Grafting microcapsules of genetically modified cells: Therapeutic potential in spinal cord injury

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Introduction

There are approximately 2 million people worldwide who are living with spinal cord injury (SCI), and in the Unites States alone, 11,000 new cases are reported each year . The average age at injury is 28.7 years, and most injuries occurred between the ages of 16 and 30, 78% of then in men www.spinalcord.uab.edu (2006). Injury causes disruption of communication between the brain and other parts of the body, resulting in total or partial loss of sensation and movement below the level of injury. The extent of disability depends on the severity and the level at which injury occurs, but in all cases requires prolonged and expensive rehabilitation. There are many factors influencing central nervous system (CNS) regeneration including retrograde cell death of neurons that cannot be replaced, poor regenerative capacity of surviving neurons, lack of neurotrophic factors at the site, and a hostile, damaged CNS environment including glial scar formation and associated growth inhibitory molecules Murray (2001). Delivery of the required growth factors, and provision of a growth-permissive environment is a challenge. Grafting of genetically modified cells expressing therapeutic products (fibroblasts secreting BDNF, FB/BDNF)) is a promising strategy in spinal cord repair, Grill (1997), Liu (1999). To promote graft survival requires strict immunosuppression protocols with considerable attendant detrimental effects. We utilized immunoprotective alginate-poly-L-ornithine microcapsules for FB/BDNF grafted into a subtotal cervical hemisection and showed partial recovery of forelimb usage without immunosuppression, Tobias (2001, 2005). We reasoned that developing a construct which could bridge the gap in the spinal cord injury, and creating a permissive substrate for growing axons to attach and differentiate would enhance this strategy. We report here studies on modification of an alginate gel surface by covalent attachment of YIGSR peptide, and construction of hydrogel strings that have the potential to be used as a growth-permissive bridge across a spinal cord lesion.

Materials and Methods

FB/BDNF were cultured in 10-cm tissue culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ), harvested with trypsin (Life Technologies, Grand Island, NY), washed three times with sterile 0.9% NaCl, and resuspended at a concentration of $3*10^6$ cells/mL in a solution of filter sterilzed, low-viscosity sodium alginate (Keltone- LV), a gift from The Nutrasweet Kelco Company (Chicago, IL), to obtain a final alginate concentration of 1.5% (w/v). To form capsules, the FB/BDNF-alginate mixture was loaded into a sterile 5-mL syringe fitted with a custom-made jethead (alcohol sterilized), mounted in a Sage syringe pump (model 355; Orion Research Inc., Beverly, MA), and sprayed using a coaxial stream of sterile air (2 slpm), into a solution of 1.3% (w/v) autoclaved $CaCl_2$ through a 22 G needle in the jethead. Spherical microcapsules were formed due to the cross-linking of the alginate droplets by Ca^{2+} ions. The microcapsules were allowed to harden in the cross-linking solution for 1 hour, after which they were washed twice with 0.9% sterile saline (pH 7.35). The microcapsules were then coated with a 0.5 mg/mL filter-sterilized solution of poly-L-ornithine (molecular weight 15,000–30,000; Sigma Chemicals, St. Louis, MI). To form strings, $3*10^6$ cells per ml of 1% (w/v) alginate solution were loaded into the syringe fitted with a non-beveled 22G needle. The syringe was mounted on syringe pump, the flow rate set at 63 ml/min and diameter set at 44 mm. The tip of the needle was dipped into 450 ml of sterile 1.3% $CaCl_2$ solution in a tall 500 ml beaker (autoclaved). Calcium cross-linked alginate strings were

