Three-Dimensional Collagen Gel Networks for Neural Stem Cell-Based Neural Tissue Engineering

Wu Ma,*[1] *Silvia Chen*,[2,3] *Wendy Fitzgerald*,[2,3] *Dragan Maric*,[4] *Hsingch J. Lin*,[1] *Thomas J. O'Shaughnessy*,[1] *Jeremy Kelly*,[1] *Xiu-Huai Liu*,[5] *Jeffery L. Barker*[4]

[1] Center for Bio/Molecular Science and Engineering, Naval Research Laboratory, Washington, D.C. 20375, USA
Fax: 202-767-9598; E-mail: wma@cbmse.nrl.navy.mil
[2] Wyle Laboratories and NASA/NIH Center for Three Dimensional Tissue Culture, Bethesda, MD 20892, USA
[3]Laboratory of Cellular and Molecular Biophysics, National Institute of Child Health and Human Development, NICHD, NIH, Bethesda, MD 20892, U.S.A.
[4] Laboratory of Neurophysiology, National Institute of Neurological Disorders and Stroke, NIH, Bethesda MD, 20892, USA
[5] Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda MD 20814, USA

Summary: Stem and progenitor cells isolated from the embryonic rat cerebral cortex were immobilized by matrix entrapment in three-dimensional (3D) Type I collagen gels, and cultured in serum-free medium containing basic fibroblast growth factor. The cells trapped within the collagen networks actively proliferated and formed clone-like aggregates. Neurons were the first differentiated cells to appear within the aggregates, followed by generation of astrocytes and oligodendrocytes. In addition, necrotic cores were developed as the aggregate diameter increased and cell viability declined significantly after 3 weeks in culture. To overcome these problems, the cell-collagen constructs were transferred to Rotary Wall Vessel bioreactors for up to 10 weeks. In the rotary culture, the collagen gels compacted 3-4 folds and a long-term growth and differentiation of neural stem and progenitor cells was dynamically maintained. Remarkably, the cell-collagen constructs formed a complex two-layered structure that superficially emulated to a certain extent the cerebral cortex of the embryonic brain in architecture and functionality. The engineered 3D tissue-like constructs displaying characteristic properties of neuronal circuits may have potential use in tissue replacement therapy for injured brain and spinal cord.

Keywords: biopolymers; bioreactor; collagen gels; matrix; neural stem cell

Introduction

Tissue engineering that combines living cells and polymer scaffolds may generate functional three-dimensional (3D) constructs to serve as replacement tissue or organs. There is a particular need for engineered neural tissues since the mammalian central

 DOI: 10.1002/masy.200550933

nervous system (CNS) has little capacity for self-repair after injury. Because neurons are not capable of proliferating and are short-lived in culture, there remain significant challenges for neural tissue engineering. Recent advances in neural stem cell biology have shown that stem cells and progenitors can be isolated from the embryonic and adult CNS and are capable of self-renewal and differentiation into both neurons and glia. These CNS stem cells have the potential to be a valuable source of specific neural cell types, which could be used for engineering of neural tissue. [1-3]

It is well established that mammalian neural stem and progenitor cells and their progeny are anchorage-dependent, and they must attach to a solid surface for survival, growth and differentiation. [4] Therefore, polymer scaffolds play a critical role in neural tissue engineering. Among polymer scaffolds, hydrogels are attractive because of their highly porous and hydrated structure that allows cells to assemble spontaneously and become organized into a recognized tissue, and permit the infusion of nutrients and oxygen and transfer of waste products and CO_2 out of the cells. In the present study, collagen was chosen because it is not only the major class of insoluble fibrous protein in the mammalian extracellular matrix but also a biologically derived hydrogel.

Neural stem cells actively proliferate in collagen gel networks

Stem and progenitor cells were isolated from the embryonic day 13 rat cerebral cortex and then added to pre-gel solutions of collagen (Rat tail tendon, Type I, Boehringer-Mannheim Corp., Indianapolis, IN) prior to casting the material into molds where gelation occurred. [5-6] As a result of crosslink formation, the cells were trapped within the networks and scattered throughout the gel. The cell-collagen constructs were cultured in neurobasal medium (NB, Gibco BRL, Gaithersburg, MD) supplemented with B27 (Gibco), and containing 30 ng/ml of recombinant human basic fibroblast growth factor (bFGF, Intergen, Purchase, NY) for up to 10 weeks. Cell viability was determined by staining immobilized cells with a Live/Dead Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR). Cell proliferation was quantified by counting the number of 5-bromo-2'deoxyuridine$^+$ ($BrdU^+$, proliferative marker) vs. propidium iodide$^+$ (PI^+, stains total cells) cells. Using the 3D scanning ability of a spectral confocal microscope (Leica TCS NT, Leica Microsystems Heidelberg, Germany), we were able to assess neural stem and progenitor cell growth in 3D collagen gels. In response to bFGF, the collagen-entrapped neural stem and progenitor cells actively divided and formed clone-like aggregates

scattered throughout the gel. The proliferating cells sharply increased from ~15% to ~80% over the first week. The proliferating index peaked around 10 days in culture and then declined progressively over the next two weeks.

