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Entrapment of Migrating Hippocampal Neural Cells in Three-Dimensional Peptide Nanofiber Scaffold

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ABSTRACT

Isolation and expansion of self-renewing neural cells *ex vivo* are required for neural tissue repair in regenerative medicine. Neurogenesis occurs in restricted areas of postnatal mammalian brain including dentate gyrus and subventricular zone. We developed a simple method to entrap migrating neural cells (potential neuroprogenitors) from postnatal hippocampal organotypic cultures in three-dimensional (3-D) peptide nanofiber scaffolds. A few hours after placing the hippocampal slices in culture, cell proliferation activity at the "interface zone" between the tissue slice and the membrane culture surface was observed. Pulse–chase experiments using 5-bromodeoxyuridine (BrdU), which measures mitotic activity, showed that a number of cells incorporated BrdU at the interface zone. The number of $BrdU^+$ cells increased exponentially during the first 3 days of exposure to the label. The $BrdU^+$ cells also stained positive for glial fibrillary acidic protein (2.2 ± 0.5%), a marker for astroglia; and for βIII tubulin (7.3 ± 2.8%) and nestin (2.7 ± 0.9%), markers for neural progenitors. When hippocampal slices were cultured on a peptide nanofiber scaffold layer (~500 μm thick), a more extended interface zone between each tissue slice and the scaffold was formed. Moreover, the migrating $BrdU^+$ cell population entrapped in the 3-D peptide scaffold was readily isolated by mechanically disrupting the scaffold and then used for conventional 2-D culture systems for further studies. This simple method may be useful not only in developing technology for neural progenitor cell isolation and enrichment *in vitro*, but also for expanding cells for cell-based therapies of regenerative medicine.

INTRODUCTION

DISCOVERY AND DEVELOPMENT of new biological materials and methods are often required to advance scientific and medical technologies. We have previously described a class of peptide scaffold consisting of >99.5% (w/v) water content made through spontaneous assembly of ionic self-complementary peptides that form well-ordered nanofibers.[1–5] Several peptide scaffolds supporting cell attachment and function of a variety of mammalian primary and tissue culture cells as well as differentiation of adult liver-derived stem cells[2–4,6] were reported. One of the peptide scaffolds, RAD16-I, supports the differentiation of rat PC12 cells and also rat hippocampal neurons that form functional synapses, when cultured on the peptide scaffold surface.[3] When the same peptide was used to culture liver-derived stem cells in three-dimensional systems, the stem cells differentiated into functional hepatocyte-like cells.[6] This class of molecularly designed biological material has several advantages: (1) the nanofiber network mimicks the extracellular matrix context and likely provides a truly 3-D

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environment for cells where they can grow, migrate, divide, or extend processes (this is usually differentiation, not proliferation); (2) the peptide scaffolds can be naturally degraded over time in tissues producing only amino acids; (3) the constituent peptides are chemically synthesized so that they are free of pathogens, growth factors, viruses, and contaminants. The use of molecularly engineered materials is the key element for future applications in reparative and regenerative medicine. The combination of stem cell biology and bioengineered biological material is an emerging area for future cell-based therapies.[6–8]

Several independent studies have reported putative progenitor cells in adult tissues, which implied that these progenitor cells may play a role in cell renewal and replacement of injured or senescent tissues.[9–11] For instance, some reports suggested that these adult progenitor cells eventually differentiate into various cell types comprising not only the specific tissue in which they reside, but even different, unrelated tissues.[12–16] For instance, when exposed to a fetal environment, adult hematopoietic stem cells can be reprogrammed to behave like embryonic hematopoietic stem cells.[17] Subsequent studies in various animals have indicated that the adult CNS contains a subset of somatic neural stem cells (NSCs) that are able to undergo self-renewal.[18–22] These multipotential cells originate in two specific areas of the brain: the subventricular zone (SVZ) and the dentate gyrus area of the hippocampus in animals.[18–20] In addition, it has been reported that oligodendrocyte precursor cells within the CNS might undergo in certain conditions cellular reprogramming, producing multipotent stem cells that can not only self-renew but also give rise to neurons and glial cells, including oligodendrocytes.[22] Moreover, it was reported that astrocytes from the SVZ can also reprogram, giving rise to immature precursors and neuroblasts and thus acting as neural stem cells.[23]

We report here the development of a 3-D culture and cell entrapment system using the self-assembling peptide scaffold RAD16-I for culture of hippocampal slices and isolation of potential neuroprogenitor cells from the dentate gyrus region. The migrating cell population was readily enriched and entrapped by culturing the rat hippocampal slices on a layer (~500 μm) of peptide scaffold that supported tissue growth and enhanced cell migration. From 1-week cultures, glial cells and neurons progressively invaded the peptide scaffold, 400–500 μm from the edge of the tissue slice. Cells entrapped in the migration zone were readily collected with a micropipette and then used to initiate cultures on laminin-coated glass coverslips covered by growth medium. The entrapped neural cells maintained their mitotic activity for ~3 days, as assessed through incorporation of 5′-bromodeoxyuridine (BrdU) into their DNA, after which they were shown to be a mixed population of glia and neurons.

MATERIALS AND METHODS

Hippocampal organotypic slice cultures

Hippocampal slices were prepared according to the technique previously described.[24] Hippocampal tissue slices were isolated and prepared from 7.5-day-old postnatal rats. Each experiment was repeated three times and in each case was performed with 6 hippocampal slices collected from 2 or 3 different postnatal rats (total of 12–18 slices). The basic hippocampal organotypic slice culture technique consists of placing a hippocampal slice, 200 μm thick, or individual hippocampal region tissues (termed area I) on top of a culture membrane insert (Millipore Millicell-CM, 30-mm culture plate insert with 0.4-μm pore; Millipore, Bedford, MA)[25]; medium is then added at the bottom of the culture plate well to prevent disruption of the tissue slice. In the present work, we developed a new hippocampal organotypic slice culture technique consisting of placing the tissue slices on top of a preformed peptide hydrogel layer (~500 μm thick) equilibrated with the same culture medium (Fig. 1). Briefly, the peptide RAD16-I (AcN-RADARADARADARADA-COH_2; 3DM, Cambridge, MA) was resuspended in deionized water at a final concentration of 0.5% (w/v). The solution was sonicated for 10 min and then loaded on a culture insert until a homogeneous layer formed. Self-assembly of the peptide was accelerated by adding culture medium to the bottom of the culture well. To clarify, the medium diffused through the membrane insert and induced the peptide to gel. The hydrogen was equilibrated in this way with medium for 1–2 h in the cell culture incubator before placing the tissue slices on top. Medium composition (250 mL) was as follows: minimal essential medium (MEM), 194 mL; horse serum (heat inactivated), 50 mL; 1.4 mM L-glutamine; 30 mM D-glucose; 5 mM $NaHCO_3$; 1 mM $CaCl_2$; 2 mM $MgSO_4$; 30 mM HEPES; 30 mg of ascorbic acid; 0.25 mg of insulin; the pH was adjusted to pH 7.3 with NaOH. Slices were then incubated at 37°C in a humidified incubator chamber equilibrated with 5% CO_2 for up to 2 weeks.

