CHAPTER 8

Isolation and Culturing of Glial, Neuronal and Neural Stem Cell Types Encapsulated in Biodegradable Peptide Hydrogel

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Summary

The mammalian central nervous system (CNS) has no or very little capacity to selfrepair. Neural tissue transplantation is impractical because it is hard to find a brain donor, and because of the low survival rate of grafted neural cells. Therefore, the need for constructing a functional brain tissue is very critical. In this study, isolated neonatal or prenatal rat brain cortical slices or cultured primary neuronal-glial cocultures were encapsulated into Puramatrix peptide hydrogel scaffold as "drop" cultures. Cell viability, proliferation, migration and dendrite outgrowths were characterized by Live/Dead cell assay, BrdU incorporation, and immuno-cytochemical detection of glial- and neuronal-specific markers by phase contrast and immuno-fluorescence microscopy. The tissue-engineered "drop" culture yielded 3D constructs of neuronal-glial cell aggregates consisting of 60% viable cells (rapidly multiplying cells). The BrdU positive cells were also positive for glial fibrillary acidic protein (GFAP). The aggregates stained positive for neuronal-specific and progenitor-specific marker proteins. The encapsulation of rat brain neuronal-glial cells inside Puramatrix peptide hydrogel as "drop" culture represents a novel method of neural tissue-engineering which may be extrapolated to grow human neural tissue that can be used in neural cell replacement therapy.

KEYWORDS: Brain tissue, astrocytes, neurons, neural stem cells, neural tissue engineering, neural 3-D constructs, nanofiber peptide hydrogel scaffolds

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Topics in Tissue Engineering, Vol. 4. Eds. N Ashammakhi, R Reis, & F Chiellini © 2008.

Introduction

Tissue engineering has revolutionized the current cell culture technology. By applying the principles across the fields of bioengineering, material science and life sciences, tissue engineering has become a pioneering interdisciplinary field in the areas of biomedical research [1]. The main goal of tissue engineering is to create a living tissue construct that is functionally, structurally and mechanically similar to that naturally occurring in our body so that the latter when becomes impaired, can easily be replaced by the newly constructed tissue. Very little success has been achieved in the neural tissue engineering field mainly due to the complexity of the mammalian central nervous system (CNS). The mammalian CNS tissue damaged either by brain injury or by neurodegenerative diseases requires the transplantation of exogenous neural tissue, because the CNS has no or very little capacity to selfrepair. The neural tissue transplantation is impractical for two main reasons: first it is hard to find a brain donor, and second, the low survival rate of grafted neural cells. Therefore, the need for alternative ways to construct a functional brain tissue by tissue engineering is very critical.

The first and foremost challenge in the neural tissue engineering is the need for neural cells that can grow into mature neurons, since adult neurons are not capable of proliferating and they are short-lived in culture. Recent studies have shown that neural progenitor or stem cells isolated from the embryonic or adult CNS can proliferate into functional neurons [2,3,4,5]. Thus, the neural progenitor or stem cells may represent an ideal cell type for the neural tissue engineering.

The second challenge is the need for an extracellular matrix (ECM) which mimics that of a natural ECM *in vivo* for the neural cells to adhere to and grow [6]. Upon transplantation, the engineered neural tissue should be able to integrate with the existing brain cells by shedding the ECM without causing any toxic effects to the transplanted neural tissue or to the host brain cells.

Recent studies have used various polymer scaffolds such as collagen, to grow neural cells in 3dimension and have proved to meet some of these challenges [7,8,9]. Additionally, the biodegradable synthetic hydrogels, including polyethylene glycol and peptide hydrogels have overcome some of the challenges and proved to be very attractive candidates in some non-neural tissue engineering applications [10,11, 12]. The Puramatrix peptide hydrogel is a

synthetic matrix, consisting of standard amino acids (1% W/V) and 99% water, has been used to grow tissue-engineered hepatocytes [10], rat pheochromocytoma (PC12) cells [13], and organotypic cultures of hippocampus [14].

The main purpose of this study was to develop and characterize a three-dimensional (3-D), rat embryonic neuronal-glial "neurospheres", encapsulated in Puramatrix peptide hydrogel with an intention to engineer the growth of human neural stem cells in these peptide hydrogels. The results show that rat embryonic neural cell outgrowth was observed from cortical tissue, or dissociated rat brain cells that are encapsulated inside the peptide hydrogel "drop" culture. The cells formed 3-D aggregates, consisting of live cells which incorporated BrdU and stained positive for, glial fibrillary acidic protein, a cytoskeletal marker protein, specific for glial cells. These aggregates also consisted of cells that were stained positive for a neuronal-specific marker, neurofilament protein. Furthermore, preliminary results demonstrate that human neural stem cells, that are growing as undifferentiated colonies on a bed of supporting cells may be grown as 3-D constructs encapsulated in the Puramatrix peptide hydrogel free of any supporting cells.

Materials and Methods

Puramatrix hydrogel scaffolds

In this study, the BD Puramatrix peptide hydrogel (BD Biosciences, Discovery Labware, MA, USA), was used as a scaffolding material**.** It is a synthetic peptide, made up of 16 standard amino acids of repeating amino acids Arginine (R)-Alanine (A), and –Aspartate (D) (RAD16). *PuraMatrix*TM's nanometer-sized peptide (1% W/V) in 99% water, used as a scaffolding material, has been shown to provide a 3-D architecture, allowing a defined neural cell culture condition, cell migration, nutrient diffusion, and cell harvesting. Under physiological conditions, the peptide component of BD Puramatrix peptide self-assembles into a 3-D hydrogel with a nanometer scale fibrous structure (10). The BD Puramatrix peptide hydrogel has been shown to provide a 3D environment to promote the growth and differentiation of hippocampal neurons in an organotypic culture (14), rat

pheochromocytoma cells (PC12; 13), and hepatocyte progenitor cells (10). In this study, a 1% (W/V) BD Puramatrix Peptide Hydrogel (BD Biosciences, Bedford, MA) was diluted to 0.5% to encapsulate rat brain slices, primary cells, mainly neurons with the glial counterparts as 3-D "drop" cultures using the manufacturer's protocol with some modifications as described in the following section.

Isolation of neonatal or prenatal rat brain regions and culture conditions

Rat brains were isolated either from 1-2 day-old Sprague Dawely rat pups or from E-15 rat embryos by surgical procedures that were approved by the Institutional Animal Care and Use Committee (IACUC) of Texas Southern University. The flow diagram in figure 1 shows the step by step procedure on the isolation and culture conditions used to grow neuronal-enriched, pure astrocytes in traditional 2-D or in 3-D architecture encapsulated in Puramatrix peptide hydrogel. Using a dissecting stereomicroscope, the hippocampus, and cerebral cortical regions, free of white matter and meninges, were isolated, sliced into cubes and used directly in organotypic cultures as described in the next section.

