopic fields with certain BrdU indices for low-density cultures (striped columns) and for culture areas at the wound site (open col-

umns). X axis: BrdU index. Y axis: number of fields with specified BrdU index divided by the total number of fields investigated ($n=120$) $\times 100$ [%]. The significant difference in BrdU-index between the scratch-wounded and control low-density cultures was confirmed by Wilcoxon-test ($P \leq 0.05$). **C-F**: The frequency of BrdU-labelled cells in various regions of injured and control cultures are plotted against cell density. Representative data obtained from three identically treated sister cultures from one set of experiments are shown.

DISCUSSION

We studied the injury-induced activation of mitosis and locomotion of astroglial cells in an in vitro "scratch-wound" model. Scratching the monolayer created a set of mechanically insulted cells, but the majority of astrocytes remained physically undisturbed. Astrocytes in confluent monolayers are coupled to each other and junctional connections render a quick intercellular communication pos-

sible throughout large culture areas (Giaume and McCarthy; 1996, Venance et al., 1997). Injury to a confluent monolayer of astrocytes, therefore, was expected to affect not only the directly wounded cells, but larger populations in the dish. Signals, spreading through the intercellular junctions (Lin et al., 1998) or via released factors (Hassinger et al., 1996) could have resulted both in altered proliferative and locomotory properties.