General immunostaining

The tissue slices were washed with phosphate-buffered saline (PBS) and fixed in 2% paraformaldehyde in PBS (pH 7.4) at room temperature for 2 h with slow shaking. The slices were washed several times in PBS and subsequently treated with 0.1% Triton X-100 in PBS for 2 h at room temperature. At this time, blocking buffer (20% bovine calf serum, 0.1% Triton X-100, and 1% dimethyl sulfoxide [DMSO] in PBS) was added to the samples, which were then incubated for 4 h at 4°C with slow shaking. Wherever possible, fluorescently conjugated monoclonal primary antibodies (diluted in the same blocking buffer) were used to minimize cross-reactivity. The an-

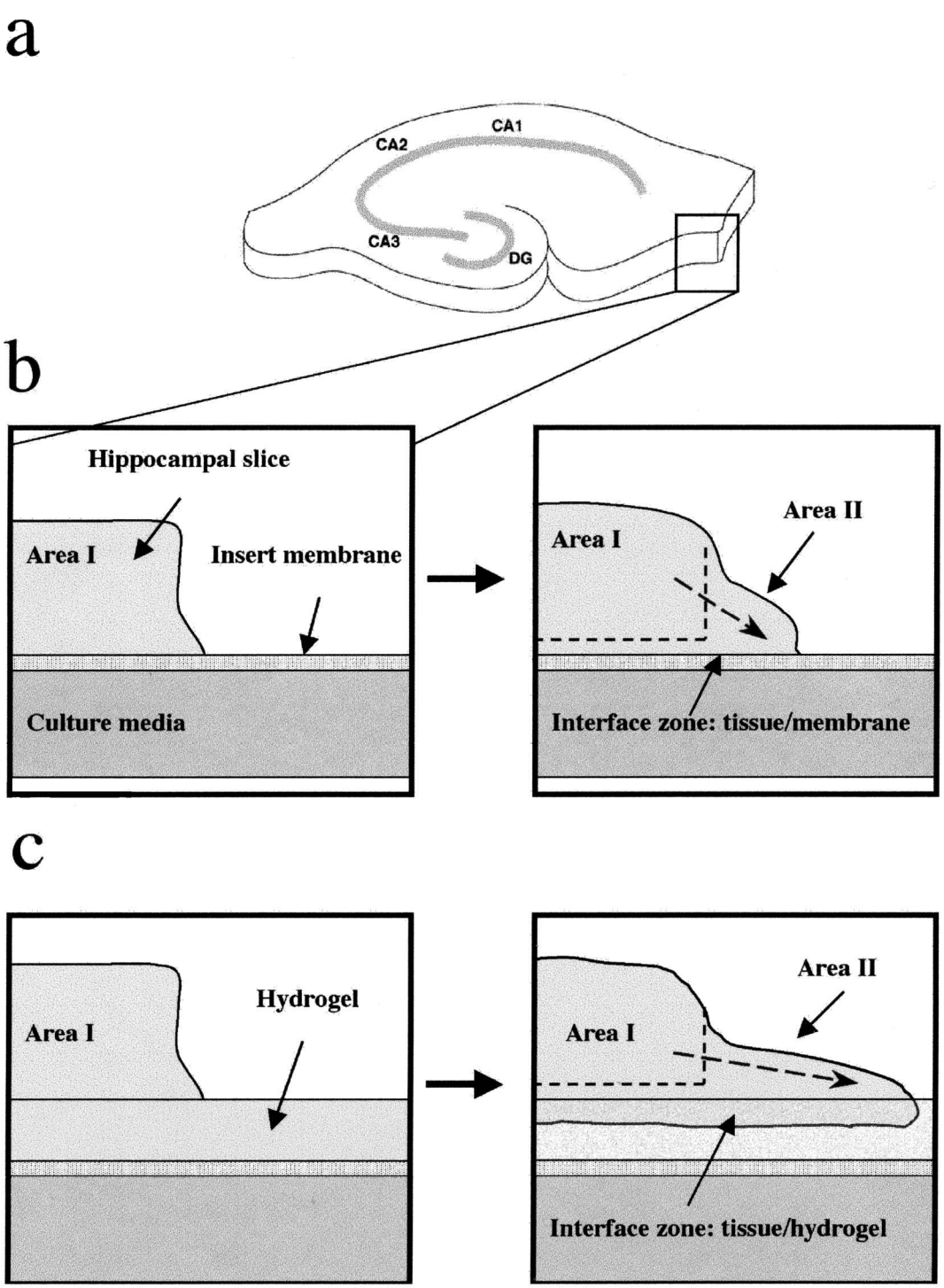

FIG. 1. Layout of interface zone defined between the tissue slice and the Millipore culture membrane in a typical organotypic hippocampal slice culture. The organotypic cultures were performed as previously described.[26] (**a**) Schematic representation of a hippocampal slice with the main defined regions: dentate gyrus (DG), CA1, CA2, and CA3. (**b**) Hippocampal slice placed on top of a Millipore membrane. (**c**) Hippocampal slice placed on top of a layer (~500 μm) of self-assembling peptide scaffold. Left panel in (**b**) and (**c**) represents a cross-section of the hippocampal tissue edge in each case immediately after preparation. The right panel represents the same cross-section after days in culture, during which the cellular scaffold has been formed, delimiting extended area II away from area I. Area I is defined as the original tissue placed on top of the culture membrane whereas area II represents the new cellular scaffold or "interface zone" extended away from the edges of area I. Dashed arrows indicate the direction of cellular scaffold extension and growth.