produced as the alginate was extruded from the syringe, and then allowed to sit in the $CaCl_2$ solution to harden for an hour followed by extensive washing with sterile saline. The strings were then coated with poly-L-ornithine, molecular weight 15,000 - 30,000, at a concentration of 0.5mg/ml of alginate for 6 minutes under gentle shaking. The strings were washed 3 times with 200 ml of HEPES buffer to remove any unreacted PLO. The washed strings were contacted with 100 ml of 1% (w/v) YIGSR modified alginate solution (sterile filtered) for 15 minutes, and washed three times with sterile HEPES buffer to remove excess unreacted modified alginate. The strings were between 350-450μm in thickness as seen in fig.1 panel G. Strings made from 1ml volume of alginate were placed in each well of flat bottom twelve well culture plates. For modified alginate YIGSR peptide was covalently attached to the alginate using an adaptation of aqueous carbodiimide chemistry, resulting in the formation of an amide bond between the carboxylic acid groups of the alginate and the amine terminus of the peptide. Alginate was dissolved in MES buffer (0.1 M MES, 0.3 M NaCl, pH 6.5) to obtain a 1% (w/v) solution, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was added to activate the carboxylic acid groups. The amount of EDC added was such that 5% of the carboxylic acid groups of the alginate are activated (50 mg EDC/g alginate). This was followed by the addition of sulfo-NHS in the molar ratio 1:2 to EDC (28 mg sulfo-NHS/g alginate). The solution was stirred for 15 min. to allow the activation to proceed , following which the appropriate amount of YIGSR peptide was added. The conjugation reaction was allowed to proceed for 24 h at room temperature under gentle stirring. The reaction mixture was then dialyzed for 4 days against about 20 liter of deionized water to remove buffer salts, reaction byproducts, and unreacted peptide using Spectra/Por dialysis tubing (MWCO 3500). The purified YIGSR-alginate conjugate solution was transferred to 50 mL polypropylene tubes, and lyophilized. The final fibrous product was then stored in airtight tubes at 20°C for future use. A 1% (w/v) solution in MES buffer was prepared from the lyophilized modified alginate, which was used to coat the strings. The two cell lines that were investigated, a human neuroblastoma cell line SHSY5Y and NB2a, a mouse line, were cultured, in cell culture medium containing DMEM with 2 mM glutamine, penicillin (100 IU/ml), streptomycin (50 μg/ml) and 10% fetal bovine serum (FBS), in 100 mm polystyrene tissue culture plates in a 37°C incubator with 5% CO_2. Cells from passage number 2-5, passaged at approximately 70-80% confluency were used for the experiments to study attachment and differentiation. Cells were harvested from culture using 0.25% trypsin, centrifuged, counted and resuspended in serum free medium made up of DMEM , 10% serum replacement, 2mM L-Glutamine. The strings were seeded with 500,000 cells per well and incubated at 37°C at 5% CO_2. After the cells had attached the medium was removed and replaced with differentiation medium consisting of serum free medium, 0.1% FBS, 5μM Retinoic acid for SHSY5Y and added 0.1% FBS, 10μM Dibutryl cyclic adenosine monophosphate for NB2a. This was replaced daily.

Results

Figure 1 compares capsules and strings containing Fb/BDNF. The capsules containing Fb/BDNF were uniform in size and approximately 400–600 μm in diameter. Forty-eight hours after encapsulation Fb/BDNF could be recognized as individual cells (Fig. 1B), and most stained positively for β-galactosidase, the product of the reporter gene, (Fig. 1C and D) indicating that the BNDF was being successfully transcribed. Encapsulated Fb/BDNF continued to divide and formed spheroid cell clusters, which filled the capsules by week 4 (Fig. 1E), and maintained β-galactosidase expression for at least 8 weeks (Fig. 1F). Fb/BDNF behaved in a similar fashion when encapsulated in strings, continuing to grow into spheroid clusters and to express active BDNF (Fig, 1 G - I). The spheroids were evenly distributed through the strings.