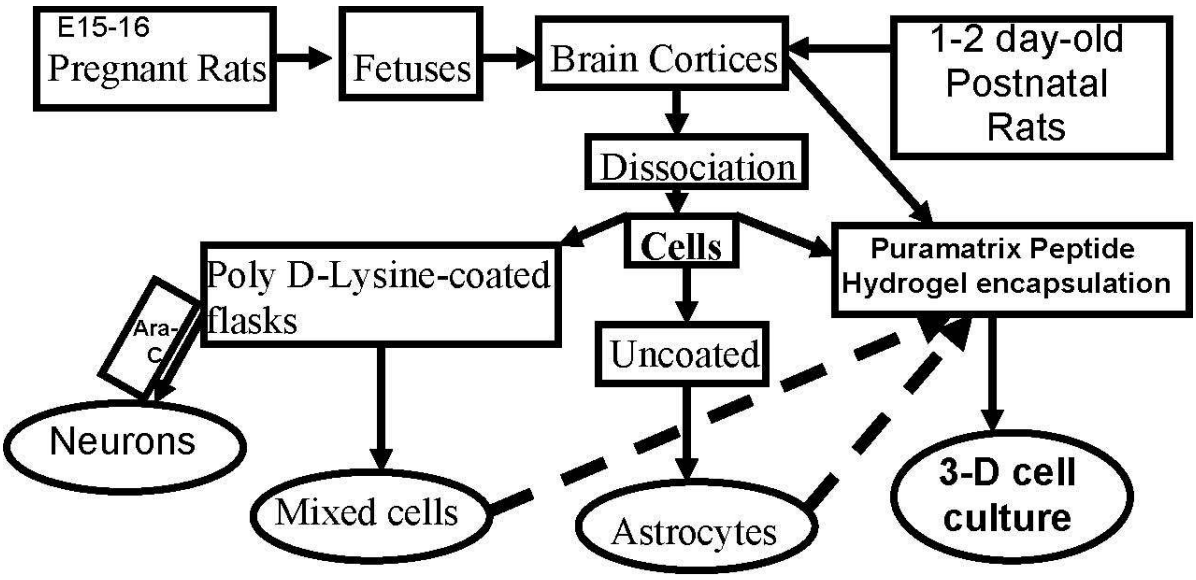

Fig.1. Flow diagram for the isolation and culturing of brain cells in organotypic, 2-D or 3-D architecture. E-15 fetuses or 1-2 day-old rat pups, were isolated from anesthetized Sprague Dawley pregnant rat according to the protocols approved by the Institutional Animal Care and use of Committee of Texas

Southern University. Immediately, brains were removed into the ice-cold dissecting medium (DMEM + 20% fetal bovine serum + penicillin/ streptomycin, 100 U/ml and 100 µg/ml). Cerebral cortex or hippocampus, were dissected under a dissecting stereomicroscope. The meninges were removed and the brain regions were sliced into 1-2 mm thick cubes or enzyme-digested (trysin 0.15%w/v) to dissociate cells. The cells were pelleted by centrifugation and then plated onto either poly D-lysine coated plates (neurons), or uncoated plates (astrocytes) to grow as 2-D cultures. The brain slices or the dissociated cells were encapsulated (see methods section for details) in Puramatrix peptide hydrogel scaffolds as "drop" culture in a 3-dimensional architecture in appropriate growth medium. For neurons and mixed cells, neuronal growth medium and for astrocytes, glial feed was used.

Rat cortical/hippocampus slice encapsulation procedure

The rat brain cortical or hippocampus slices were rinsed two times in 10% sucrose solution to remove any salt, which may interfere with the assembly of hydrogel. The slices were suspended in 0.5% diluted BD Puramatrix Peptide Hydrogel in 10% sucrose solution. The suspension was quickly dispensed as 50ul "drops" into a 100 mm Petri dish containing 15 ml neural growth medium (NGM; DMEM containing 20% heat inactivated horse serum and 100U/ml penicillin and 100µg/ml streptomycin (Sigma–Aldrich, MI USA)), which initiated the assembly of the hydrogel. The medium promoted instantaneous gelation of the Puramatrix Peptide Hydrogel and the slices became "encapsulated" within the BD Puramatrix "drop". Since BD Puramatrix Peptide Hydrogel has low pH, the acidic medium was carefully aspirated after 5 minutes and fresh medium was added to bring the pH back to 7.2. The medium was changed twice within the next 30 min and then incubated at 37° C in a humidified chamber equilibrated with 5% CO_2 for up to three weeks by changing 50% of the medium twice a week.

In some cases, the cortical or hippocampus slices were digested for 10 min at room temperature with 0.15% trypsin and 1 mM EDTA in PBS to dissociate cells and used to grow glial cells and neurons. The enzyme digestion was stopped by adding Dulbecco's modified Eagle medium (DMEM) containing 20% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals, USA). The brain cells were dissociated by trituration using a Pasteur pipet with "fire-polished" tip. The resulting homogeneous cell suspension was passed through a 70nm strainer to separate the brain cells from any small blood vessels and tissue pieces. The filtrate was centrifuged for 10 min at 800x g in a refrigerated centrifuge and the cells were suspended in DMEM containing 20% FBS and 100U/ml penicillin and 100µg/ml streptomycin (Sigma –Aldrich USA) to obtain a cell density of $1x10^6$ /ml and seeded on tissue culture flasks (75 cm2, Nunc, USA) as described previously [15,16] or encapsulated

into Puramatrix Peptide Hydrogel as described in the following section.

Rat cortical neural-glial cell encapsulation culture procedure

Freshly dissociated brain cell suspension from prenatal or postnatal brain cortex or primary cultures of glial-neural mixed cells that were scraped off the flasks, were centrifuged for 10 min at 600Xg, and the pellet was suspended in 10% sterile sucrose solution and centrifuged once more. The cell pellet was suspended in 10% sterile sucrose solution to a final concentration of $1x10^6$ cells /ml. During the cell preparation, a 1% solution of BD Puramatrix peptide hydrogel was placed in a bath sonicator for 30 minutes to lower the viscosity. An equal volume of 1% peptide hydrogel and cell suspension was mixed thoroughly but gently minimizing air bubbles and then quickly pipeted as "drops" of 25 μL into a 100 mm Petri dish containing 15 ml of growth medium, which initiated the assembly of the hydrogel. Following pH equilibration, the encapsulated brain cells were incubated at 37°C in a humidified incubator chamber equilibrated with 5% CO_2 for up to three weeks by changing 50% of the medium twice a week. The encapsulated cells in "drop culture" rapidly used the nutrients after an initial 5-day lag period.

Cell Viability Assay

The cell migration and morphological characteristics were periodically examined using a Motic inverted phase contrast microscope and digital images were captured using a digital camera equipped with Motic 2.0 software. The cell viability was assessed using the Live/Dead cell Viability/Cytotoxicity Kit for animal cells (Invitrogen-Molecular probes, USA). The kit contains two probes, Calcein AM and ethidium homodimer-1, which measure intracellular esterase activity and plasma membrane integrity, respectively, the two recognized parameters of cell viability. Calcein AM is a fluorogenic esterase substrate that is hydrolyzed by the intracellular esterase activity to a green fluorescent product (Calcein), and thus the green fluorescence is an indicator of live cells. Ethidium homodimer-1 is a red fluorescent nucleic acid stain that is only able to pass through a leaky membrane of a dead

cell. The green and the red fluorescence can be detected using the green or red filters, respectively. The 3D encapsulated peptide hydrogel neurospheres were washed three times to remove serum contents and were incubated in dark for 10 min with a mixture of component A and B in the assay Kit, diluted in PBS and the fluorescence in a particular field was detected using the green filter or the red filter in a Nikon fluorescence microscope.