tibodies used here were as follows: goat polyclonal IgG anti-glial fibrillary acidic protein (GFAP) (sc-6170; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal IgG1 antibody anti-neuronal nuclei (NeuN) (MAB377; Chemicon International, Temecula, CA), mouse monoclonal IgG1 antibody anti-nestin (60051A; BD Biosciences Pharmingen, San Diego, CA), and mouse monoclonal antibody anti-βIII tubulin (G7121; Promega, Madison, WI). For single labeling, the primary antibody was added to the fixed slices and incubated overnight at 4°C with gentle shaking. A long period of washes (for a minimum of 6 h) with four changes of blocking buffer were performed the following day. The secondary antibody (respectively: donkey anti-goat IgG, rhodamine conjugated [Santa Cruz Biotechnology; sc-2094], and goat anti-mouse IgG, rhodamine conjugated [Santa Cruz Biotechnology; sc-2029]) was then preincubated in blocking buffer at room temperature for 1 h (1:5000 dilution), subsequently added to the washed slice samples, and left overnight at 4°C with slow shaking. The slices were then washed three times the following day with blocking buffer over the course of 2 h, and a final wash in PBS was performed for an extra 1 h. When double immunostaining was performed, the same procedure was followed after the single labeling, taking into consideration the cross-reactivity of each secondary antibody with the primaries in order to avoid false costainings. The tissue slices were monitored under a Nikon TE 300 inverted microscope with phase contrast and fluorescence, using an OpenLab data acquisition system mounted with a Hamamatsu digital video camera. The images obtained represent a single optical layer (average optical error, $\sim$1 μm) observed with phase contrast and fluorescence for fluorescein isothiocyanate (FITC) or rhodamine. In addition, negative controls for immunostaining were performed. In the single immunostaining experiment, the negative control consisted of omission of the first antibody to see whether or not the secondary antibodies presented any background staining against the tissue. In the double-immunostaining experiment, one of the primary antibodies was omitted in each experiment to test the cross-reactivity of the secondary antibodies to the nonspecific primary antibody.

5′-Bromodeoxyuridine incorporation and staining

To study cell proliferation in the hippocampal slice cultures, 5′-bromodeoxyuridine (BrdU) was added (to a final concentration of 10 μM) to the culture medium for a period of 2 h. Pulse–chase experiments were also performed. In this case, a 2-h pulse of BrdU incubation was performed, followed by different time incubations (24, 48, and 72 h) after washing the hippocampal slices with fresh medium. After BrdU exposure (or chase), the hippocampal slices were washed with PBS several times and fixed in 2% paraformaldehyde in PBS (pH 7.4) at room temperature for 2 h with slow shaking. The slices were washed several times in PBS and subsequently treated with 0.1% Triton X-100 in PBS for 2 h at room temperature. To fragment cellular DNA, the slices were treated with 2 M HCl in PBS for 30 min at 37°C. After the HCl treatment, several washes were performed with PBS until pH 7.4 was recovered. At this time, blocking buffer (20% bovine calf serum, 0.1% Triton X-100, 1% DMSO in PBS) was added to the samples, which were then incubated for 4 h at 4°C with slow shaking. Anti-BrdU mouse monoclonal antibody IgG1, FITC conjugated (33284X; BD Biosciences Pharmingen), was preincubated in blocking buffer for 1 h at 4°C (1:40 dilution) and then added to the samples overnight at 4°C with slow shaking. After the incubation period, the slices were washed three times with blocking buffer over the course of 2 h, and a final wash in PBS was performed for an extra 1 h. In the case of double immunostaining for a specific cellular marker in combination with BrdU, first the immunostaining against the particular cellular marker was performed (as described above) and then the BrdU label was added to the slice. The treated hippocampal slices were monitored under a Nikon TE 300 inverted microscope with phase contrast and fluorescence, using an OpenLab data acquisition system mounted with a Hamamatsu digital camera. The pictures obtained represent a single optical layer observed with phase contrast and fluorescence.

Entrapment of migrating cells from scaffolds and culture. In the rat brain, area I is the hippocampal slice itself and area II is the proximal region where cells migrate. Area III is the distal region where cells extend into the peptide scaffold. (These areas were visualized under a microscope, aspirated separately with a micropipette, and resuspended in hippocampal organotypic slice medium as described in the previous section.) The collected scaffold containing entrapped migrating cells was disrupted mechanically with a micropipette by up–down aspirations until about 50–70% of the cells were extracted as determined by phase-contrast microscopy. The suspension was placed in a regular cell culture dish containing laminin-coated coverslips (BD Biosciences Discovery Labware, Bedford, MA) and incubated overnight in the same medium at 37°C equilibrated with 5% CO_2. The following day, scaffold remnants were removed from attached cells by washing the culture dish with fresh medium. Attached cells were used for BrdU uptake and *in situ* immunofluorescence assays as described above.

RESULTS AND DISCUSSION

Mitotic activity at the interface zone between the rat hippocampal slices and the culture membrane

Our interest in studying mitotic activity in hippocampal tissues was inspired by previous studies describing

the formation of a natural scaffold through 3-D reorganization of glial cells in hippocampal organotypic slice cultures.[24] We cultured the hippocampal slices on Millipore membrane inserts to examine hippocampal tissue from rats at postnatal day 7.5 (Fig. 2). We first monitored cell migration from the tissue slice edge to the culture membrane during the first few days of culture. Twenty-four hours after initial culturing, a cellular scaffold projecting outward from the tissue slice edge was observed (Fig. 1b and Fig. 2a).

To analyze the cellular events observed in greater detail, we divided the slice culture system into two distinctive areas: area I and area II. Area I is the tissue contained within the perimeter of the original hippocampal slice. Area II is the cellular scaffold formed after culturing the slice that extends beyond the original tissue edge (Fig. 1b and Fig. 2a). We then assessed mitotic activity at different times after slice preparation in the dentate gyrus area from 4 h to 4 days (96 h) in organotypic culture. Slices were incubated with a 2-h pulse of 5′-bromodeoxyuridine (BrdU) to specifically label cells in S phase. They were subsequently examined for incorporation of BrdU into DNA by immunostaining. The number of $BrdU^+$ nuclei was counted and calculated by cell density area (1000×1000 μm, or 1×10^6 μm^2).

A small fraction of $BrdU^+$ nuclei (4.2 ± 1.4 $BrdU^+$ nuclei per 1×10^6 μm^2) was observed after 4 h in culture in the dentate gyrus region, suggesting that cells in the hippocampal slice exhibited some mitotic activity (Table 1). $BrdU^+$ nuclei were observed mainly in the dentate gyrus area, consistent with previous reports that neural progenitor cells naturally reside in this region.[15–19]

Unexpectedly, after 12 h in culture, the amount of $BrdU^+$ nuclei in area II increased over time (32.6 ± 14.8), suggesting that during cellular scaffold formation, more cells, progressively underwent cell division and/or migrated into area II (Table 1 and Fig. 1). The $BrdU^+$ cells from 3-day-old organotypic culture could be clearly observed in cross-sections of area I and area II immunostained for BrdU (Fig. 2b–d). It is interesting to observe the accumulation of $BrdU^+$ nuclei at the interface zone, as a result of an induction of cell division in the area and/or

