Diverse Microglial Motility Behaviors During Clearance of Dead Cells in Hippocampal Slices

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ABSTRACT We used two-channel three-dimensional time-lapse fluorescence confocal imaging in live rat hippocampal slice cultures (1–7 days in vitro) to determine the motility behaviors of activated microglia as they engage dead and dying cells following traumatic brain tissue injury. Live microglia were labeled with a fluorescently conjugated lectin (IB4), and dead neurons were labeled with a membrane-impermeant fluorescent DNA-binding dye (Sytox Orange or To-Pro-3). Tissue injury during the slicing procedure induced neuronal death and microglial activation, but the density of dead cells diminished ~10-fold by 7 days in vitro as resident microglia cleared dead cells. In time-lapse movies (4–20 h long), activated microglia exhibited varying levels of motile and locomotory activity. The motility of microglia could change abruptly following contact by other microglia or death of nearby cells. When neighboring cells died, some microglia rapidly moved toward or extended a process to engulf the dead cell, consistent with a chemotactic signaling response. Dead cell nuclei usually were engulfed and carried along by highly motile and locomoting microglia. The mean time to engulfment was ~5 times faster for newly deceased cells (33 min) than for extant dead cells (160 min), suggesting that the efficacy of microglial phagocytosis in situ might vary with time after cell death or mode of cell death. These observations demonstrate that activated microglia are heterogeneous with respect to motile activity following traumatic tissue injury and further indicate that cell motility in situ is temporally regulated at the single cell level, possibly by direct cell-cell contact and by diffusible substances emanating from nearby dead cells. © 2004 Wiley-Liss, Inc.

INTRODUCTION

One of the hallmarks of traumatic brain injury is a rapid microglial response, termed activation (Thomas, 1992; Kreutzberg, 1996; Gonzalez-Scarano and Bartucz, 1999; Raivich et al., 1999). Activating signals produced by injured or dying neurons can induce a dramatic structural transformation in microglia. Activation following severe tissue injury involves a morphological transformation from a highly branched resting state to a more amoeboid or rounded reactive form (Davis et al., 1994). Such morphological changes likely reflect changes in movements and functional behaviors of activated microglia, including motility, migration, and phagocytosis. Indeed, studies in dissociated cell cultures have shown that activated microglia are capable of highly active movements (Booth and Thomas, 1991; Ward et al., 1991; Haapaniemi et al., 1995; Tomita et al., 1996; Takeda et al., 1998). However, the behaviors of activated microglia in situ are still poorly understood.

To gain a better understanding of motile behaviors and cell-cell interactions of microglia in situ, it is essential to investigate these events in a tissue context.
because other resident glia and neurons regulate microglial morphology, motility, and phagocytosis (Siev

ers et al., 1994; DeWitt et al., 1998; Smith and Ho

erner, 2000; Chan et al., 2001; Hailer et al., 2001). Brain slice preparations have become particularly attractive because microglia retain normal anatomical relationships to other neuronal and glial cells, and cells within the tissues are optically accessible. Indeed, time-lapse imaging studies in live tissue slices of mouse corpus callosum showed microglia-like amoeboid cells that could rapidly migrate to the slice surface where they used velum-like processes to screen and phagocytose dead/damaged cells and exogenous erythrocytes (Brockhaus et al., 1996). Similarly, in slices of rat facial nucleus following peripheral axotomy, Schiefer et al. (1999) found that microglia at the slice surface were capable of phagocytosing cells, debris, and contaminating bacteria, although microglia within the tissue depth did not show velum-like pseudopods or phagocytosis. In developing hippocampal slices, ramified microglia withdraw their branches and become highly motile within hours after tissue slicing (Stence et al., 2001), sometimes crawling along blood vessels (Grossmann et al., 2002). These studies in acutely prepared tissue slices demonstrate that recently activated microglia can mobilize and engage dead/dying cells. However, the motile behaviors and phagocytic activities of microglia on subsequent days following tissue injury have not been examined. Do microglia sustain their ability to engage and phagocytose dead/dying cells rapidly? Do microglial motility behaviors change over time? Do all activated microglia behave similarly?