BrDU incorporation

The cell growth/proliferation was assessed by incubating encapsulated organtoypic cultures or the cells with bromodeoxy uridine (BrdU) (10 microM) for 24 hr. followed by a chase for 48 hr. The samples were fixed with 4% formaldehyde solution for 10 min at room temperature, washed with 1x PBS, and incubated with 2M HCl for 1 hr at 37°C. The excess acid was washed off with borate buffer pH 8.00 and the samples were incubated with 5% BSA for 15 min at room temperature, followed by anti-BrdU antibody solution in 2.5% BSA (1:200 dilutions) for 30 min at 37°C. After incubation, samples were washed three times with 1X PBS. The samples were then incubated for 15min at 37°C in dark with a secondary antibody conjugated with FITC, washed three times with 1X PBS and then incubated for 30 min with DAPI. The samples were placed on a microscope slide for imaging (apply coverslip to spread/flatten the material).

Neuronal- and glial-specific marker protein expression

The cells growing from cortical/hippocampus slices or in mixed cell cultures encapsulated in the 3-D hydrogel were identified by immunocytochemical staining of neuronal-specific marker protein, neurofilament or glial-specific marker glial fibrillary acidic protein (GFAP), using the polyclonal anti-NFL (Santa Cruz, Biotech. USA), and anti-GFAP antibodies (Alpha Diagnostics, USA), respectively. The cortical/hippocampus organotypic or the cell drop cultures were carefully transferred to a 35 mm Petri dish containing 1X PBS using a wide mouth pipette tip. The gel capsules were washed three times in 1X PBS. The washed gel capsules were fixed in 4% paraformaldhyde for 20 min at room temperature followed by a10 min, methanol-fixation. The cells that were incorporated with BrdU were incubated for 1 hr with 2M HCL at 37°C. After the incubation the samples were neutralized by washing (thrice)

with 0.2M borate buffer pH8.0. The cells were suspended in 1XPBS and blocked for 1 h with 5% BSA (Bovine Serum Albumin) in PBS. Subsequently, cells were incubated with glialspecific polyclonal rabbit anti – mouse GFAP IgG, (dilution 1:200) or neuronal-specific polyclonal anti-mouse NFL IgG (dilution 1:200) in 2.5% BSA in PBS. After 1hr incubation at 37°C, the cells were washed and then probed with the appropriate secondary antibodies (1:500 dilution) conjugated with TRITC (for GFAP) and FITC (for NFL). After 30 min incubation at 37°C in dark, cells were washed and stained with DAPI-10μg/ml (4, 6-Diamidino-2-phenylindole). To rule out any nonspecific staining, controls were incubated with only the secondary antibodies. The fluorescence was viewed using a Nikon Inverted Fluorescence Microscope ECLIPSE TS100 equipped with CCD camera. Images were captured using Metavue software (Meta Imaging Software, Molecular Devices Corporation, Sunnyvale, CA, USA). The Percentage of cells showing colocalization of GFAP and NFL, anti-BrDU, were counted by merging the images using Metavue Software.

Cell recovery from Puramatrix Peptide Hydrogel and sub-culturing procedure

The cell aggregates from Puramatrix Peptide Hydrogel were recovered after the desired growth period by gently aspirating the hydrogel up and down with a pipette. The cell aggregates and broken pieces of peptide hydrogel mixture were transferred to a tube and centrifuged for 5 min at 800 x g (800 rpm). After centrifugation, the tube containing cell aggregates at the bottom were separated from the Peptide hydrogel and medium layers by aspiration. The cell pellet was resuspended in desired amount of medium, plated onto a dish, and the cell growth was monitored by BrDU incorporation, or used for analysis of DNA, RNA and /or proteins and enzymatic assays.

Human neural stem cell culture

The SC30 human neural progenitor cells (hNPCs) were obtained from National Human Neural Stem Cell Resource (Children's Hospital of Orange County, CA, USA) and plated onto fibronectin-coated plates and grown in the medium supplemented with EGF, EF-2 and

PDGF-AB as previously described [17]. The cells were passaged on a weekly basis and considered as one passage. Once the stably expanding passage is established the cells were cryopreserved under liquid nitrogen for future use. When needed, Neurosphere formation was indued by plating cells on uncoated plates. Proliferating cultures were differentiated by replacing 50% of the medium daily, with 1:1 GCM: DGA supplemented with 1% FBS, 100nM all-trans retinoic acid, 20ng/ml BDNF and 20ng/ml neurotrophin3 as previously described [17]. The cells were characterized by immunocytochemical analysis for nestin, GFAP and MAP-2, as markers for progenitor cells, glial cells and neurons respectively.

Results

Glial and Neural Cell Morphological Characteristics in 2D-monolayer cultures

Phase contrast microscopic and fluorescence imaging analyses revealed that glial cell cultures were consisted of flat polygonal shaped cell population that were stained positive for glial fibrillary acidic protein, a marker for astrocytes (Fig.2, panels A, B). Panel C shows phase contrast photomicrograph of 2-D monolayers of 12-day-old Ara-C (10μM 24 hrs) treated neuronal-enriched cultures. Neurons show extensive dendrite processes with bright aggregates of cell bodies, both of which stained positive for neural filament protein, a marker for neurons (Fig.2 Panel D).