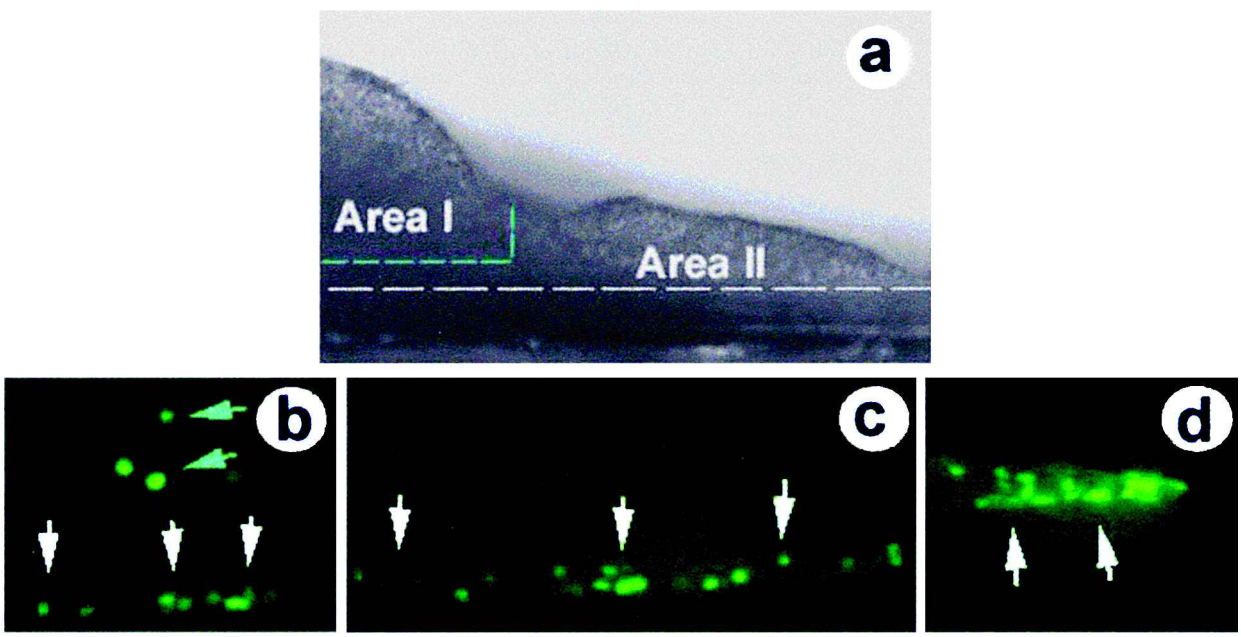

FIG. 2. Mitotic activity at the interface zone of hippocampal tissue slices. Hippocampal organotypic slice cultures from 7.5-day-old postnatal rats were performed on regular Millipore membrane inserts and incubated in a six-multiwell plate at 37°C in a humidified incubator chamber equilibrated with 5% CO_2. Three-day-old hippocampal organotypic slices (six slices) were incubated with 5′-bromodeoxyuridine (BrdU) for 2 h, fixed, and immunostained for BrdU to detect dividing cells (at S phase) in the tissue. The samples were analyzed with an inverted stereomicroscope (Nikon TE 300) by phase-contrast and fluorescence microscopy. (**a**) Cross-section of dentate gyrus region (×40 magnification) showing area I and area II. The dashed line at the bottom of the picture indicates the border between the tissue slice and the culture membrane. The upper dashed line represents the border between area I and area II. (**b**) Cross-section of dentate gyrus region after staining for BrdU (green; original magnification, ×200). (**c**) Area II extended from dentate gyrus region after staining for BrdU (green; original magnification, ×200). (**d**) Final edge of area II from dentate gyrus region, showing high density of dividing cells at the migrating area (original magnification, ×200). White arrows indicate dividing cells lined out at the interface zone, and blue arrows point out dividing cells into the tissue slice away from the interface zone. Color figure available online.

Table 1. Mitotic Activity of Hippocampal Tissue at the Interface Zone during the First 4 Days in Culture[a]

Culture time (h)	*Total BrdU$^+$ cells*[b]
4	4.2 ± 1.4
12	32.6 ± 14.8
24	41.8 ± 10.2
48	50.33 ± 26.9
72	62.4 ± 18.0
96	78.3 ± 22.5

[a]Hippocampal organotypic slices were cultured for various times (hours) and then they were incubated for 2 h in the presence of BrdU. The samples were used to assess the occurrence of BrdU$^+$ cells at the interface zone of the hippocampal region dentate gyrus by immunostaining (see Materials and Methods).

[b]After immunostaining the samples, BrdU$^+$ cells were counted from four selected areas of 1×10^6 μm^2 of each selected region in the dentate gyrus from six different slices. The experiment was repeated three times. Data are expressed as median values and standard deviation (SD).

migrating cells with mitotic activity from internal regions of the slice (Fig. 2b–d). A cross-section of the dentate gyrus region showed that BrdU not only incorporated into nuclei at the interface zone, but also into the tissue an appreciable distance from the cellular scaffold (Fig. 2b). Thus, this observation suggests that the immunostaining procedure allowed detection of nuclei with mitotic activity in internal parts of the tissue (Fig. 2b).

To characterize the behavior of the dividing cell population at the interface zone, we performed a "BrdU pulse–chase" experiment. Briefly, 24-h-old hippocampal slices were incubated for 2 h with BrdU (pulse) and then washed with medium and cultured for an additional 3 days (chase). Samples were stained every 24 h for quantitative analysis of the amount of BrdU$^+$ cells. The amount of BrdU$^+$ cells increased over time nonlinearly, suggesting that the majority of the BrdU$^+$ cells initially labeled during the short pulse continued dividing over time with a tendency toward symmetric cell division (Table 2) and with a division time of approximately 32 h.

Characterization of the cell populations present at the proliferation interface zone

To analyze the cell-type composition of the dividing cells at the interface zone or area II, the hippocampal slices incubated for 2 h with BrdU and chased for 48 h were double immunostained for BrdU and glial fibrillary acidic protein (GFAP), an astrocyte marker (class I glia). Because glial cell migration and proliferation in neural tissue remodeling is a well-known phenomenon,[26] we decided to test the possibility that in our experimental system some remodeling process is also occurring. Thus we double immunostaining the tissue slices for GFAP in combination with BrdU. We detected a dense population of elongated GFAP-positive (GFAP$^+$) cells that extended processes throughout the interface zone (Fig. 3d). In addition, a considerable number of BrdU$^+$ cells at the interface zone consistently costained for GFAP during the time course of the experiment (Fig. 3d–f and Table 3). It was also observed in the pulse–chase experiments that the population of BrdU$^+$ cells increased over time (Table 3). Our observations are consistent with previous reports that under conditions of tissue remodeling, glia can undergo migration and proliferation simultaneously.[27]