Here we use dual fluorescence labeling and two-channel time-lapse confocal imaging to examine the dynamic microglial behaviors mediating clearance of intrinsic dead cells over the first week in brain slice cultures. Our observations show that activated microglia exhibit diverse motility behaviors that can change suddenly in response to cell-cell contact or cell death. Moreover, even several days after traumatic tissue injury, some microglia maintain a high level of motility and can rapidly respond to spontaneous neuronal death, leading to phagocytic clearance of dead cells within minutes.

Materials and Methods

Brain Slice Preparation and Culturing

The in vitro brain slice preparation (Gähwiler et al., 1997) has served as an attractive model for numerous studies of parenchymal microglial activation and migration (Coltman and Ide, 1996; Hailer et al., 1996, 1997; Bechmann and Nitsch, 1997; Heppner et al., 1998; Czapiga and Colton, 1999; Stence et al., 2001; Grossmann et al., 2002). Preparation of organotypic tissue slice cultures was previously described in detail (Dailey and Waite, 1999). In brief, early postnatal (P4–P6) Sprague-Dawley rats (Harlan) were decapitated and brains were removed in a sterile environment. Hippocampi were removed and sliced transversely (400 μm thick) using a manual tissue chopper (Stoelting). Tissue slices were attached to cover glasses and cultured in HEPES-buffered medium by the roller tube technique (Gähwiler et al., 1997; Dailey and Waite, 1999). Cover glasses (11 x 22 mm) were precleaned (xylene, acetone, alcohol, and distilled water series) and coated with neutralized isotonic Vitrogen 100 (Collagen, Palo Alto, CA). Fresh tissue slices were secured to cover glasses using a plasma/thrombin clot. Tissue slices were cultured for 1–8 days prior to staining and imaging.

Fluorescent Staining

Labeling microglia with FITC-IB₄

To stain microglia vitally, we used a fluorescein-conjugated plant lectin (FITC-IB₄). In CNS tissues, FITC-IB₄ selectively labels microglia as well as endothelial cells of blood vessels (Streit, 1990). Details of the staining procedure were described previously (Stence et al., 2001; Grossmann et al., 2002). Briefly, cultured tissues were incubated for 45 min to 2 h in HEPES-buffered media containing FITC-IB₄ (10 μg/ml; 1:20 dilution from the stock solution in distilled water). During the staining period, some samples continued to roll in the roller drum to promote aeration while others were kept static with tissue face down in the FITC-IB₄-containing media. The latter approach yielded better staining. Staining with FITC-IB₄ even for several days, does not adversely affect microglial behavior (Dailey and Waite, 1999).

Labeling dead cells with membrane-impermeant fluorescent nucleic acid stains

Cells that are dead or dying lose plasma membrane integrity (Majno and Joris, 1995; Sastry and Rao, 2000), permitting nuclear access for small molecules. Consequently, plasma membrane-impermeant nucleic acid stains have been used extensively to label nuclei of dead cells selectively in hippocampal tissue slices (Pozzo Miller et al., 1994; Laake et al., 1999; Hailer et al., 2001; Liu et al., 2001). Four different dead cell markers (all from Molecular Probes, Eugene, OR) were used in these experiments at the following dilutions: DEAD Red, 1:500; Sytox Orange, 1:10,000; Toto3, 1:10,000; or To-Pro3, 1:10,000. The best results, including consistent and rapid penetration of stains into tissues, were obtained using Sytox Orange or To-Pro3, so most experiments (n = 29) utilized one of these dyes. The dyes where diluted in HEPES-buffered media, were bubbled onto the cover glasses (0.50–0.75 ml) following removal from roller tubes, and maintained (10–25 min) in a sterile environment at room temperature. Tissues were then mounted in either fresh
HEPES-buffered media or fresh media containing the working concentration of the nucleic acid stain.

In some experiments, the dyes were included with the imaging media in order to label any cells that died during the imaging session. First, we determined the time course of dye penetration and nuclear labeling in live tissue slices to confirm that dead cell markers could be used to examine neuronal cell death in real time. When fluorescent DNA-binding dyes were applied to slices during a time-lapse imaging session, labeled nuclei appeared at progressively deeper tissue levels over the first few minutes following application. Within 15–30 min, fluorescent nuclei were observed to a depth of > 75 μm. This indicated that the dyes readily penetrate the brain tissue slices and, when present, dead cells are rapidly labeled. The rapid kinetics of nuclear labeling of the DNA-binding dyes suggested that, if maintained in the chamber media, these dyes could be useful for visualizing cell death (loss of membrane integrity) as it occurred. Indeed, we observed spontaneous labeling of individual nuclei in several of our time-lapse sequences. Nearby labeled nuclei in the same optical sections were usually evident for several hours with little or no change in intensity prior to appearance of a new cell nucleus, supporting the idea that the rapid appearance of new nuclei was due to a sudden change in cell membrane permeability (i.e., cell death) rather than to dye accessibility or to focal plane drift.

