Nanoparticles in Optic Nerve Trauma: Nanoscaffolding, Visualization and Regeneration

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LEARNING OBJECTIVES

1. Understanding that the optic tract can be regenerated and that this process is dynamic.
2. How nanotechnology is beginning to impact medicine and the eye.
3. Size changes ADME and can also impact toxicity.

CME QUESTIONS

1. How does size change the properties of drugs and materials?
2. How does time impact the ability to regenerate the optic tract and nerve?
3. How would this type of material change the delivery, targeting, and dose regime of a treatment?

KEYWORDS

1. Self-assembled systems
2. CNS regeneration
3. Nanotechnology
4. fMRI
5. Vision

INTRODUCTION

Within the emerging field of stem cells there is a need for an environment that can regulate cell activity, to slow down differentiation or proliferation, *in vitro* or *in vivo* while remaining invisible to the immune system.

The ability to keep the cells forever young during implantation is very important to the reconstitution of tissue or organs being replaced. In the emerging field of stem cells there is a need for an environment that can be tuned to the needs of the cells to slow down the differentiation or proliferation in vitro or in vivo while remaining invisible to the immune system. We have shown that when cells are placed into a defined system we can delay their proliferation, differentiation and maturation depending on the density of the cell population, density of the matrix, and the local environment. This is done in a three dimensional environment that allows the benefits of cell therapy to be more fully realized.

Each time in history where there is a power of 10 increase in resolution it is followed by an exponential increase in the number of discoveries[1]. We are now sitting at that threshold, with the dawning of nanotechnology and our ability to manipulate molecules in the body and build nanodevices. These devices will allow for a higher resolution of analysis. This symposium is to outline what is fact today and where things are going in the near future.

Nanotechnology is often associated with materials fabrication, microelectronics, and microfluidics. Until now, the use of nanotechnology and molecular self assembly in biomedicine to repair injured brain structures has not been explored. In order to achieve axonal regeneration after injury in the central nervous system several formidable barriers must be overcome, such as scar tissue formation after tissue injury; gaps in nervous tissue formed during phagocytosis of dying cells after injury and the failure of many adult neurons to initiate axonal extension[2].

Using the mammalian visual system as a model, we previously demonstrated that a designed self-assembling peptide nanofiber scaffold creates a permissive environment not only for axons to regenerate through the site of an acute injury, but also to knit the brain tissue together. In experiments using a severed optic tract in the hamster, we showed that regenerated axons were able to reconnect to target tissues with sufficient density to promote functional return of vision, as evidenced by visually elicited orienting behavior. The peptide nanofiber scaffold not only represents a new nanobiomedical technology for tissue repair and restoration, but also raises the possibility of effective treatment of central nervous system and other tissue or organ trauma[2].

There are four distinct issues that need to be addressed in order for successful central nervous system (CNS) regeneration to occur[3] (Figure 1):

1. ***Preserve*** – How can neurons with damaged axons be kept alive long enough to regrow and obtain functional regeneration?
2. ***Permit*** – How can a permissive environment be created that will allow axons to grow through the site of the lesion?

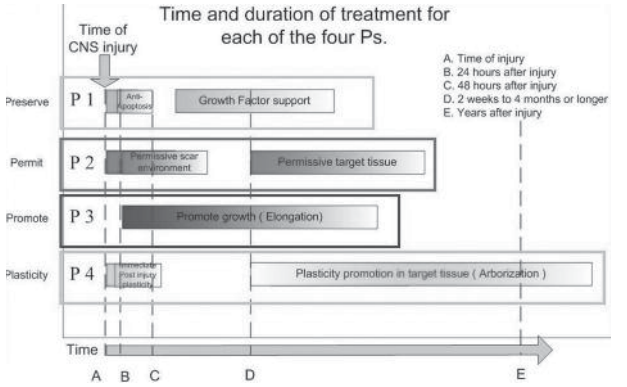
   Using the mammalian visual system as a model, we showed that a designed self-assembling peptide nanofiber scaffold (SAP), in combination with Chondroitenase-ABC creates a permissive environment not only for axons to regenerate through the site of an acute injury, but also to knit the brain tissue together in both young and adult animals.

3. ***Promote*** – How can axon growth be promoted in the correct direction coincident with the onset of the permissive environment?

   The combination of SAP and CNTF is shown to offer an effective new means of creating a permissive environment for axonal growth while CNTF promotes growth of axons across this environment in tissue disruptions caused by traumatic injury to the CNS, allowing regrowth of axons into the SC and functional return of vision.

4. ***Plasticity*** – How can changes in morphology *in vivo* be promoted so the axon connections are functional in the target, mapped correctly and are in sufficient amount to drive behavior?

FIGURE 1. Each of the four Pís of regeneration is represented by horizontal bars depicting the time of treatment and the duration the treatment must remain active. P1 is preserving cells and is broken down into two parts: the anti-apoptosis and anti-necrosis treatment and the growth factor support during elongation. P2 is also broken into two parts: the first treatment is to create a permissive environment around the site of injury; then the target tissue must be made permissive to the reinnervating axons. P3 is the promotion of growth of the axons in an elongation mode. P4, plasticity, has two parts: the immediate post-injury plasticity must be reduced so filling-in is minimal and, while axons are reconnecting in the target tissue, plasticity must be up-regulated. This function can be accomplished by factors or training. Timeline runs from left to right and starts at the time of injury. A is the time of injury, B is 24-hours post injury, C is 72 to 96 hours post injury, D is 2 weeks to 4 months and can extend longer, and E is years.