To explore the possible existence of a neuroprogenitor cell population at the interface zone, we immunostained hippocampal slices for nestin, a known neural stem cell marker, in combination with BrdU (2-h incubation). An anti-nestin monoclonal antibody was used for this analysis. A positive nestin signal was detected throughout the interface zone, suggesting the plausible presence of neuroprogenitors in area II (Fig. 3a–c). Moreover, a small fraction of nestin$^+$/BrdU$^+$ cells (2.7 ± 0.9%) was also observed at the interface zone through-

Table 2. Cell Division Kinetics at the Dentate Gyrus Interface Zone of Organotypic Hippocampal Slice Cultures[a]

Culture time (h)	*Pulse*	*Chase*	*Total BrdU$^+$ cells*[b]	*Growth rate (%)*
24	+	–	51.6 ± 5.2	100
48	–	+	98.8 ± 11.2	190
72	–	+	172.2 ± 22.1	330
96	–	+	340.9 ± 33.7	560

[a]Hippocampal organotypic slices (24 h old) were incubated for 2 h in the presence of BrdU and then chased with medium for 3 days (from 48 to 96 h). The samples were used to assess the occurrence of BrdU$^+$ cells at the interface zone of the hippocampal region dentate gyrus by immunostaining (see Materials and Methods).

[b]After immunostaining the samples, BrdU$^+$ cells were counted from four selected areas of 1×10^6 μm^2 within the dentate gyrus region as described in Table 1. The total amount of BrdU$^+$ cells present in each selected area after the BrdU pulse of 2 h was considered as 100%. The values obtained from each region were combined to estimate the average occurrence of BrdU$^+$ cells. Data are expressed as median values and standard deviation (SD).

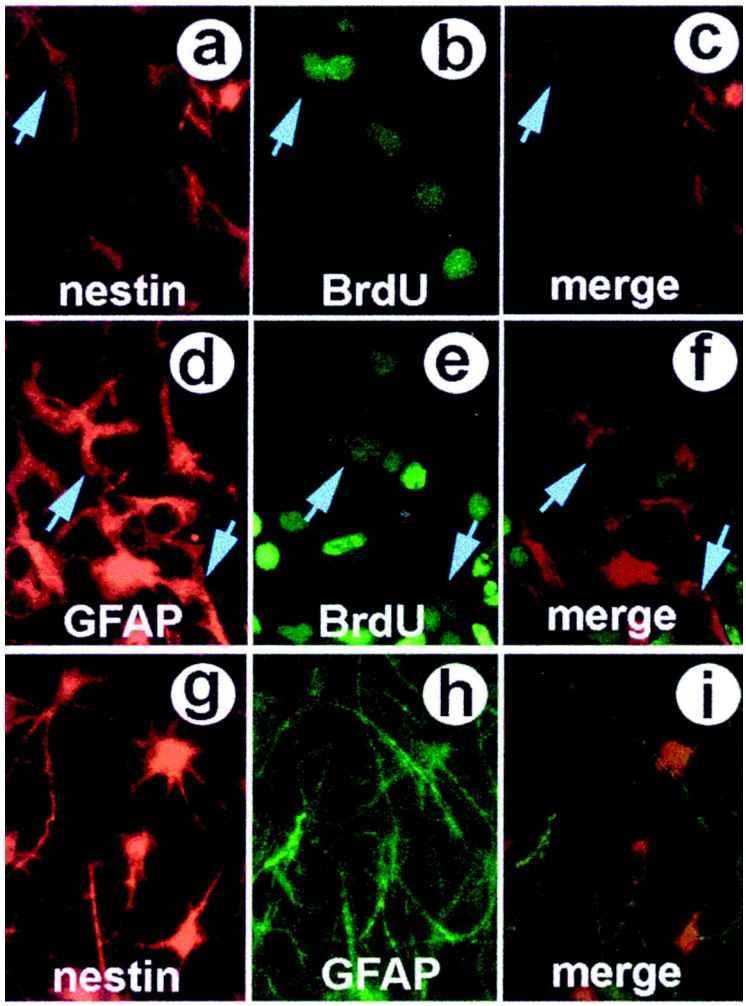

FIG. 3. Glial and progenitor cells at the interface zone undergo cell division. Hippocampal tissues were incubated for 2 h in the presence of BrdU to detect actively dividing cells. Tissue slices were stained for BrdU in combination with one or two other markers including nestin (for detection of progenitor cells) and GFAP (glial cells). Images were obtained from a single optical layer under a fluorescence stereomicroscope as described in Fig. 2. Data were collected from six independent slices and repeated three times. (**a**) Dentate gyrus section immunostained for nestin (red); (**b**) same optical layer as (**a**), immunostained for BrdU (green); (**c**) merging of layers (**a**) and (**b**). (**d**) Another section immunostained for GFAP (red); (**e**) same optical layer as (**d**) immunostained for BrdU (green); (**f**) merging of layers (**d**) and (**e**). (**g**) Another section immunostained for nestin (red); (**h**) same optical layer as (**g**), immunostained for GFAP; (**i**) merging of layers (**g**) and (**h**). Arrows indicate examples of a $BrdU^+$ nucleus associated with the corresponding cell marker used. Original magnification (**a–i**): ×200. Color figure available online.

out the dentate gyrus region. In addition, many $nestin^+$ cells (>50%) at the interface zone were costaining for GFAP (Fig. 3g–i). Previous reports showed similar correlation during nervous system development, neural tissue injury, or after neural grafting process,[26–28] thus further suggesting a remodeling process in area II. We also observed that the cells double stained for GFAP and nestin presented different morphology from $GFAP^+$ $BrdU^+$ cells (compare Fig. 3g–i with Fig. 3a–c). It is plausible that the differences are due to the fact that the cell types in the glial population might be different and/or they are transiently undergoing different cellular processes such as proliferation, migration, or differentiation.

In addition, βIII-tubulin, a cellular marker expressed in immature neurons,[29] was used in combination with BrdU staining (2-h incubation) to reconfirm the presence of dividing neuroprogenitor cells in area II of 3-day cultures. In general, the entire interface zone stained strongly for βIII-tubulin, suggesting an immature state in the neuronal lineage (data not shown). Furthermore, βIII-$tubulin^+BrdU^+$ cells (7.3 ± 2.8%) were detected in the interface zone after staining, suggesting the presence of a

Table 3. Percentage of Neuronal Phenotypes Detected in BrdU$^+$ Cells[a] at the Interface Zone of Hippocampal Region Dentate Gyrus

	BrdU incubation time (h)		
Cell marker	*Pulse*	*Chase*	*Marker$^+$/BrdU$^+$ cells (%)*
Nestin	2	—	2.7 ± 0.9
βIII-Tubulin	2	—	7.3 ± 2.8
NeuN	2	—	ND[b]
	2	24	ND
	2	48	ND
GFAP	2	—	2.2 ± 0.5
	2	24	4.7 ± 0.9
	2	48	9.0 ± 1.8

[a]Three-day-old organotypic hippocampal slice cultures were incubated (for 2 h) in the presence of BrdU and used to assess the occurrence of various neuronal markers in BrdU$^+$ cells at the interface zone of the hippocampal region dentate gyrus. In some cases, a BrdU pulse (2 h) and chase experiment was also performed (see Materials and Methods). After immunostaining the samples, BrdU$^+$ cells were counted from four selected areas (200 × 200 μm) of each hippocampal region. From the same area, doubly immunostained cells for each neuronal marker and BrdU (Marker$^+$/BrdU$^+$) were also counted and used to calculate the percent occurrence of doubly positive cells in each case. The total amount of BrdU$^+$ cells present in each selected area was considered as 100%. The values obtained from each region were combined to estimate the average value of occurrence of double immunostaining (Marker$^+$/BrdU$^+$) throughout the interface zone of the hippocampal region dentate gyrus. Data are expressed as median values and standard deviation (SD).