Double labeling of microglia and dead cells

For many experiments, tissue slices were stained with both FITC-IB₄ and a dead cell marker. Dead cell stains sometimes seemed to reduce stainability of microglia with IB₄, so the labels were applied sequentially: IB₄ staining was performed first, then nucleic acid stain was applied, as indicated above.

Time-Lapse Confocal Imaging

Methods for confocal imaging in live brain tissue slices are described elsewhere in detail (Dailey and Waite, 1999; Stence et al., 2001). We used a Leica TCS NT scanning laser confocal microscope equipped with a 20×/0.7 PL APO objective and two epifluorescence channels (Leica, Heidelberg, Germany). FITC-IB₄-labeled microglia were examined using an Argon laser (488 nm), and dead cells were examined with either a Krypton (568 nm) or Helium-Neon laser (633 nm), depending on the particular stain. For simultaneous two-channel imaging, we used one of two fluorescent filter configurations. To image FITC-IB₄ and Sytox Orange/DEAD Red, we used an FITC-TRITC filter configuration that utilized a double-dichroic primary beam splitter (488/568 nm). The fluorescent emission was split into two channels (580 nm beam splitter), and barrier filters (530/30 nm band pass or 590 nm long pass) were positioned in front of the channel 1 or 2 detector, respectively. To image FITC-IB₄ and To-Pro-3/Toto-3, a similar filter arrangement was used except that in channel 2 the 590 nm barrier filter was replaced with a 645 nm long-pass barrier filter. There was significant bleed-through of the most intense Sytox Orange signal into the FITC channel (appearing as yellow in merged two-channel images), but these signals were nevertheless clearly distinguishable since there was no bleed-through from FITC (green) into the TRITC (red) channel. No cross-talk or bleed-through was observed between the FITC and To-Pro-3 signals.

All experimental observations reported here were made in hippocampal area CA3, but comparable observations were made in other hippocampal regions. Stacks of images were collected at 2- to 4-min intervals and contained 5–10 optical sections (512 × 512 pixel array; average of three scans per optical section). Optical sections were spaced approximately 5–8 μm apart, with each stack of images spanning 20–72 μm of depth. As a compromise between fluorescence throughput and axial resolution, the confocal (detector) pinhole aperture was set to 1.7–2.0 Airy disks. Slices were examined at 1–8 days in culture. Sequences were typically imaged for 2–11 h. Slices were often mounted and viewed in HEPES-buffered media that contained a small concentration of the nucleic acid stain. The present results are based on analysis of 40 separate time-lapse experiments spanning a total of 257 h of observation.

Quantifying Cell Death

To quantify the number of dead cells, projection images of through-focus stacks were examined at the 1-h time point in each time-lapse series. Most dead cell nuclei were labeled after 20–30 min of staining. By waiting 1 h, this ensured that the nucleic acid stain had ample time to penetrate the tissue. Stacks of optical sections were flattened into a two-dimensional projection image, converted into a threshold image, and analyzed for number of dead cells using the particle count feature in Image-J (Scion, Frederick, MD). Counts were normalized for tissue volume (i.e., the total number of dead cells in each projection image divided by the total volume of tissue imaged). Where dead cells are most dense, the measure may be a slight underestimate since the analysis was performed on two-dimensional projection images and nuclei at different tissue depths may be overlapping and thus counted as one cell.

Generating Two-Channel Three-Dimensional Time-Lapse Movies

Digital time-lapse movies were made as described in Stence et al. (2001). Using Scion Image, separate projection images of each channel were made for each time
The FITC-IB₄ projection image was pseudocolored green and the projection image of the dead cell nuclear stain was colored red. Then, for each time point, the red and green images were merged into a single RGB image, and the series of images was imported into Adobe Premiere and compiled into a digital movie.