We have previously shown in a rat model that encapsulated Fb/BDNF are protected from rejection after transplantation into a cervical cord injury site by the capsules and promoted behavioral recovery Tobias (2005). The encapsulated Fb/BDNF also elicited axonal growth, but rubrospinal

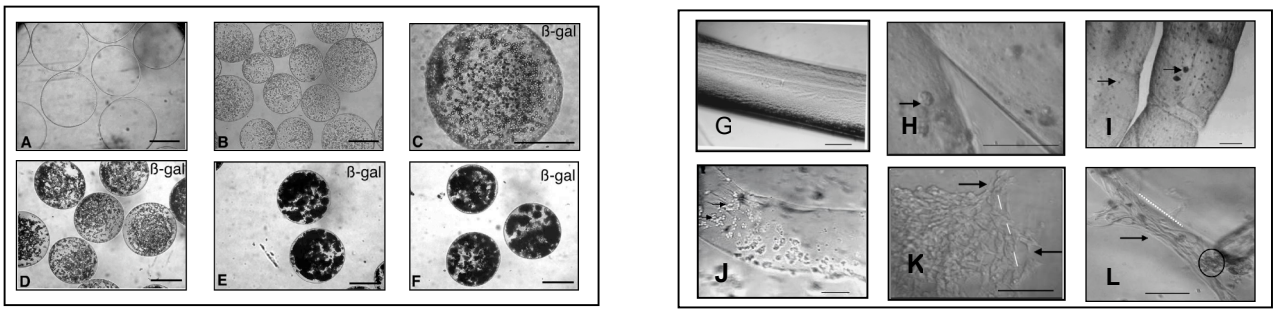

Figure 1: Encapsulated Fb/BDNF, growth and reporter gene expression. Microcapsules over 2 months in vitro (Left): *Phase contrast microscopy* A) cell free capsules, B) immediately after encapsulation. *β-galactosidase staining* C) and D) high and lower-power view 48 h after encapsulation E) at 4 weeks and F) at 8 weeks after encapsulation. Bar = 250 µm.
Strings (Right): G) empty string H) string with Fb/BDNF @ 2 days, I) *β-galactosidase staining* of encapsulated Fb/BDNF after 2 weeks (arrows show spheroid aggregates), J) neurite adhesion (arrows) of NB2a cells on modified alginate-coated strings K) SHSY5Y differentiation on modified alginate string 2 days post addition of differentiation medium, line demarks edge of string L) SHSY5Y neurites extend along string 7 days post addition of differentiation medium. Dashed line demarks edge of string. Bar = 100 µm.

axons did not regenerate. In contrast, grafted unprotected Fb/BDNF were lost without immune suppression. Encouraged by these results, and as a prelude to using the strings as a bridge in a similar spinal cord model, we investigated the potential to develop a growth-permissive coating on the strings that would encourage and enhance cell attachment and growth along the string. We chose to chemically attach the pentapeptide YIGSR to alginate and add this as a final coat after the PLO coating. Peptides such as the YIGSR peptide of laminin have been shown to promote neurite activity, are commercially available, and can be covalently linked to alginate via aqueous carbo-diimidechemistry. Laminin is an extracellular matrix protein found in basement membranes and is both a structural component as well as a biologically active one. We hypothesize that this process could be used to increase the growth permissive nature of alginate strings. EDC, a water-soluble, zero-length alginate alone (Plain, S for SHY5Y cells and N for NB2a cells), or to strings coated with only PLO (PLO S and PLO N). The SHSY5Y cell adhesion to the modified alginate strings was found to be about 26.4% of the initial cells added and for the NB2a cells about 34.8% ofcells initially added attached. Cells that did not adhere to the strings settled to the bottom of the wells and at the end of 24 hours were removed with the medium while the strings were transferred to a new culture plate. NB2a cells, which grow more rapidly and produce more confluent and robust growth, had crosslinker was used to form amide linkages between the amine terminus of the peptide and the carboxyl groups on the alginate. EDC equivalent to 5% uronic acid activation was used to minimize side reactions associated with increasing amounts of EDC, which could affect the gelation properties of the alginate and could have undesirable effects *in vitro* as well as *in vivo*. Both SHSY5Y and NB2a cells in culture attached to strings that were coated with YIGSR modified alginate. Fig. 1 panel J shows a modified alginate string with adherent NB2a cells. When cells were contacted with strings of different compositions, and the numbers adhering after 24 hours assessed after trypsinizing the strings and staining the released cells with 0.4% typan blue and counting using a haemocytometer, it was found that only strings coated with modified alginate showed any significant cell count. Figure 2A shows that neither cell line adhered to strings of alginate alone (Plain, S for SHY5Y cells and N for NB2a cells), or to strings coated with only PLO (PLO S and PLO N). The SHSY5Y cell adhesion to the modified alginate strings was found to be about 26.4% of the initial cells added and for the NB2a cells about 34.8% of cells initially added attached. Cells that did not adhere to the strings settled to the bottom of the wells and at the end of