[b]ND, Nondetection of doubly positive cells under the experimental conditions tested.

population of immature neural cells with mitotic activity (possible neural stem cells) at the interface zone that expressed the neurodevelopmental marker βIII-tubulin (Table 3). In addition, NeuN (neuron-specific nuclear protein) was used to characterize the presence of neurons in the region. Cells staining positive for NeuN were also detected in area I but not at the interface zone or area II, suggesting that the entire new tissue framework is likely formed with glia and neuroprogenitor cells (Table 3).

Enhanced tissue scaffold extended from hippocampal slices into peptide scaffolds

We were encouraged by the above-mentioned observations and decided to culture hippocampal slices over the peptide scaffold, where the migrating cells in the extended tissue framework could invade a recoverable support. It therefore would be possible to "entrap" migrating cells for further isolation, subculture, expansion, and differentiation. We used a support culture of the self-assembling RAD16-I peptide scaffold and rat hippocampal slices (7.5 days postnatal) were cultured either on insert membranes (controls), or over an ~500-μm-thick layer of peptide scaffold previously equilibrated with culture medium (Fig. 1).

First, we monitored tissue invasion, by microscopy, from the borders of the slice into the scaffold during the first few days of culture. Twenty-four hours after initiating the cultures, little tissue invasion was observed in the control culture slices (Fig. 4a and c). However, active migration into the scaffold of elongated cells was observed in all the areas of the hippocampus including the dentate gyrus, CA1, CA2, and CA3 (Fig. 4). Cell migration of elongated cells continued for an additional 2 days and then was followed by a wave of small round-shaped cells (Fig. 4d). To analyze the migrating cell type composition, we immunostained the slices with two neuronal markers, including NeuN (Fig. 4g and h) to detect neurons and GFAP for astrocytes (Fig. 4e and f). A dense population of elongated GFAP$^+$ cells extending processes in the direction of migration was observed in the control and peptide scaffold cultures in all three areas of the hippocampus (CA1, CA2, and CA3). These observations suggest that the glial cell was the aggressive migratory cell type during the first few days (Fig. 4e and f).

Cell migration into the scaffold after 3 days in culture was considerably higher than into control cultures (compare Fig. 4e and g with Fig. 4f and h). On average, the area extended into the scaffold from the original border of the slice was ~500 μm compared with ~150 μm in the control experiments (Fig. 4). In addition, these observations also suggest that the peptide scaffold might be suitable for encouraging neural tissue growth and extension, as well as neural proliferation, and therefore eventually could be used as a tool for neuroreparative medicine.

Isolation and reculture of neural cells entrapped in the peptide scaffold

We observed that cells at the interface zone and migrating into the peptide scaffold were less adhesive to each other, likely because of their attachment onto the peptide scaffold. Thus, enzymatic treatment (such as trypsinization and collagenization) may not be needed to dissociate the cells from the scaffold because the nanofiber network has low mechanical strength with >99.5% water. It could be easily disrupted to release the cells. This characteristic would provide an additional advantage in the use of peptide scaffolds for future culture and isolation of migrating cells.

The hippocampal cultures from control membrane inserts and peptide scaffolds were used to isolate cells from the interface zone to culture on 2-D coverslips for further characterization. After 3 days in culture, cells in area II were collected by aspirating the scaffold with a mi-