RESULTS

Neuronal Death in Slice Cultures

The preparation of live brain slices from early postnatal rats induces tissue trauma leading to neuronal cell death (Pozzo Miller et al., 1994). To assess the time course and pattern of cell death in our rat hippocampal tissue slice cultures, we used a membrane-impermeable fluorescent DNA-binding probe (Sytox Orange or To-Pro-3) to label dead cell nuclei in live tissue slices at successive stages over the first week in culture. Most of the dead cell nuclei were confined to neuronal cell body layers in areas CA3, CA1, and the dentate gyrus, consistent with their identification as principal neurons. We quantitatively assessed neuronal cell death in area CA3, which showed prominent cell death. As shown in Figure 1, the density of labeled dead cell nuclei was highest on the first day in vitro (DIV; 449 ± 180 cells/mm³ × 100) and diminished about 10-fold by 7 DIV (43 ± 15 cells/mm³ × 100).

Our time-lapse observations indicated that very few cells die between 1 and 7 days in vitro, suggesting that the dead cell staining patterns observed on these days largely indicate remnants of cells that died within the first few hours in culture rather than ongoing cell death. Interestingly, a small fraction of dead cells persisted indefinitely in slice cultures (> 6 DIV), and these cells usually had elongated nuclei and often were organized in linear arrangements outside of neuronal cell body layers. Double labeling of tissue slices with a dead cell marker and with IB₄, which labels microvessels as well as microglia, showed that the persisting dead cells were closely associated with microvessels. These cells were immunonegative for the ED2 antigen, a marker of perivascular cells (data not shown), and are likely en-
Diverse Motile Behaviors of Activated Microglia

Resident microglia that are activated in tissue slices likely play a major role in clearing dead cells, but the ongoing dynamic behaviors of individual microglial cells have not been examined in traumatized tissues. Previously, we showed that some activating microglia in tissue slices can withdraw all extant branches and begin to locomote within a few hours after tissue slicing (Stence et al., 2001). Here, we further assessed microglial responses quantitatively and found a diversity of activation responses and rates of activation. We classified microglia according to their dynamic behaviors, assessed in time-lapse sequences, using the following three categories: immotile microglia, i.e., cells that did not significantly remodel existing processes or that withdrew existing processes but did not produce new processes within the elapsed time (corresponding to R- and W-stages of Stence et al. (2001)); motile-only microglia, i.e., cells that resorbed extant processes and extended new processes but where the cell body did not translocate a distance greater than one cell body diameter (corresponding to T- and M-stages); and motile and locomotory microglia, i.e., cells showing dynamic extension and retraction of cell processes and where the cell body moved a distance greater than one cell body diameter (corresponding to L-stage). Quantitative analysis of microglial behaviors (n = 86 cells in three tissue slices) at 8 h following tissue slicing showed a distribution of microglial cells within these motility categories: 43% ± 5% immotile; 19% ± 5% motile only; and 38% ± 8% motile and locomotory. Even after 7 days in vitro, when most microglial cells showed an activated morphology, there was a similar range of microglial motility behaviors (Fig. 2). These time-lapse observations reveal a diversity of microglial motility behaviors during and after activation, even among cells with a similar morphology. Moreover, they indicate that morphological appearances in static images are not necessarily accurate predictors of cell motility behaviors.

Time-lapse sequences also showed that motile activity of microglia could change abruptly, sometimes changing after contact by other motile microglia. Figure 3 shows an example of two highly motile and locomotory microglia moving among other quiescent immobile microglia. Following contact by the locomotory microglia, the formerly quiescent microglia showed either increased motile activity or signs of shrinking and blebbing, which may indicate cell death. It is noteworthy that newly activated microglia showing enhanced motile activity seemed, in turn, capable of inducing higher levels of motility in neighboring microglia. Not all cell-cell contact events induced changes in cell shape or motility. These observations suggest that motility behaviors of individual activated microglia are regulated dynamically over time, and that this may involve cell contact-mediated signaling mechanisms.
Rapid Response of Activated Microglia to Spontaneous Cell Death

The above observations describe interactions between activated microglia and extant dead cells that presumably had died at the time of tissue slicing, an event occurring from 1 to 8 days earlier. We next explored the response of microglia to acute spontaneous cell death as it occurred in real time. In 25% of time-lapse sequences (10 of 40 slices imaged), at least one cell nucleus became labeled with a dead cell marker during the period of observation. In 95% of these instances, there was a total of 1–5 dead cells that appeared during the observation. In two sequences, there was massive cell death in which hundreds of cells became labeled, which likely indicates deterioration of overall slice health. Excluding these two sequences, we observed 16 dead cell nuclei appear spontaneously in 8 different slices, all on days 3–6 in culture. The new dead cells first appeared from 1 to 9 h (mean, 5.4 h) after the start of the time-lapse observation. No dead cell nuclei appeared spontaneously during imaging sessions on the first two days in culture, despite ~70 h of imaging in the presence of dead cell indicators (n = 10 slices).