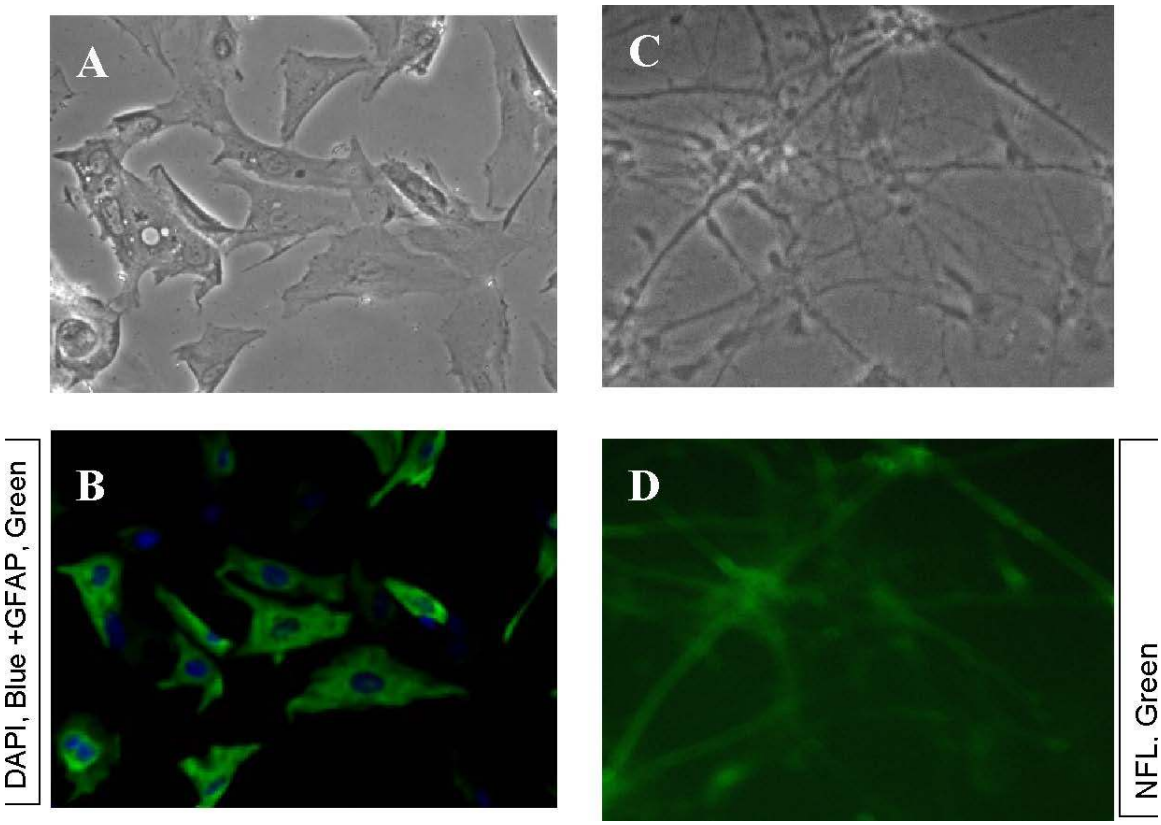

Fig. 2. Primary 2-D cultures of (20x-magnification) rat brain cortical cells. A. Phase contrast micrograph of cortical type 1 astrocytes, 21 days after isolation. B. Immunocytochemical detection of glial fibrillary acids protein (GFAP) a cycloseletal marker for astrocytes. The nuclei, which were stained by DAPI appear as blue fluorescence. C. Phase contrast micrograph (20 x magnifications) of a typical neuronal-enriched culture 12 days after isolation, showing extensive dendrite processes and a central cell body. D. Immunocytochemical detection of neurofilament protein, a neuronal-specific marker for neurons.

Figure 3, panel A shows a phase contrast photomicrograph of a typical Puramatrix peptide hydrogel drop with no tissue slice/cells encapsulated in it. Panel B shows the encapsulation of embryonic brain cortical slice inside the Puramatrix Peptide Hydrogel. Panel C shows cell migration from the tissue. Panel D shows the extension of cellular processes into the Puramatrix peptide hydrogel, indicating that the peptide hydrogel promoted cellular outgrowth and extension of neuronal dendrite processes freely through nanometer size pores.

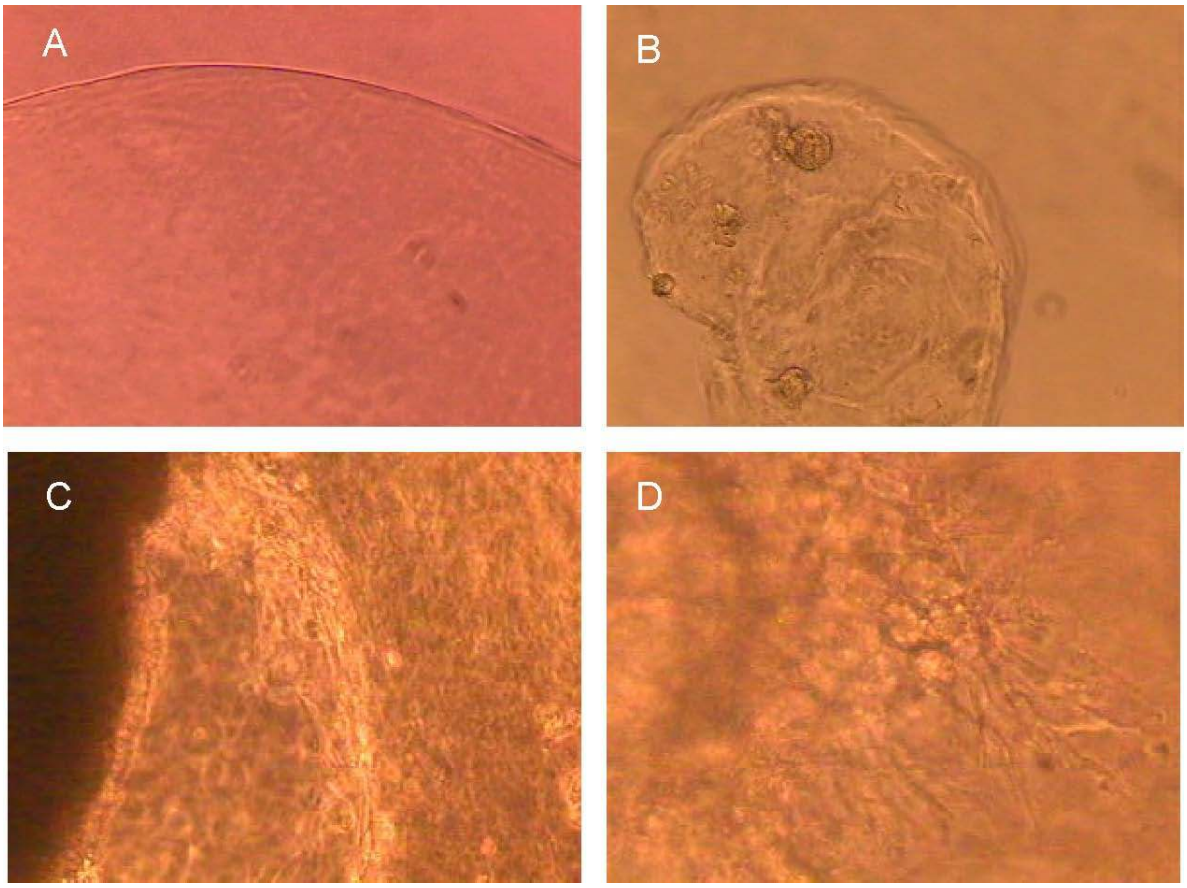

Fig. 3. Cortical organotypic slice cultures grown encapsulated as a "drop" cultures in Puramatrix peptide hydrogel. A. Phase contrast micrograph (20X magnification) showing part of the "drop" of peptide hydrogel without any brain cells/slices. B. Phase contrast photomicrograph showing a typical peptide Hydrogel: drop" culture encapsulating brain cells/slices (10 x magnification). C. Rat brain cortical slice encapsulated in peptide hydrogel "drop" from which developed extended cellular out growth. D. Phase contrast micrograph of a portion of the tissue outgrowth at higher magnification (40x) showing cells bearing extensive dendrite processes.

Figure 4 is the phase contrast photomicrographs showing the encapsulation of rat brain cells in Puramatrix peptide hydrogel. Encapsulation of pure glial cells formed a 3-D "cobble-stone"-like architecture (Fig.4, panel A), whereas the mixed cortical cells exhibited a 3-D cell aggregate with an outgrowth of the cell processes extended outside the aggregate (Panels B, C and D). In some instances the processes from one aggregate formed synapse with the neighboring cell aggregate (panel C).