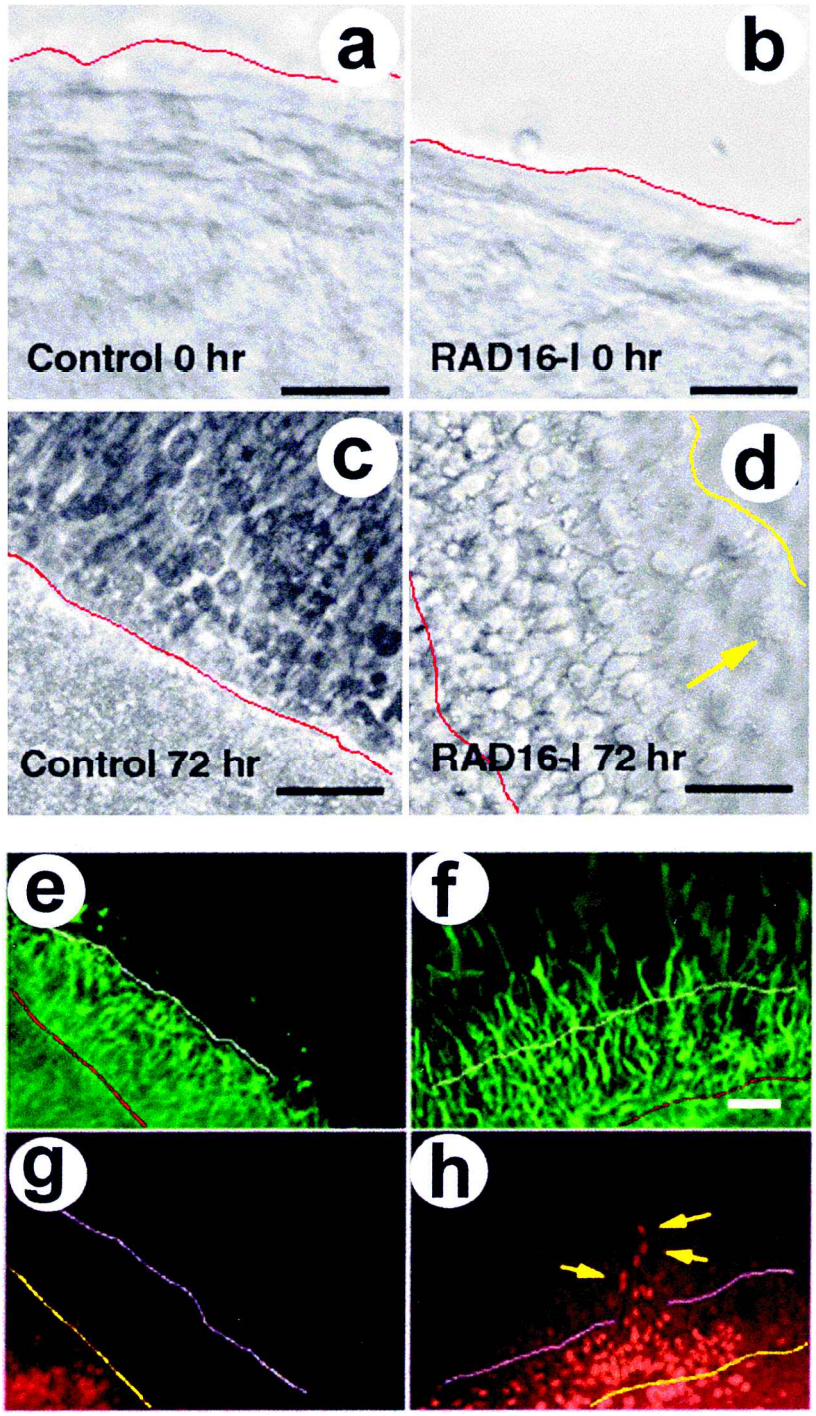

FIG. 4. Hippocampal organotypic slice cultures on peptide scaffolds develop extended tissue scaffolds. Hippocampal slices were cultured organotypically either on control membrane or on RAD16-I peptide scaffolds layers (~500 μm thick). Time-lapse experiment was carried out to monitor tissue scaffold growth from the perimeter of the dentate gyrus region. (**a**) Time 0 (0-h) control slice culture; (**b**) time 0 (0-h) scaffold slice culture; (**c**) 72-h control slice culture; (**d**) 72-h scaffold slice culture. The red line indicates the original border of the tissue slice; the yellow line in (**d**) indicates the extended tissue scaffold. The yellow arrow in (**d**) indicates the direction of tissue scaffold growth and extension. Scale bars: 100 μm. (**e**) Seventy-two hour control slice culture immunostained for GFAP (glial cell marker, green); (**f**) 72-h scaffold slice culture immunostained for GFAP (green); (**g**) same optical layer as (**e**), immunostained for NeuN (neuron marker, red); (**h**) same optical layer as (**f**), immunostained for NeuN (red). The red line in (**e**) and (**f**) and the yellow line in (**g**) and (**h**) indicate the original perimeter of the tissue slice. The white line in (**e**) and (**g**) indicates the extended tissue scaffold in control cultures. The white line in (**f**) and (**h**) is used to compare the overextension obtained on peptide scaffold cultures. Yellow arrows in (**h**) indicate neuron cells staining with NeuN (red) migrating into area II of scaffold slice cultures. Scale bar in (**f**), for (**e**–**h**): 100 μm. Color figure available online.

cropipette and then resuspended in neural medium (Fig. 5e). These cells were easily dissociated by pipetting up and down several times and then used to initiate primary 2-D cultures on laminin-coated coverslips. The cells were indeed not strongly attached to the peptide scaffold. After overnight culture, the medium containing the peptide scaffold was removed, and the attached cells were cultured with fresh neural medium for several days, in some cases for 1 week. In contrast, cells in area II of control cultures were strongly associated with the tissue slice and it was not possible to dissociate them by simple up–down pipetting, indicating the clear advantage in using the peptide scaffolds to entrap neural cells present at the expanding tissue (not shown).

To assess the mitotic activity of cells isolated from the peptide scaffold, cultures were incubated with BrdU for 2 h and then double stained for BrdU and NeuN. We observed that some cells stained positive for BrdU but negative for NeuN, suggesting that a fraction of cells underwent DNA synthesis even after several days in culture (Fig. 5b–d, blue arrows). Interestingly, another fraction of cells exhibited a high level of NeuN, but were negative for BrdU incorporation (Fig. 5b–d, white arrows). This observation suggests that some neuroprogenitor cells isolated from the scaffold may differentiate into neuronal cell lineage (Fig. 5b–d, white arrows). The dividing cells also independently stained positive for the neural marker α-internexin, thereby verifying their neuronal lineage (Fig. 5f–h).

Implication of entrapment and enrichment of migrating cell

At present it is extremely difficult to entrap and enrich migrating cells in 2-D adherent cell culture without altering cell physiology because cells directly attach onto the surface of the culture dish. Specifically, cell surface receptors interact with ligands on the material coated on the substratum. It is nearly impossible to entrap and enrich neural cell types in cell culture without substantial cellular damage because they form complicated neural networks. Our unexpected finding, that the cell population with mitotic activity in the new tissue scaffold formed between the brain tissue slice and the culture membrane, the interface zone or area II (Fig. 1 and Fig. 2), suggests that cells exhibit different behaviors in the scaffold and on the solid surface.

Neural cell isolation was achieved by culturing the hippocampal slices on a layer of self-assembling peptide scaffold that supported tissue growth and enhanced cellular migration. The characterization of the dividing cellular fraction suggested that the major cell types are glial and neuroprogenitor cells. The formation of a tissue scaffold was enhanced by ~3-fold in peptide scaffold cultures, suggesting their potential use for migrating cell entrapment and enrichment. A hydrogel thickness of 500 μm was important before placing a tissue slice on top, in order to allow enough space underneath for cells to migrate and grow. Nevertheless, a thickness of 100 μm could work as well: the tissue slice placed on top could mechanically displace the layer of hydrogel below, making it thinner. The event of cell migration was observed along the perimeter of all regions of the hippocampus slice, including dentate gyrus, CA1, CA2, and CA3.

Glial and neuroprogenitor cells from 1-week peptide scaffold cultures progressively invaded the peptide matrix ~500 μm from the edge of the slice, in contrast to ~150 μm in control cultures. Cells in the scaffold–migration zone were easily collected with a micropipette and then used to initiate cultures on laminin-coated coverslips. Cells maintained their mitotic activity for 2–3 days as evaluated by incorporation of BrdU into DNA, after which they may differentiate into glia and neurons (Fig. 5).

We are currently exploring the possibility of lifting the tissue slice to assess whether some cells are left in the hydrogel layer. We observed that at early culture times, for example, 2–3 days, some cells are retained in the hydrogel after we detached the tissue slice from the hydrogel, but if we wait for longer cultures times (e.g., 1 week) the tissue slice takes up all of area II. This finding suggests that after 2–3 days in culture the synthesis and/or accumulation of extracellular matrix in area II is enough to keep the newly formed tissue attached to the original slice. In any case, the cells retained in the hydrogel could be an interesting, highly migratory population of cells that can be subcultured for analysis of their expansion capacity, their progeny, and lineage potential.