Our time-lapse observations revealed changes in behaviors of nearby microglia when cells died. Cell death often, but not always, occurred near a spheroid microglial cell. The cell death event shown in Figure 5 occurred coincident with a transient shape change (rounding) of an adjacent microglial cell. Over half of the dead cell nuclei that appeared spontaneously during a time-lapse session were rapidly engulfed by microglia. In some cases, this occurred by extension of a microglial cell branch directly toward the labeled nucleus. The nucleus was engulfed and transported back toward the cell body of the microglia as the process retracted (Fig. 6). The rapid and directed extension of microglial processes following immediately after nuclear labeling is consistent with the possibility that the microglia were responding to a...
chemoattractant signal emanating from the dead cells.

Our impression was that microglia engulfed newly deceased cells more readily than extant dead cells. To investigate this quantitatively, we determined the time to nuclear engulfment following initial microglial contact. Engulfment of extant and spontaneously appearing dead cells was assessed in the same fields of view at the same postslicing time points. For those spontaneously appearing dead cells that were engulfed during the imaging session, the average time to engulfment was $33 \pm 4.5$ min ($n = 7$). This was significantly ($P < 0.0001$) faster than clearance of extant dead cell nuclei, likely killed during the initial slice preparation, which were engulfed on average $160 \pm 13.2$ min ($n = 23$) following microglial cell contact. These data suggest that the efficacy of microglial clearance of dead cells might decrease with increasing time after cell death. Alternatively, differences in rates of phagocytosis might be related to different modes of target cell death.

Fig. 3. Motility state of individual microglial cells can change rapidly. Time-lapse sequence shows several IB$_4$-labeled microglial cells in a tissue slice cultured for 7 days. A: Cells were false-colored to highlight differences in motility states. Two active, locomotory cells are shown in green (1 and 2). White cells are quiescent microglia showing little or no motility. Cell 1 contacts a quiescent immotile cell (3) at 38 min. Subsequently, cell 3 shrivels, blebs, and dies (red in 100–180 min). Blebs (arrows, 140 min) are characteristic of apoptosing cells. Cell 1 returns to ingest part of the remnants of cell 3 (arrow, 180 min). Meanwhile, cell 2 contacts a quiescent cell (4 at 38 min) that rapidly shows greatly enhanced motility following contact. In turn, cell 4 contacts a neighboring cell (5 at 140 min), which subsequently also shows enhanced motility. In the time-lapse movie, the impression is that a motility stimulus is passed in series from cell 2 to cell 4 to cell 5.

B: Difference images showing changes in motility of microglial cells shown above. To highlight movements of cells, images from sequential time points were digitally inverted and subtracted from each other. Dark regions in the images correspond to differences in pixel intensity between two time points and thereby identify locations where cell structure is changing rapidly. Initially, only cells 1 and 2 were changing significantly and thus showing prominent dark regions at the expanding leading edges (arrowheads, 21 min) and at the retracting trailing edges (arrows, 21 min). Subsequently, cell 4 (arrowheads at 66–111 min) and then cell 5 (arrowheads at 142–175 min) become more active. Motility changes can be best appreciated by viewing the three-dimensional supplementary video, presented as red-green stereo images. These observations indicate that microglia with an activated form can show very different motility behaviors, that these behaviors can change suddenly, and that cell-cell contact among microglia may induce these transitions in some instances.
Fig. 4. Time-lapse sequences showing phagocytic engulfment of dead cell nuclei by motile and locomotory microglia. 

A: Merged two-channel image of FITC-IB4-labeled microglia (green) and To-Pro-3-labeled dead cell nuclei (red) in a live brain slice culture (P6 + 2 DIV). The image represents a composite of nine optical images spanning 40 µm in depth. 