Neural stem cells cultured in collagen gel networks recapitulate CNS stem cell differentiation

To evaluate the differentiation of multipotent neural stem and progenitor cells along neuronal and glial lineages, collagen-entrapped cells were examined by immunocytochemistry with well-known cell markers, including TuJ1 for neuronal progenitors and immature neurons, MAP-2 for mature neurons, synapsin I for synaptic vesicles, GFAP for astroglial cells and O4 for developing oligodendrocytes. The appearance of these markers represents major maturational events of neural progenitor cells. Although $TuJ1^+$ neuronal progenitors were already present on the first day of culture, most of the cells were immunostained for nestin, an intermediate filament protein characteristic of neural stem and progenitor cells. $GFAP^+$ and $O4^+$ cells did not appear until after 10 days in culture. The collagen-entrapped progenitors also efficiently developed normal neuronal polarity, neurotransmitters, ion channels/receptors, and excitability. Ca^{2+} imaging showed that differentiation of neuronal progenitors was accompanied by a shift in expression of functional receptors for neurotransmitters from cholinergic and purinergic to predominantly GABAergic and glutamatergic. Spontaneous postsynaptic currents were recorded by patch-clamping from progenitor cell-derived neurons and these currents were partially blocked by 10 μM bicuculline, and completely blocked by additional 10 μM of the kainate receptor antagonist CNQX, indicating an appearance of both GABAergic and glutamatergic synaptic activities. These demonstrated that neural progenitor cells cultured in 3D collagen gels recapitulate CNS stem and progenitor cell differentiation.

A cell-collagen-bioreactor system maintains long-term growth and differentiation of neural stem cells

A major challenge for growing cell-collagen constructs is that a necrotic core invariably develops in the center of cell aggregates. The necrotic core contains heavily-packed dead cells, caused by a restricted diffusion of oxygen and nutrients. In addition, the

differentiated neurons in the constructs are typically short-lived, similar to those cultured on 2D surfaces. To overcome these difficulties, simulated microgravity created by a NASA-designed Rotating Wall Vessel (RWV) bioreactor was used in the present study. The RWV bioreactors offer two beneficial factors: low fluid shear stress, which promotes cell-cell contacts and initiation of differentiating cellular signaling, [7] and randomized gravitational vectors, which affect intracellular signal transduction and gene expression. [8] The cell-collagen constructs were cultured in RWV bioreactors for up to 10 weeks. After an initial lag phase of cellular growth and differentiation in the first week, rotary cultures generated large cell aggregates without necrotic cores and accelerated growth and differentiation. The enhanced cell survival and growth could be maintained for up to 10 weeks, while the cells cultured under static conditions declined after 3 weeks (Figure 1).

Figure 1. Time-course of collagen-entrapped neural stem and progenitor cell growth under rotary (RWV) or static (static) culture conditions. Cells were seeded at the same density in collagen gels and cultured in the defined medium with bFGF, which supports survival, proliferation and differentiation. Cells cultured under static conditions manifest a significant growth in the first week and spurt over the next two weeks, and decline gradually in number after third week. Cells cultured in RWV bioreactors show a delay before they proliferate and generate a similar number of progeny as those cultured under static conditions. However, cells cultured in RWV bioreactors maintain the similar number of progeny over the ensuing weeks.

Compared to static cultures, a significantly larger number of TuJ1$^+$ and MAP2$^+$ neurons and synapsin I$^+$ puncta were generated, followed by vigorous gliogenesis in the rotary cultures (Figure 2).

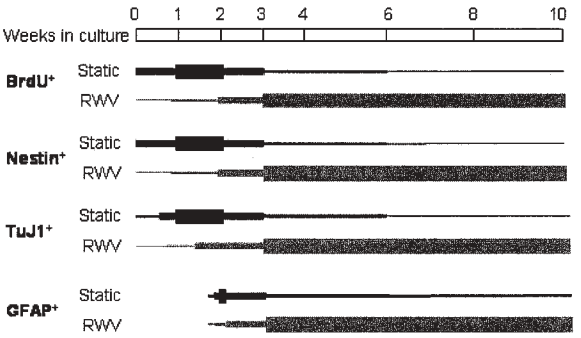

Figure 2. Chronology of BrdU, nestin, TuJ1 and GFAP expressions of collagen-entrapped neural stem and progenitor cells cultured under static conditions or in RWV bioreactors. The relative expressions of BrdU to identify proliferative cells, nestin to mark immature cells, TuJ1 to identify neuronal progenitors and neurons and GFAP to mark astroglial cells, are depicted over time in culture. The thickness of the line depicting marker expression is proportional to the number of cells expressing the markers. Cells cultured in gels under static conditions exhibit proliferative and differentiation markers that are most abundant during the first three weeks. Only a minor population of $GFAP^+$ astroglial cells survival for 10 weeks. In contrast, cells cultured in RWV bioreactors are slightly delayed in their expressions of the same markers compared to cells under static conditions, but all of the markers are maintained throughout 10 weeks.