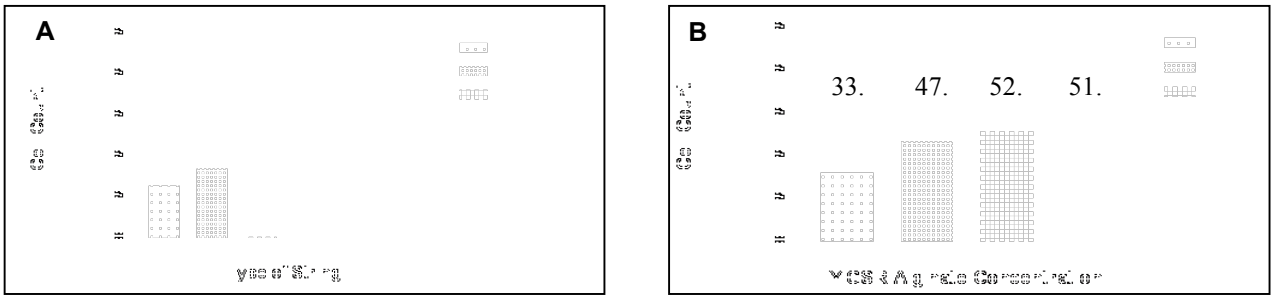

Figure 2. Adhesion of cells to modified alginate strings. A) Neuronal cell attachment to different types of string. B) Effect of increasing peptide loading on NB2a cell adhesion to modified alginate.

24 hours were removed with the medium while the strings were transferred to a new culture plate. NB2a cells, which grow more rapidly and produce more confluent and robust growth, had statistically significant higher adhesion. In a quantitative comparison of numbers adhering, as the amount of peptide used to modify the alginate was increased it was found that a maximum of 52.5 % of NB2a cells adhered when 3 mg/g of alginate were used. This is shown in figure 2B.

Having established adhesion, we also show differentiation. Differentiation medium was added to cells adhered to alginate strings. Fig. 1. Panel L shows neurites extending from SHSY5Y cell bodies and growing along a modified string, an excellent starting point for *in vivo* experiments.

Conclusions

Encapsulation of genetically engineered fibroblasts provides an excellent construct for delivery of neurotrophic factors to the injured spinal cord. Capsule protects the cells from the immune system, while allowing the fibroblasts to grow and continue to express BNDF. The construct can be fashioned into strings, providing a better support to bridge the gap at the injury site, but alginate itself has no inherent cell adhesive property. To enhance neuronal adhesion and regeneration, alginate was modified with the basement membrane peptide YIGSR. Upwards of 30% of the cells added to the grafts adhered when alginate was modified with 1mg of YIGSR/ gm of alginate, and over 50% with 2 mg. We have shown similar results on alginate discs (Dhoot 2004) and Yu (2005), using laminin derived peptide modified HEMA grafts to show that chick embryo dorsal root ganglia cells (DRGs) adhered to these peptide modified surfaces but not control polymers with no peptide.

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