B: Time-lapse sequence shows phagocytic clearance of dead cell nuclei (arrowhead) by a motile locomotory microglial cell (arrow). Note that the microglial cell maintains a rapid rate of locomotion as it sweeps over and picks up the dead cell nucleus. 

C: Another locomotory microglial cell (arrow, 0 min), moving at a speed of ~ 80 µm/h, extends a 50 µm long process (arrow, 6 min) directly toward a target nucleus (arrowhead). The process contacts and engulfs the nucleus, which is then gathered into the microglial cell body (arrowheads, 33–64 min) as the cell continues to move forward rapidly. See the supplemental video movie.

Fig. 5. Microglia rapidly engulf cells that die spontaneously in slice cultures. Some cells die during the imaging session, thus becoming labeled when nuclear dyes are present. These dead cells often appear near spheroid microglia and are usually engulfed by microglia within 30–60 min. This series shows a dead cell appearing spontaneously (arrowhead at 41 min). Note that a spheroid microglia (open arrow at 41 min) already contains an engulfed nucleus and is directly adjacent to a newly emerged dead cell (arrowhead at 41 min). The time-lapse movie shows that the adjacent microglial cell, which was formerly motile, ceases motility and rounds up before the dead cell nucleus appears. A second, ameboid-like microglia (solid arrow at 0 and 34 min) moves in and engulfs the dead cell nucleus (arrowhead at 75 min) about 40 min after its first appearance. The spheroid microglial cell then resumes its motile activity, and the two microglia (solid and open arrows, respectively, at 163 through 346 min) now move away from each other, each carrying an engulfed nucleus. Images are selected composites of four optical sections representing 20 µm of depth. Slice culture is P5 + 3 DIV. The first time point (0 min) is approximately 20 min from the start of imaging and 70 min from the start of the staining procedure. See the supplemental video movie.
Transfer of Phagocytosed Nuclei Between Microglia

Typically, whole nuclei were engulfed and were carried intact by locomotory microglia. The size of engulfed nuclei usually diminished very slowly. Some nuclei showed little change after being engulfed and were still evident within moving microglia after 10 h of imaging. Some microglia ingested more than one nucleus, occasionally even undergoing a mitotic division and partitioning the nuclei to different daughter cells. Thus, while engulfment of deceased cells could be quite rapid (within minutes), degradation of ingested nuclei often was relatively slow (several hours). Only twice did we see a labeled dead cell nucleus that fractured into smaller pieces before being engulfed and eventually disappearing within the microglia (data not shown).

Although most engulfed nuclei remained within the phagocytosing microglial cell over the observation time period, in a few instances the nucleus was shuttled from one microglial cell to another. In the example shown in Figure 7, a single nucleus is rapidly transferred between two adjacent motile microglia. Such a transfer was observed even after engulfment of a newly deceased cell nucleus, occurring within 1–2 h after the initial engulfment. This indicates diverse means by which dead cell components may be routed to microglia, either directly by phagocytosis or indirectly by transfer from other phagocytosing microglia.

DISCUSSION

In this study, we examined the real-time dynamics of cell death and microglial phagocytosis of intrinsic dead cells in mammalian brain tissues. We show that motile behaviors vary among activated parenchymal microglia in situ, and that individual microglial cells can rapidly change their motile activity in response to death of nearby cells or following contact by neighboring microglial cells. These data suggest that microglia motility behaviors supporting rapid clearance of dead cells are regulated in time on an individual cell basis. Regulation of motile behaviors may involve direct cell-cell contact as well as diffusible substances released from nearby dying cells.