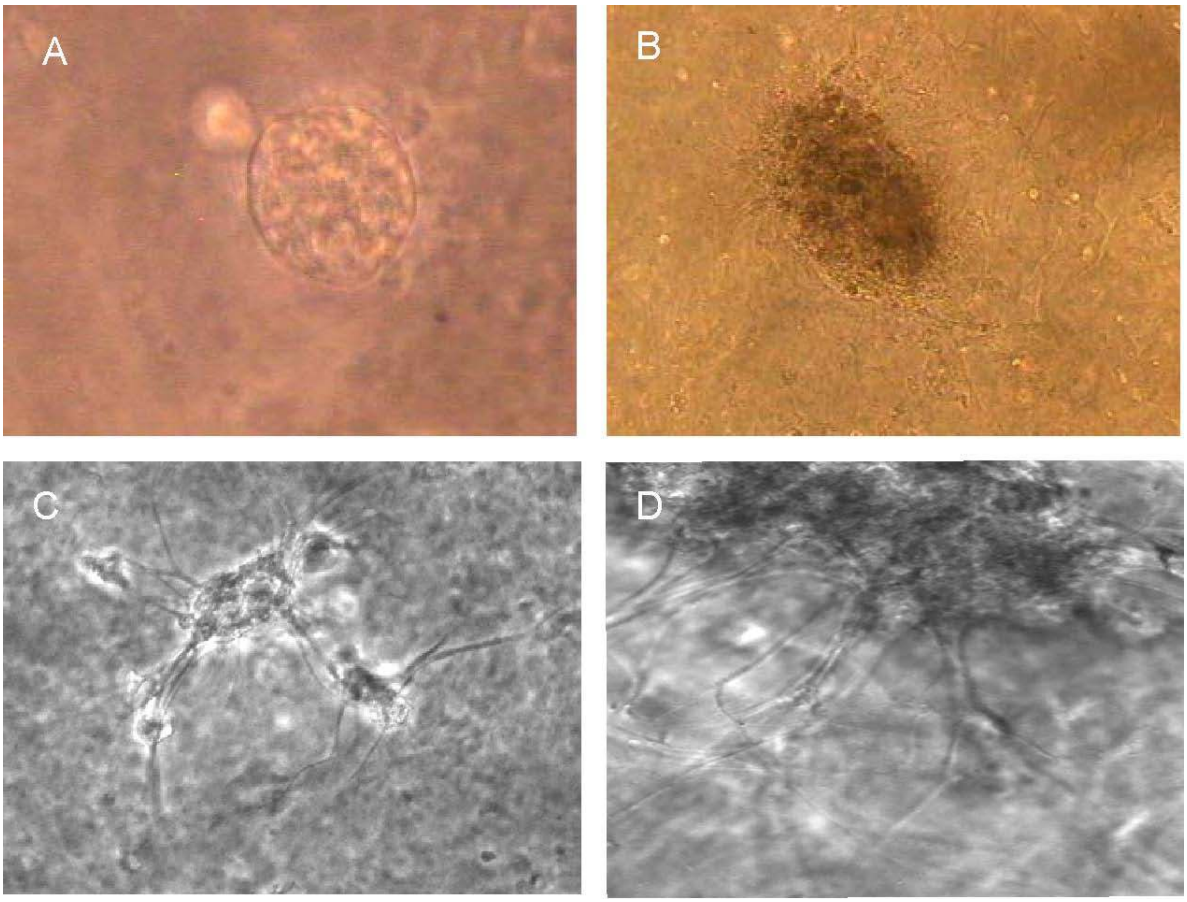

Fig. 4. Phase contrast photomicrophs showing variations in cell growth in 3-D architecture encapsulated in Puramatrix peptide hydrogel drop culture. A. Phase contrast photomicrograph of a typical cell drop culture (10x magnification) showing varying sizes of astroglial cell aggregates in 3-D without any processes extending outside the cell aggregate. B. Phase contrast photomicrograph showing the encapsulation of cortical neuronsglial cell aggregates (20X magnification) bearing extensive dendrite outgrowths. C. Phase contrast photomicrograph showing multiple neuronal-glial aggregates connected to each other via dendrite outgrowth. D. Phase contrast photomicrograph of a typical neuronal-glial aggregate at higher magnification showing the details of neurotic processes.

The Live/Dead Cell Assay studies showed that encapsulated cells were viable (Fig. 5 green fluorescence, panels B, C and D) and remained viable for more than 3 weeks. However, a significant number of dead cells were also found around the live cells (red fluorescence, panels E and F). Panel F shows the merged images containing an inner core of live green cells surrounded by an outer layer of dead cells. The fluorescence microscopic analysis indicated that the 3-D aggregates contained more live cells than dead cells as

assessed by Live/Dead Cell staining assay. Furthermore, at higher magnification revealed that that the live neurons and glial cells were often in physical contact with each other through their processes (Panel C) in a 3-D architecture, exhibiting glial-neuronal interactions.

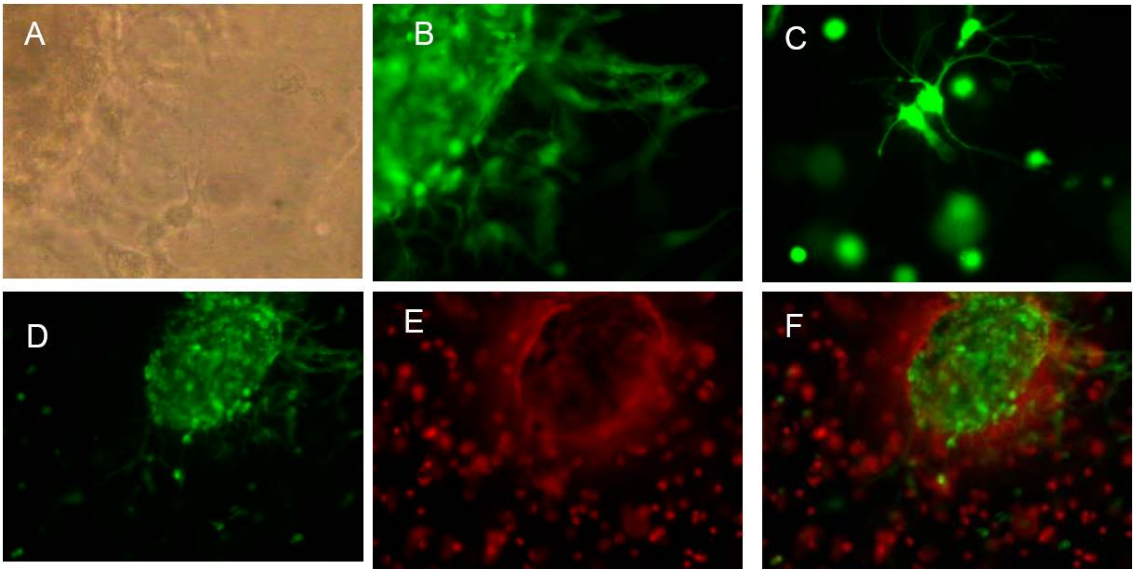

Fig. 5. Immunoflourescent detection of cell viability in a typical 3-D mixed cell drop culture by live/dead cell assay. A. Phase contrast micrograph showing a portion of the mixed cell aggregate (20x magnification) with extensive neurite outgrowth in the drop culture. B. Immunocytochemical detection of green fluorescence (calcein) labeled viable cells in a typical 3-D mixed cell aggregate. C. Immunocytocehmical images of free viable cells near the aggregate at higher (40X) magnification. D and E. Immunocytochemical double staining of live (green) and dead (red) cells, respectively. F shows the merged images of D and E showing an inner core of live cells surrounded by an outer layer of dead cells.