Differentiating neural cells are polarized and organized into a functional embryonic brain tissue-like structure

Cultured in bioreactors for 4 weeks, developing neural progenitor cells and their progeny in 3D collagen gels began to be polarized. The surface layer is composed primarily of neural progenitor cells, which predominantly express nestin, vimentin, PCNA and GFAP. The cells in the deeper layer were mostly stained for TuJ1 and GFAP. Ca^{2+} imaging showed that cells in the surface layer expressed functional neurotransmitter receptors for cholinergic and purinergic agonists, while in the deeper layer, differentiating cells mainly respond to GABAergic and glutamatergic agonists and to veratridine. Staining of cells in the deeper layer with the endocytotic marker FM1-43 demonstrates active synaptic vesicle recycling occurring among differentiating neurons (Figure 3).

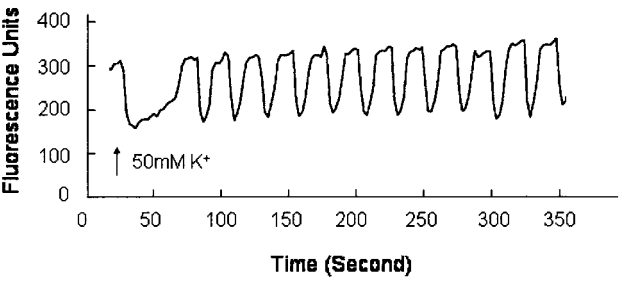

Figure 3. Maturation of synaptic vesicle recycling in collagen-entrapped neurons. Cells in the deeper layer of a rotary culture-produced construct were stained with 10 μM FM1-43, a fluorescence dye, which can image synaptic activity by measuring synaptic recycling in neuronal circuits. This plot shows the time-course of FM1-43 release recorded from a representitive synaptic terminal. FM1-43 was incorporated into the pre-synaptic membrane by endocytosis, and an application of depolarizing solution (50 mM K^+) results in a destaining of FM1-43-labeled synaptic terminals by stimulating exoxytosis. A characteristic oscillation of FM1-43 fluorescence intensity may reflect a dynamic recycling of synaptic vesicles in the local presynaptic terminals. The fluorescence staining is indicated by relative fluorescence intencity (fluorescence units).

This complex two-layered structure superficially emulates the cerebral cortex of the embryonic brain in architecture and functionality. The surface layer cells were nestin$^+$, vimentin$^+$ and PCNA$^+$ reflecting the immature nature of proliferating phenotypes, which correspond to the neuroepithelial cells in the ventricular and subventricular zones of the embryonic brain. In addition, some of surface layer cells were GFAP$^+$. This is consistent with the recent finding that GFAP-expressing astrocytes of the subventricular zone can self-renew as well as generate neurons. [9-10] The deeper layer cells were TuJ1$^+$ and GFAP$^+$ and correspond to the cortical plate where differentiated neurons and glial cells were seen.

Conclusion

We demonstrate that 3D matrices constructed from mammalian type I collagen do support CNS stem and progenitor cell growth and differentiation. The stem cell-collagen-bioreactor system provides at least three major advantages: 1) neural stem cells hold promise as an unlimited source of cells for transplantation therapies; 2) collagen networks provide scaffolds for cell survival and expansion; and 3) bioreactors promote long-term growth and differentiation of neural stem cells in a 3D collagen matrix and generate neural tissue-like constructs without necrotic cores, which resemble polarized cortical tissue.

This strategy could provide a 3D neural tissue-like structure assembled *in vitro* using patient or donor cells and collagen scaffold, which could then be implanted once it has reached a designed developmental stage. Therefore, this strategy presents the promise of utility of tissue engineered constructs not only for use as an *in vitro* model for studies into neural stem cell biology, but also for use as a possible replacement to repair damaged tissue with functional cells and circuits.

[1] F. H. Gage, Current Opinion in Neurobiology **1998**, 8, 671.
[2] R. D. G. McKay, Science **1997**, 276, 66.
[3] L. J. Fisher, *Neurobiology of Disease* **1997,** 4, 1.
[4] E. Ruoslahti, *Science* **1997**, 276, 1345.
[5] S. M. O'Connor, D. A. Stenger, K. M. Shaffer, W. Ma, *Neuroscience Letters,* **2001,** 304, 189.
[6] S. M. O'Connor, D. A. Stenger, K. M. Shaffer, D. Maric, J. L. Barker, W. Ma, *Journal of Neuroscience Methods*, **2000**, 102,187.
[7] T. J. Goodwin, W. F. Schroeder, D. A. Wolf, M. P. Moyer, *Proc. Soc. Exp. Biol. Med.* **1993**, 202, 181.
[8] J. M. Jessup, T. J. Goodwin, G. Spaulding, *J Cell Bioch.* **1993**, 51, 290.
[9] J. Dietrich, M. C. Easterday, *Trends in Neuroscience* **2002,** 25, 129.
[10] F. Doetsch, *Nature Neuroscience* **2003,** 6, 1127.