Cell Death in Tissue Slices

We used in vitro slice cultures as a model of traumatic cell death and clearance in CNS tissues. The timing and pattern of cell death in our rat hippocampal slice cultures is consistent with previous studies in tissue slices (Pozzo Miller et al., 1994; Behl et al., 1997; Skibo et al., 2000; Hailer et al., 2001), which together indicate that the cellular degeneration accompanying the slicing procedure has ended by 7 DIV. In these assays, dead cells become labeled with membrane-impermeant fluorescent markers following membrane lysis (Macklis and Madison, 1990; Majno and Joris, 1995). Our data show that most cell death occurred on the first day in vitro because very few new dead cells appeared spontaneously in time-lapse sequences collected on subsequent days (2–7 DIV). In many previous studies, morphological features have been used to help distinguish between necrotic and apoptotic cell death. Apoptotic death often involves nuclear condensation, disintegration, and cytoplasmic boiling. In contrast, necrotic death involves plasma membrane ballooning (oncosis) and lacks nuclear breakdown and disintegration (karyorrhexis). In our images, most labeled nuclei appeared as single intact spheres, although somewhat condensed. In time-lapse sequences, nuclear disintegration was seen only rarely. Moreover, staining of slice cultures with annexin-V, a marker of cell surface phosphatidylserine thought to be necessary for phagocytosis of apoptotic neurons (Witting et al., 2000), showed only a few scattered cells (data not shown). Based on the rapid appearance of dead cells immediately following tissue slicing, the paucity of annexin-V labeling, as well as morphological features of dead cell nuclei, we conclude that most of the cell death was necrotic. However, there may be some undetected apoptotic cell death in the slice cultures that could regulate the motility and phagocytic behaviors of microglia.

Motility States of Activated Microglia

Several previous studies have shown that brain resident phagocytes (microglia) become activated in CNS tissue slice preparations, presumably in response to neuronal injury and death (Smith et al., 1990; Brockhaus et al., 1996; Hailer et al., 1996; Czapek and Colton, 1999; Koshinaga et al., 2000; Stence et al., 2001). Although by 1 DIV, most microglia had a morphological appearance of being fully activated, our time-lapse observations showed that individual microglia differed dramatically in their motile behaviors. Some microglia with an activated form were highly motile, whereas others were immotile. The immotile microglia were not dead as assessed by staining with dead cell DNA markers. In fact, microglia showing little or no motile activity could abruptly shift to a higher level of activity. This supports the idea that microglial activation following trauma is not “all or nothing” but rather encompasses distinct states or stages, here reflected in differences in motile capacity, and that individual microglia are able to switch rapidly between stages of activation. Our observations also suggest that signaling between microglia can regulate their motility and possibly their survival, and that this may be mediated by direct cell-cell contact. Future
work will need to clarify the molecular bases of these signaling events.

**Microglia Mobilization and Clearance of Dead Cells**

Although microglia must physically move and change shape in order to phagocytose dead cells, it has been unclear how they locate and engage dead cells in situ. When activated microglia are added to hippocampal tissue slices damaged by excitotoxicity, they preferentially accumulate at sites of injured and dead neuronal cell bodies (Heppner et al., 1998). This preferential accumulation could occur by directed migration (chemotaxis), by selective retention of activated microglia moving randomly (undirected) into regions of degeneration, or by local proliferation of microglia. Consistent with the possibility of long-range chemotactic signaling, time-lapse studies in the intact leech have shown that individual microglia can move directly toward sites of nerve injury (McGlade-McCulloh et al., 1989). Indeed, several studies have proposed that microglial chemotaxis and chemokinesis are regulated by secreted factors, including macrophage colony-stimulating factor (Raivich et al., 1991, 1994), macrophage inflammatory protein (MIP) 1α and 1β (Schmidtmayerova et al., 1996; Cross and Woodroofe, 1999), macrophage chemoattractant protein-1 (Calvo et al., 1996), complement factors C3a and C5a (Nolte et al., 1996; Möller et al., 1997), and epidermal growth factor (Nolte et al., 1997). Together, these studies raise the possibility that microglia are directed via chemotactic signals toward regions of degenerating cells in injured mammalian brain tissues.

Using real-time imaging of cell death and microglial movements in tissue slices, we show here that microglia in mammalian brain tissues can sense and respond directionally and rapidly (within minutes) to the death of single cells. Microglial processes extend directly toward dead/dying cells from a distance of tens of microns, indicating that the death of a single cell is sufficient to induce a directed motility response in nearby microglia. It should be emphasized that these motile behaviors occur in response to natural sources of cell death cues operating over physiological distances and

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**Fig. 6.** Motile microglia extend processes directly toward newly deceased cells during phagocytic clearance. A microglial cell (arrow, 0 min) extends a long process (arrow, 44 min) directly toward a spontaneously appearing dead cell nucleus (arrowhead at 3 and 6 min). The dead cell nucleus is engulfed about 40 min after it first appeared (at 3 min). The engulfed nucleus is then dragged away by the same phagocytic microglia (92–140 min). Note that other nearby microglial processes do not respond to the dead cell, suggesting that the capacity to respond rapidly to cell death varies among activated microglia. Images are composites of three optical sections spanning 15 μm in depth. Tissue is P6 + 3 DIV. See the supplemental video movie.