Next, incorporation of BrdU by encapsulated glial cultures was measured, to determine cell proliferation, since BrdU would be incorporated only by replicating cell DNA. Immunocytochemical studies revealed that the cobble-stone-like astroglial aggregates were consisted of a cell population that was positive for BrdU (Fig. 6 Panels C and D), which are also positive for the astrcoyte-specific marker, GFAP (panels B and D). When these images were merged together, an orange colored image showing a 100% colocalization (Panel F), indicating that the 3-D cell aggregates consisted mainly of rapidly multiplying astrocytes. The embryonic brain cortical cell aggregates consisted of process bearing cells (Fig.7 panel A) that were positive for the neuronal marker, neurofilament protein (Fig. 7 panel B) which

were not BrdU +ve.

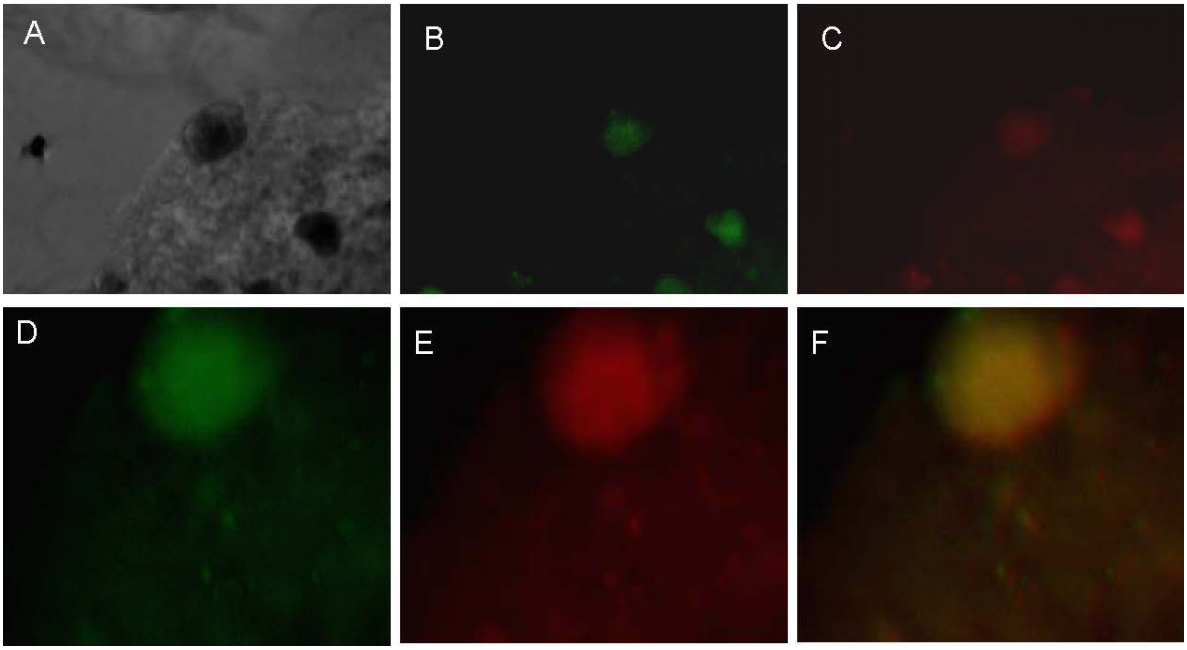

Fig. 6. Photomicrographs showing the phase contrast and double immunocytochemical cololacalization of GFAP+ve and Brdu +ve cells in a glial 3-D culture. A. Phase contrast micrograph (10x) showing glial cell aggregates encapsulated in the peptide hydrogel. B. Immunocytochemical images showing GFAP+ve cell aggregates. C. Immunocytochemical images showing BrdU+ve 3-D aggregates. D and E are GFAP and BrdU+ ve aggregates at higher (40X) magnification. F. Merged immunocytochemical images of D and E showing colocalization of GFAP and BrdU staining in the astroglial aggregates.

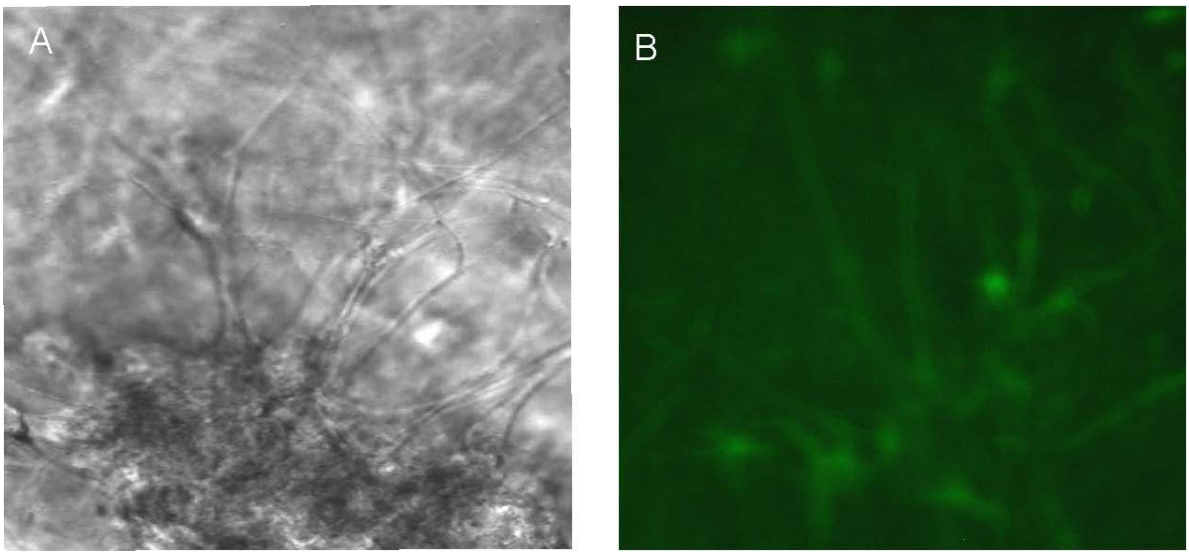

Fig. 7. Photomicrographs showing the phase contrast and immunocytochemical images of mixed cell 3-D aggregates encapsulated in Puramatrix Peptide hydrogel. A. Phase contrast photomicrograph showing a portion of a typical 3-D aggregate with extensive outgrowth of cellular processes. B. Immunocytochemical staining of NFL+ve cells in the 3-D aggregate. Note the diffuse staining in the dendrites as well as in the cell body.