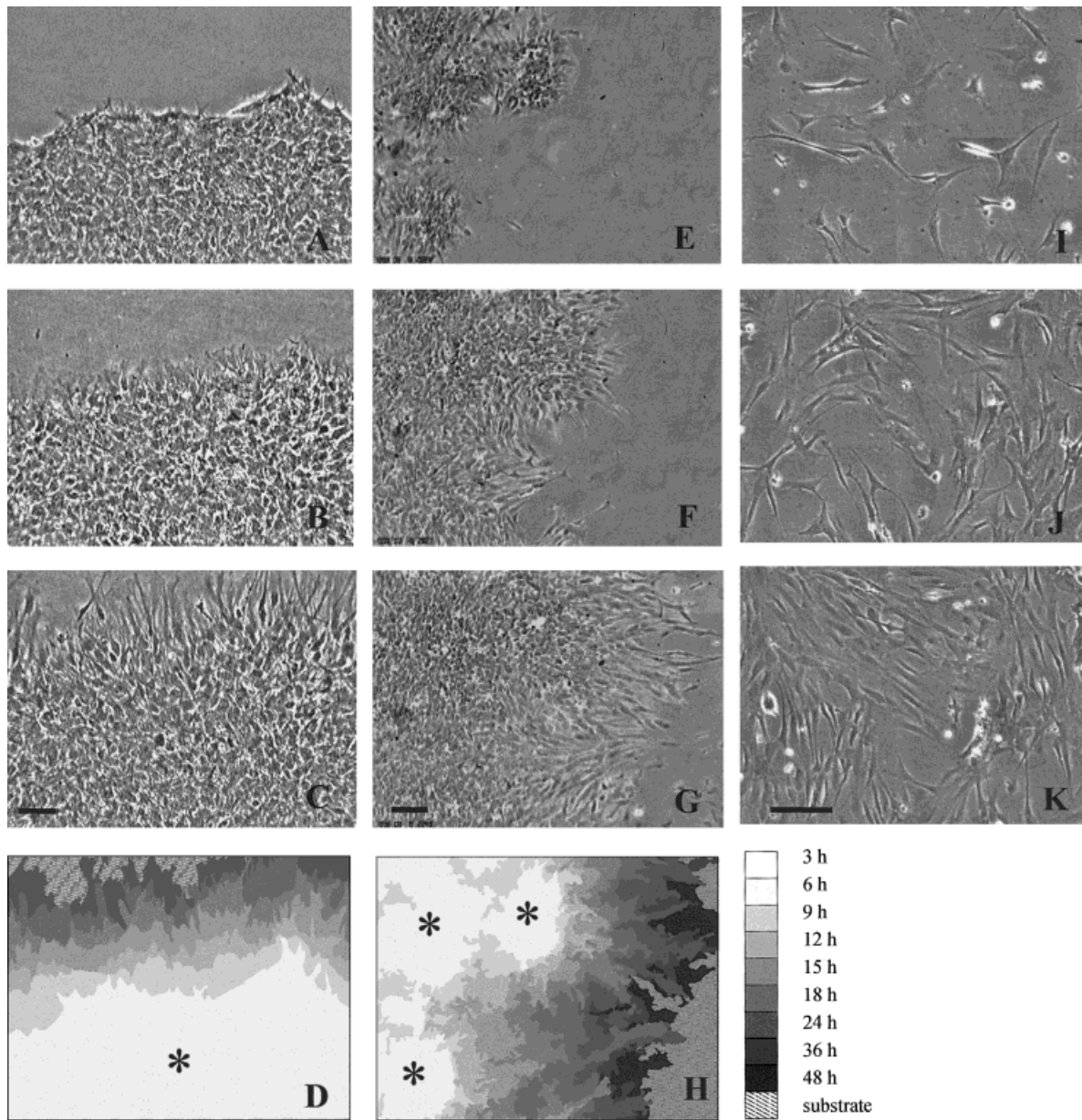


Fig. 3. Time-lapse micrographs illustrating the front advancement following mechanical injury. Images show the same microscopic fields at 0 (A), 6 (E,I), 12 (B,F), 24 (J), 48 (C,G,K) hr after scratching or plating. D,H: Graphical elaboration of digitalized data on front-propagation. The differently shaded areas represent the cell-covered surfaces of scratch-wounded monolayer (D) and of expanding aggregates (H) at various points of time after injury. Asterisk: Core of the cell assemblies. Scale bars = 50 μ m.

Individual cells, squeezed in a monolayer, responded to scratching with extension of cytoplasmic processes towards the cell-free area, resembling the *in vivo* observed shape of the hypertrophic processes of reactive astrocytes. Time lapse analyses, however, indicated that the elongation of glial processes was just an initiating step for the dislocation of the geometric centres of the cells, necessary for a normal, outward directed migration. The elevation

of the GFAP and nestin content within the extending processes could also be reasoned by the cytoskeletal changes accompanying the outgrowth of cells from compact cell assemblies (Madarász et al., 1996).

Astroglia, as many other tissue cells *in vitro*, stop migrating after reaching confluency and can be maintained in monolayer cultures for weeks. The density dependent “contact inhibition” of migration prevents overlapping of