**Fig. 7.** Engulfed nuclei can be transferred from one microglial cell to another. A: An engulfed nucleus (yellow-red, arrowheads) is transferred (93–104 min) between two adjacent motile microglia (arrows). B: Red-green three-dimensional stereo images of the same cells showing that the cell bodies of the two adjacent microglia are in different optical planes. Arrows indicate the position of the engulfed nucleus as it is transferred, moving from one optical plane to another. The original z-image stack was composed of seven optical sections spanning 35 μm. For clarity, each image here represents a composite of two optical z-sections spaced 5 μm apart. Use red–green stereo glasses to view depth. Tissue is P6 + 2 DIV. See the supplemental video movies.
time scales. Given the rapid and directed response of microglia toward actively dying cells, it is likely that microglia sense substances emanating from lysed cells. We suspect that when the plasma membrane of a dying cell becomes leaky, the dead cell marker gains access to nuclei coincident with leakage of internal cell contents into the extracellular space. These cell death cues could include nucleotides or other chemoattractants released from damaged cells and acting at microglial cell surface receptors to orient microglial movements (Yao et al., 1990; Neary et al., 1996; Honda et al., 2001).

Intrinsic microglia were able to clear all but a small fraction of dead cells in tissue slices even in the absence of signals from invading hematogenous cells. We found that activated parenchymal microglia engulfl dead cells rapidly in some instances, whereas in other instances they do so very slowly and only after extensive palpations. The time lag from first contact to engulfment of intrinsic dead cells was variable, similar to what was found that activated parenchymal microglia engulf dead cells rapidly in some instances, whereas in other instances they do so very slowly and only after extensive palpations. The time lag from first contact to engulfment of intrinsic dead cells was variable, similar to what was seen for removal of exogenous red blood cells applied to mouse tissue slices (Brockhaus et al., 1996). However, unlike the rapid shrinking and disappearance (~ 30 min) of phagosomes containing red blood cells that lack nuclei (Brockhaus et al., 1996), digestion of the engulfed dead cell nuclei (as defined by condensation of phagosomes) was very slow, occurring over a period of hours. This slow nuclear digestion did not appear to impede further phagocytic uptake or to affect other microglial behaviors adversely because individual microglial cells were able to maintain high motile activity, to translocate rapidly, and even to progress through mitotic division after engulfing several dead cell nuclei.

As noted above, not all microglia with an activated form were motile. Differences in motile activity may underlay functional differences within the population of activated microglia. Previous studies working in dissociated cell cultures, in tissue slices, and in vivo suggest that intrinsic microglia may be comprised of a diverse population of phagocytic and nonphagocytic microglia (Amat et al., 1996; Brockhaus et al., 1996; Bechmann and Nitsch, 1997; Bohatschek et al., 2001). Our time-lapse observations indicate that motile activity is another aspect of functional diversity among microglia. Although there may well be phenotypically distinct subpopulations of microglia that are especially equipped to participate in phagocytic clearance and/or degradation of dead cells, our observations also indicate that individual microglial cells can switch between motile states. The nonmotile microglia may represent a reserve population of readily activated cells that could be quickly stimulated to a higher state of activation as needed. Differences or changes in microglial motility and phagocytic behavior could depend on differences in expression or activation of cell adhesion molecules (Hailer et al., 1997; Witting et al., 2000; Hartlage-Rubsamen and Schliebs, 2001), growth factor receptors (Nolte et al., 1997), cytokines (von Zahn et al., 1997; Milner and Campbell, 2002), structural proteins (Abdel-Basset and Fedoroff, 1994; Ohsawa et al., 2000), or other signaling molecules.

In summary, our results indicate that activated microglia can persist in a highly motile state for at least several days after traumatic tissue injury. This persisting motile activity supports efficient clearance of cells injured during the initial insult and enables microglia to respond rapidly to ongoing cell death. Moreover, we found that microglial behaviors are dynamically regulated over time on a cell-by-cell basis. Future studies will need to probe the mechanistic bases of cell-cell interactions regulating microglial motility over time. However, the ability to monitor microglial behavior and function in situ at the single cell level should help elucidate the diverse functions of activated parenchymal microglia following traumatic CNS injury.

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