Disruption of the peptide hydrogel by trituration yielded a mixture of cell aggregates, containing pieces of peptide hydrogel and the cell aggregates, which were separated by centrifugation (Fig.8). The recovery of cell aggregates by trituration followed by centrifugation was greater than 65%. However, the cell aggregates were often found associated with small pieces of peptide hydrogel (panel B). The trypsinization of glial cell aggregates showed 60-70% cell viability as measured by trypan blue dye exclusion method and when these aggregates were plated on to a Petri dish the cells started migrating from these aggregates as 2-D monolayer culture (panel C) and incorporated BrdU as detected by immunofluorescence studies (panel D).

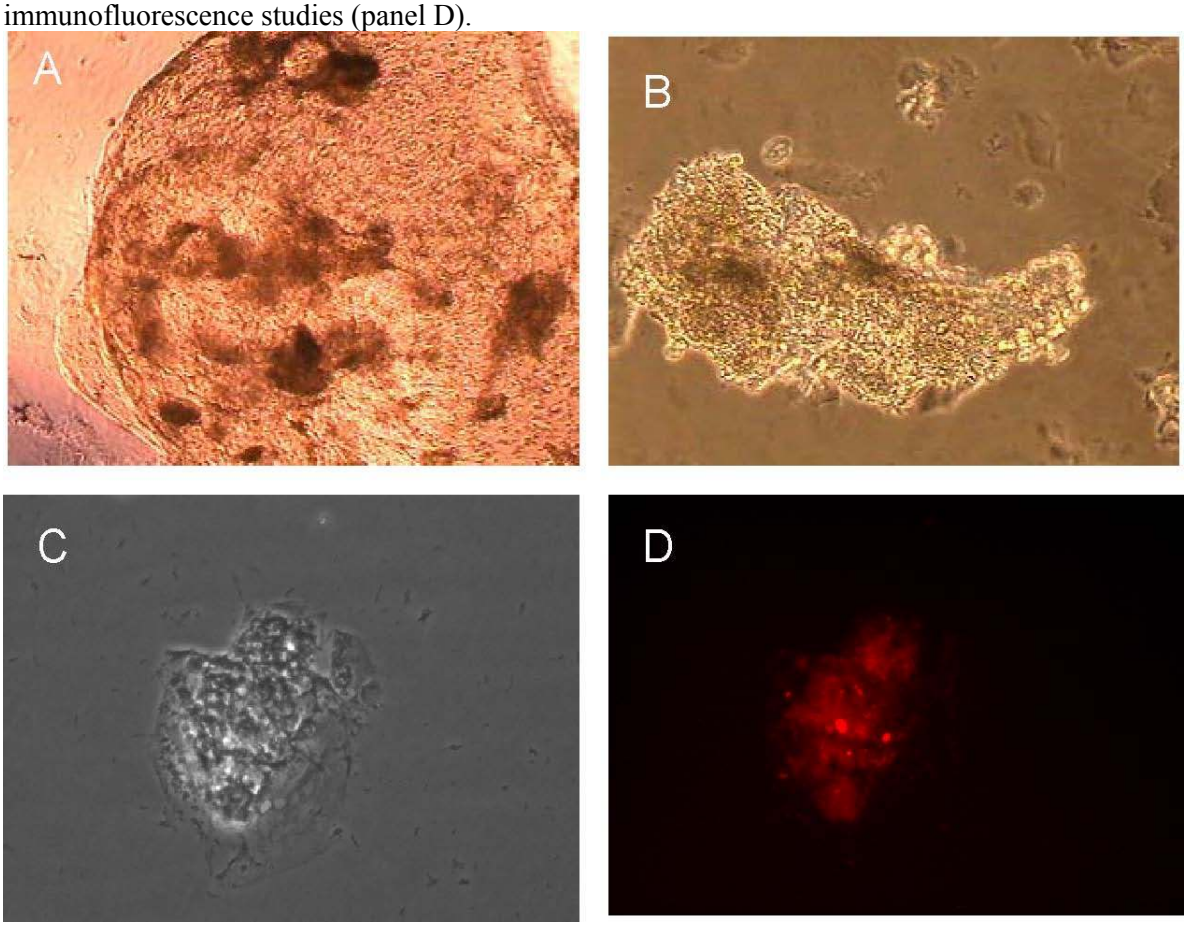

Fig. 8. Recovery of cells from the Puramatrix peptide hydrogel drop culture. A. Phase contrast photomicrograph (10X) showing a typical Puramatrix peptide hydrogel with 3-D astroglial aggregates encapsulated in the drop culture. B. A portion of the disrupted peptide hydrogel with cell aggregate after pipetting up and down. Note the cell aggregate is still attached to portions of the disrupted Puramatrix's hydrogel. C. Phase contrast micrograph of astrocytes migrating from the aggregate as 2-D monolayer. D. Imunocytochemical staining of the astrocyte monolayer showing proliferating BrdU+ve cells.

Morphological characteristics of human neural stem cells

Figure 9, Panel A shows a typical colony of an undifferentiated human neural stem cell line (SC30), cultured on a fibronectin- coated surface of a dish. Immunocytochemical staining of BrdU labeled cells with anti-BrdU antibody showed that the cells are rapidly proliferating (panel B). These cells were also positive for nestin, the cell-specific marker for progenitor cells (panel C). The colony also contained a majority of cells stained positive for MAP-2, a neuronal-specific marker (panel D), indicating the stem cell differentiation into mainly, progenitor and neural cell populations.