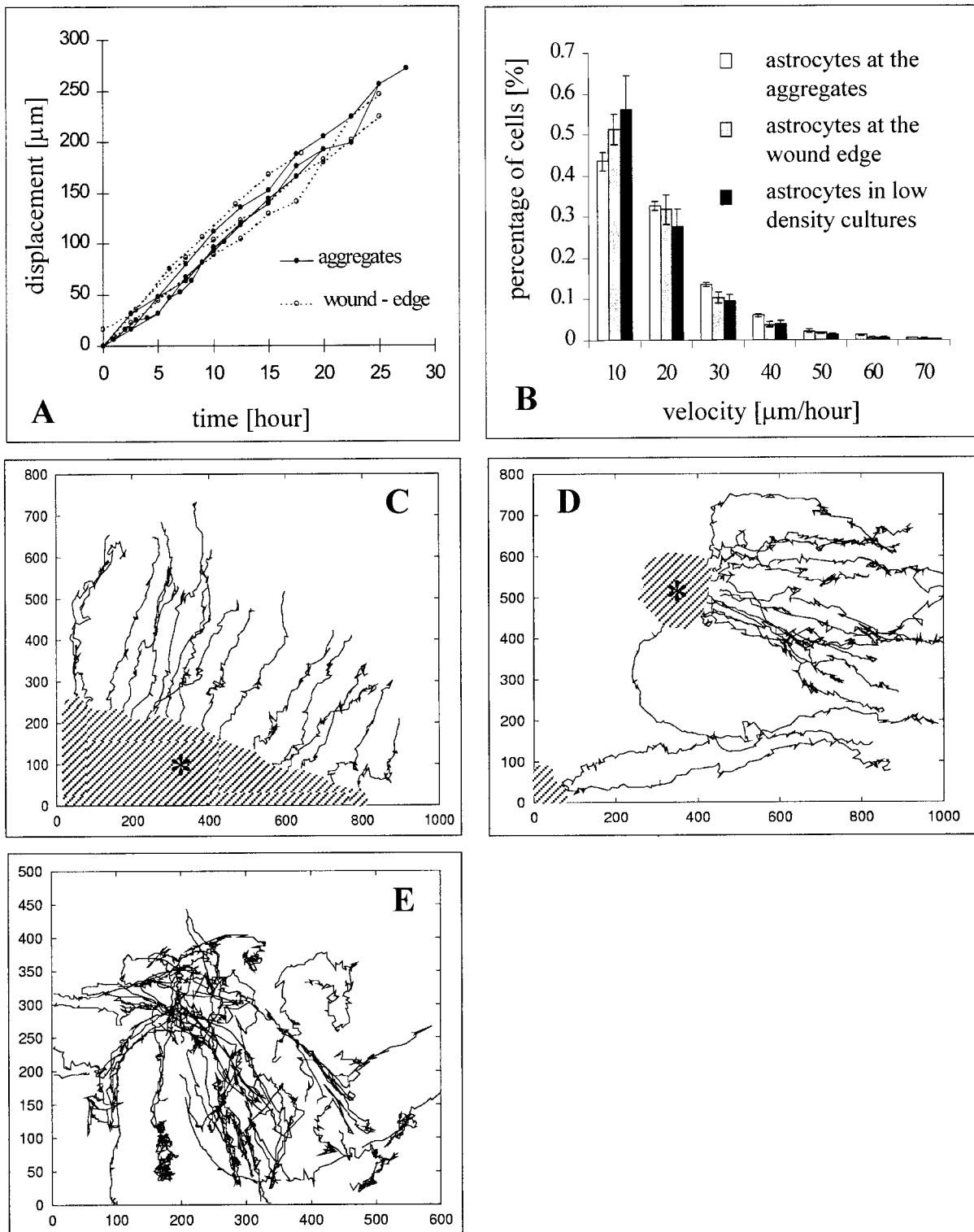


Fig. 4. Expansion velocities of cell assemblies and the migration of individual astrocytes. **A**: The displacement of the front line at the wound (dotted line) and the expansion of the aggregates (straight line) are plotted as a function of time. The zero on the X axis indicates the time-point when cell locomotion was first detected following a quiescent period (maximum 2–3 hr) after scratching or seeding. Each line represent data obtained from independent experiments. **B**: Distribution of cell velocities are shown by summarizing the velocity data (displacement of cell per hour) of individual cells. The relative occurrence of

cells within various velocity-ranges are plotted. Astrocytes at the wound edge did not move significantly faster or slower than those used as controls (Wilcoxon-test, $P = 0.05$). **C–E**: Migratory routes of individual cells. The trajectories represent 48- (C) or 68-hr-long (D) routes of a few individual cells migrating out of the densely populated areas (\star) of the monolayer (C) or of the aggregates (D). In E (68 hr) the random crawling of cells plated with low cell density is shown (E). Scale units: μm .

perykaria (Steinberg, 1973) and is thought to be mediated by cell adhesion molecules (Huttenlocher et al., 1998). Breakup of the cell-cell connections results in locomotory activation. Besides the tempting interpretation of injury-induced activation of locomotion of astrocytes by some specific growth factors and cytokines (Faber-Elman et al., 1995; Hou et al., 1995), the rather unspecific phenomenon of the liberation from contact inhibition (Abercombie et al., 1957; Steinberg, 1973) also has to be taken into account.

Data obtained on the motility of astroglial cells departing either from aggregates or the edges of scratched monolayers showed that mechanical injury did not induce specific alterations in the locomotory activity of cultured astrocytes. Cells migrating out from freshly seeded aggregates displayed a migration-pattern similar to that observed at the edges of the wounded monolayer. Moreover, analyses on the velocity parameters of cell migration failed to reveal differences between randomly crawling individual cells in sparse cultures and cells displaying ordered migration from either non-injured aggregates or wounded monolayers.

Monolayer-forming cells *in vitro* also cease dividing as soon as saturation density has been reached (Abercombie et al., 1957; Huttenlocher et al., 1998; Todaro et al., 1965). Confluent astrocytic cultures can be maintained in such a stable, quiescent state for several weeks. Cells in long-term primary cultures, however, are able to re-enter the cell cycle if released from contact inhibition via trypsinization and subsequent re-plating at small cell density (Langan and Slater, 1992). The mitotic arrest can also be interrupted if mechanical injury creates cell-free areas in the monolayer (Todaro et al., 1965). Migration from confluent areas onto a denuded area, therefore, may result in elevated proliferation both in the zones of immigration and outmigration. Liberation from contact inhibition may mask the potential proliferation-regulating effects of the mechanical injury.

Significant increase in the rate of cell proliferation was detected only within and around the wound, where cell density was decreased by the mechanical trauma. Such enhanced proliferation might be explained assuming that cells inside the scratch represented a highly motile subpopulation sorted out by traumatic conditions and displaying different cell cycle parameters (Seniuk et al., 1994). Our morphological studies, however, failed to reveal any phenotypically distinct populations of astrocytes within the wound.

Astrocytes located within the gap displayed significantly higher mitotic activity than those inhabiting non-injured subconfluent areas with comparable, low cell-densities. Enhanced proliferation in response to scratching was also observed under serum-free conditions, in an environment lacking exogenous growth factors known to regulate the proliferation of astrocytes (Yoshida and Gage, 1991; Schwartz and Nishiyama, 1994; Lotan and Schwartz, 1994; Eclancher et al., 1996; Holland and Varmus, 1998). Our findings indicated that locally increased

mitotic activity, exceeding the rate caused by the liberation from contact inhibition, was a result of the *injury-induced* production of specific, *astroglia-derived* mitogenic factors. Such agents, however, could act only in a range not greater than 1–5 cell bodies.

In summary, our data suggested that the mechanical damage in purified cultures of astrocytes—lacking immune cells responsible for activating the inflammatory cascade—was not sufficient to initiate changes in the locomotory characteristics of astrocytes but resulted in a local enhancement of cell proliferation. The findings indicated that several previously described features of astrocytes in scratch-wounded cultures could have been explained by the release of cells from the density-dependent “contact inhibition.”

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