Potential usefulness for biomedical research and cell-based therapies in regenerative medicine

Our previous studies showed that the RAD16-I peptide did not elicit measurable immune response or inflammatory problems when injected into animals (Holmes *et al.*[3]; and R. Ellis-Behnke, C.E. Semino, S. Zhang, and G.E. Schneider, unpublished results). Because the peptides can not only be chemically made, using mature GMP manufacture technology, but can also be purified to homogeneity, devoid of biological contaminates such as viruses, growth factors, and cytokines, they can be used as a defined 3-D culture system, thus providing consistent results. Furthermore, it is absolutely crucial to use scaffolds without chemical and biological contaminates for cell-based therapies. Amino acid-based peptide scaffolds may provide a complementary system to polymer scaffolds currently used in regenerative medicine, and the degradation products are pure amino acids that can be readily reabsorbed into the body.

Our findings suggest that the peptide scaffold will be

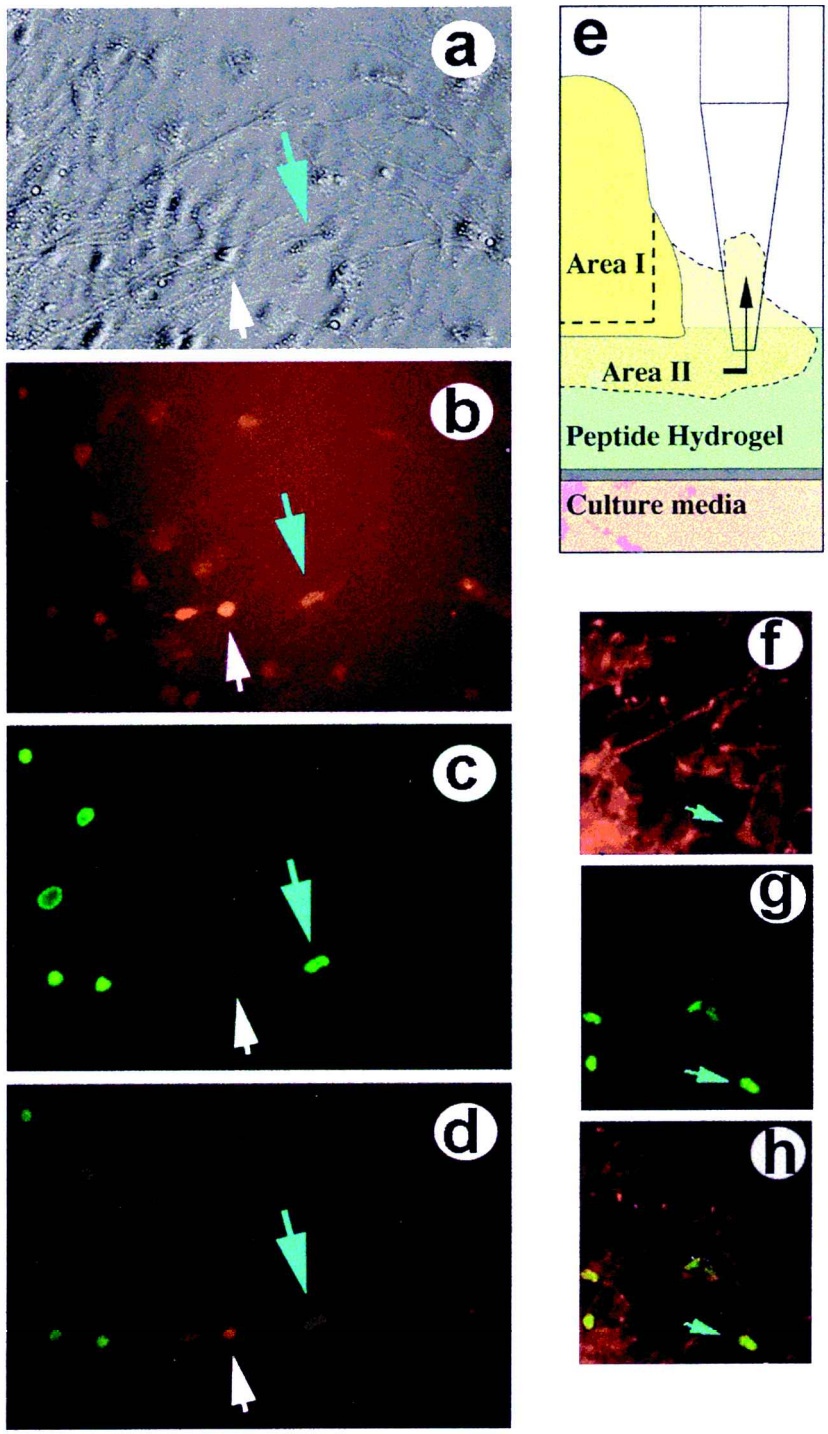

FIG. 5. Characterization of mitotic cells isolated from interface zone of hippocampal slice cultures on RAD16-I scaffold cultures. Three-day-old hippocampal organotypic slice cultures were used to isolate migrating cells from the interface zone or area II by aspiration of scaffold material with a micropipette [see Materials and Methods and (**e**)]. Cells were cultured on laminin-coated coverslips, incubated in the presence of BrdU for 2 h, and stained for BrdU and NeuN or for BrdU and α-internexin (neuronal marker). Pictures were taken from a single optical layer under a fluorescent stereomicroscope as described in Fig. 2. (**a**) Phase contrast; (**b**) same optical layer as (**a**), immunostained with NeuN (red); (**c**) same optical layer as (**a**), immunostained for BrdU (green); (**d**) merging of layers (**a**) and (**b**). (**f**) Another culture immunostained for α-internexin (red); (**g**) same optical layer as (**f**), immunostained for BrdU (green); (**h**) merging of layers (**f**) and (**g**). Blue arrows in (**a–d**) and (**f–h**) indicate the presence of a mitotic figure in a $NeuN^-$ cell and in an α-$internexin^+$ cell, respectively. White arrow in (**a–d**) indicates a $NeuN^+BrdU^-$ cell. Color figure available online.

a useful technology to enrich neural populations for a broad range of scientific studies and medical applications, including further characterizing cells in a well-controlled manner and harvesting neural cells for cell-based therapies.

Adult stem cells can provide an abundant source of immune-compatible cells for future autografts and have been used for *in vitro* differentiation into a variety of different tissues.[30–33] They may thus represent a reliable source for a wide variety of differentiated cell types useful for future applications in biomedical research. Through combining seemingly unrelated disciplines, molecular engineering, synthetic chemistry, and stem cell biology, it is possible to further advance our understanding of cell behavior in three dimensions in general and to accelerate the development of new biological materials, methods, and tools for regenerative medicine.

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†Sadly, Julia passed away on April 30, 2001.

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