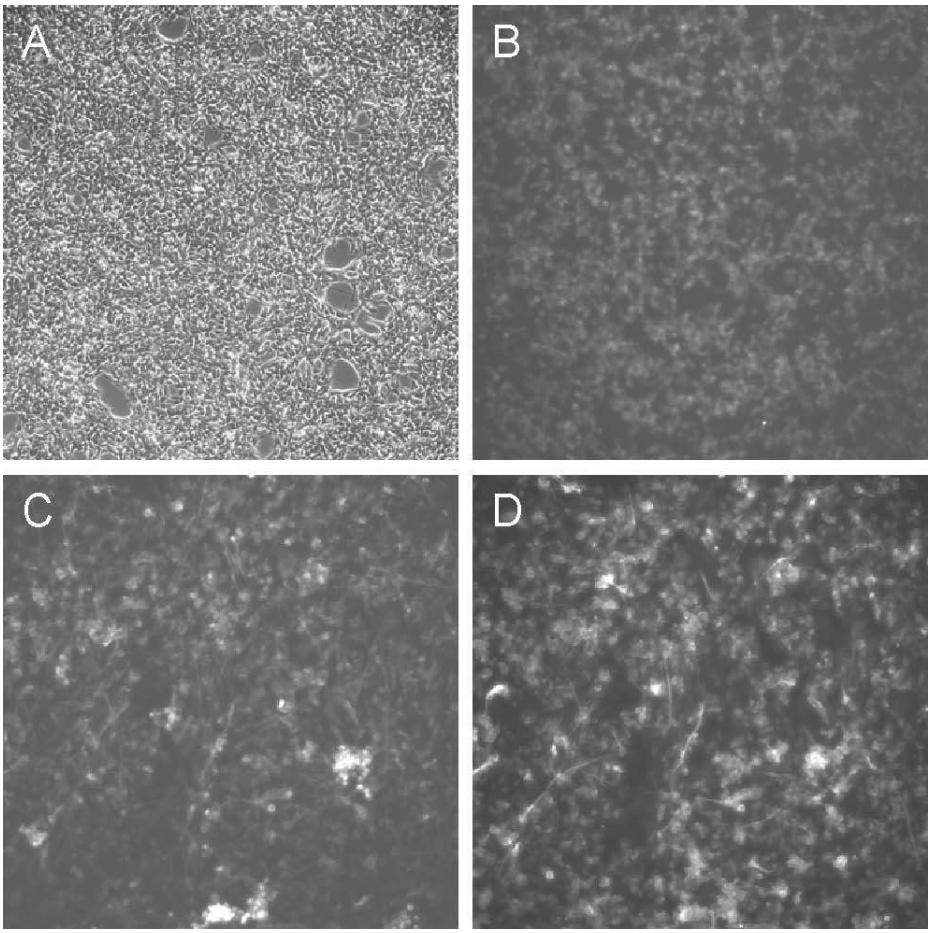

Fig. 9. Photomicrographs of human neural stem cells, SC30 showing BrdU, nestin and MAP2 immunocytochemical staining. A. Phase contrast photomicrograph of a typical colony of human neural progenitor/stem cells, SC30, growing on a fibronectin-coated surface. B. C and D. Immunocytochemical staining of a differentiating SC30 cells showing BrdU+ve proliferating (B), and nestin +ve progenitor cells (C) and MAP-2 +ve mature neurons.

Discussion

The present study examined the suitability of Puramatrix peptide hydrogel as a scaffolding material for engineering the growth of brain-like tissue. This study demonstrates for the first time the cellular growth of isolated rat brain neuronal-glial cells inside the Puramatrix hydrogel. The cell morphology and out-growth were microscopically determined by encapsulating (a) neonatal or prenatal rat brain cortical or hippocampus slices, (b) pure cortical astrocytes or (c) neuronal-glial cocultures in a 3-D

architecture.

Among the scaffolding materials, electrically conducting and biocompatible surfaces are of great importance in neural tissue engineering [10, 11]. The Puramatrix peptide hydrogel provides a highly versatile scaffolding material wherein one can control both the percentage and size of the hydrogel "drop" culture. In this study, 0.5% (w/v) peptide hydrogel solution gave the 3-D architecture with a pore-size large enough for neurons to extend their dendrites and form synapses with that of the same neuron or neighboring neuron (s) (Fig.5, panels C and D). Although a lower percent (< 0.25%w/v) Puramatrix peptide hydrogel provided a larger pore-size, the gel became very soft and fragile to handle, especially when the sizes of the "drop-culture" exceeded 25μL. On the contrary, a 1% (w/v) peptide hydrogel provided a "harder" gel and resulted in no cellular processes or cellular out-growths (data not shown). This is not surprising because neuronal growth inhibition due to narrow mesh size has been previously reported in polyethylene-based scaffolds [11, 18, 19, 20] and also in peptide-based scaffolds [21]. Thus, a 0.5 %(w/v) Puramatrix hydrogel was found optimal for the neural tissue engineering and the "drop- culture" sizes were kept between 25-50μL. The 50μL drop cultures were used to encapsulate brain slices in organotypic cultures of brain slices and 25μL drop cultures were used for encapsulating astrocytes or mixed brain cells. In this study, the 3-D cultures were maintained in the neuronal growth medium supplemented with 5% Horse serum and no additional growth factors were added that were known to influence the neurite outgrowth [19].

Apart from the size and percentage of the peptide hydrogel, it is very important to exercise caution at the following steps: (1) one should minimize the time of exposure of neural cells to this acidic environment once cells were added into the peptide solutions, because the peptide solution is very acidic, (2) work quickly to dispense the solution as 25 or 50μL drops using a wide mouth peptide tip into a growth medium of neutral pH, (3) do not introduce any air bubble, as the latter bursts open the "drop", and (4) change the growth medium at least twice within the first 10 min during gelation period without disturbing the peptide hydrogel "drops". These four steps are the key for a successful brain cell encapsulation and cell growth inside the peptide hydrogel.

The Live/dead cell assay clearly demonstrates that there is an extensive cell loss. However, each aggregate contained >50% live cells in the inner core and are extending their

dendrite processes. It is however, unclear as to why the outer layer of cells was dead in these aggregates. It is also important to note that after a 3-5 day lag period, cells started multiplying as determined by BrdU incorporation. The cellular outgrowth in the organotypic cultures were very similar to those reported previously for hippocampus slices [13, 14].

The cell recovery from the Puramatrix peptide hydrogel however yields cell aggregates that are still attached to small pieces of hydrogel even after centrifugation. It is unclear whether the peptide hydrogel contamination interferes with any quantitative measurement of cell proteins or any enzymatic assays. However, the peptide hydrogel did not interfere with the cell identification by phase contrast and immunofluorescence microscopy. The aggregates were fixed while encapsulated inside the hydrogel and immunocytochemically stained using the cell-specific marker antibody proteins, such as neurofilament (NFL) and GFAP, the markers for neurons and glial cells, respectively. Due to the 3-D nature of the peptide hydrogel, another option is to use confocal microscopy instead of fluorescence microscopy for characterizing cell morphology, and cell-cell interactions.

Finally, in this study, the preliminary results showing the growth of human neural stem cells, NSC30, suggest that the majority of neural stem cells differentiated into nestin-positive progenitor cells and MAP-2 positive neurons. Currently, the feasibility of growing these cells encapsulated in Puramatrix hydrogel is in progress in the author's laboratory. This approach may help develop neurospheres that can be implanted into human brain in future for the treatment of various chronic brain diseases similar to the one used in multiple sclerosis [22].

Future prospective

These results suggest that the neuronal-glial aggregates encapsulated in Puramatrix Peptide Hydrogel represent a 3-D bioartificial brain-like architecture that can be used as an *in vitro* model system in future biomedical research, mainly to study the effects of radiation, therapeutic drug delivery and brain tissue replacement therapy. The 3-D brain cell growth was rapid in encapsulated cell drop culture in 0.5% Puramatrix peptide nanofiber hydrogel. Cell drop culture is a novel method to grow brain cells for longer period of time encapsulated within the nanoscale scaffolds of peptide hydrogel. Encapsulated 3-D cells are readily

recovered by gentle pipetting for studying morphology, cell counting, and to perform other cell-based assays. Puramatrix peptide hydrogel is a novel nanofiber scaffold matrix for the safe growth and maintenance of brain neurons for a longer period of time *in vitro* with a potential biomedical application in brain regenerative medicine and brain tissue replacement therapy. The results in this study suggest that 3-D Puramatrix peptide hydrogel seems to be a good supporting scaffold material for the growth of neurons and glial cells. The "drop" culture represents a unique nanobiotechnology for the growth of engineered brain tissue in a 3-D architecture, to be used in drug discovery assays, clinical therapies and bioproduction.

Acknowledgements

This work was supported by Grant Number G12RR03045-17 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH) and "its contents are solely the responsibility of the authors and do not necessarily represent the official views of NCRR or NIH." The author also wishes to thank Dr. Philip H. Schwartz, Director of National Human Neural Stem Cell Resource, Children's Hospital of orange County
Research Institute, Orange, CA, USA for providing the training opportunity to culture human neural stem cells.

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