First P: preserving the cell

Until recently, the area of cell preservation was the most important area of CNS regeneration, because without the living cell, neuron regeneration is impossible. Treatments are available, such as hypothermia, which can keep CNS tissue alive or delay apoptosis but can only be used in the surgical theater.

Nanotechnology can keep cells alive[6] either through (1) enabling better delivery of a small molecule, such as methylprednisone, (2) transfections using a known viral delivery method with a nanocomplex, or (3) the promise of RNA–mediated interference (RNAi) technology that will impact the future of this field. Each treatment is new, or has better–enabled control and delivery, because of nanotechnology[3].

Second P: creating a permissive environment

Several formidable barriers must be overcome to achieve axonal regeneration after injury in the CNS. These obstacles are (1) scar tissue formation after tissue injury, (2) gaps in nervous tissue formed during phagocytosis of dying cells after injury, (3) factors that inhibit axon growth in the mature mammalian CNS, and (4) failure of many adult neurons to initiate axonal extension.

Through reducing or overcoming the first two and possibly the first three of the barriers described above and creating a permissive environment for axonal regrowth using a synthetic biologic nanomaterial, with components that break down into beneficial building blocks and produce no adverse effects in the CNS, functional behavioral recovery becomes a reality.

Three avenues are available through which a permissive environment can be created: (1) blockade of scar formation; (2) removal of the scar and accompanying growth inhibitory signals, and (3) use of bridge materials that are grafts or artificial self–assembling peptides.

The inactivation of inhibitory signals in the scar creates a local permissive environment at the site of injury. However, the permissive environment is not limited to the scar and the area surrounding the injury, but extends to the target–tissue inhibitory factors, such as ephrins and Eph receptors, that block or cause axon growth cone collapse. In the past, regeneration was the removal of barriers to axon growth.

Neurons that are normally able to grow may not be able to grow through an injury, depending on the conditions in the local environment of the injury site. The goal is to attack the local environment in a way that will permit neurons to grow through to the target tissue. If the path is not permissive, neurons will stop growing and produce retraction bulbs and a cavity[3].

Third P: promoting neuronal growth

Once a permissive environment has been created, the next task is to restore the CNS neuronís ability to elongate and promote growth. This difficult problem has two components that must be addressed in a temporally distinct manner. The ëëclock needs to be turned backíí on the neuron and reset to early development, which means turning down transmission and turning up elongation while ensuring that the environment through which the neurons must grow is permissive.

Previously, regeneration was simply the removal of the inhibitory signals, but this has proven to be inadequate to achieve functional recovery in the CNS. Early in development, during the elongation phase of growth, many CNS neurons are able to grow through a permissive environment without any promotion. However, after early development this ability seems to be lost. The assumption is that neurons are successfully preserved and a permissive environment has been created.

Two distinct issues must be addressed in promoting axon growth: 1) getting the axons to elongate in a similar way to the early stage of development and 2) getting the signal for growth to switch from elongation to arborisation when the target tissue is reached.

Promotion of growth for regeneration is a relatively new area. Until recently the dominant theme in regeneration was removing the scar or inhibitory molecules, which experts believed would cause regeneration to spontaneously occur. This misconception was caused by the inability to overcome the inhibitory environment at the site of CNS injury and the ëëaspiriníí approach to regeneration: one magic bullet will cure the deficit. There was also a lack of understanding that neurons are not always able to grow.

For clarification purposes, grow or growth refers to the ability of the axons to elongate or extend collaterals several microns to millimeters. Arborize, focalize, or prune refers to the ability of dendrites to make new, strengthen, or eliminate existing connections. Promotion means the elongation of axons over long distances.

The promotion of axon growth in the adult CNS has no magic bullet. The careful selection of treatments to achieve axon growth promotion in the population of injured axons desired requires understanding early development of each neuronal type and the surrounding environment, in addition to the distances each axon must grow before arborization commences.

The discovery of a non-viral transfection technique, along with the successful transfection of Bcl-2 in vivo at promising rates in a targeted neuronal population, is the first step toward successfully targeting an affected neuronal population with a growth-promoting gene.

Finally, controlling neurons as they regenerate in the CNS requires new ways to pull apart the contribution of different aspects of growth promotion[3]. The fourth, and possibly the most important aspect of regeneration and recovery of function, is plasticity.

Fourth P: plasticity

Plasticity comes into play two times after injury. First, during the immediate period after an injury, sprouting occurs by the surrounding tissue, occupying the vacated space. Second, plasticity, driven by behavior, occurs after reinnervation. Although these two plastic events are equally important in CNS regeneration, this article only addresses plasticity after reconnection to the target tissue. More plasticity is the growth of terminal arbors or the growth of interneurons over very short distances. A few microns would be the longest neurite or dendrite change, which can also mean the up-regulation of transmission at an existing synapse; however, although this fits the definition of plasticity, this measurement was not possible until a couple of years ago *in vivo*.

The four Pís framework outlines the issues that must be addressed before behavioral return of function can occur. With the advent of nanomedicine, functional return after trauma or stroke seems to be an achievable medical goal, and not the intractable problem it once was. Nanomedicine is an area where size really does matter. People are often amazed at how small things can make such a big impact, and this is also true in nanomedicine. Experts always believed that to get therapeutically relevant dosages, blood serum levels needed to be checked. However, in the realm of nanomedicine, the only relevant measures are how to impact the specific cell population, how long to continue, and how to measure it noninvasively[3].

NANOSCAFFOLDS

Scaffolds play a central role in organ regeneration. They act as a template and guide for cell proliferation, cell differentiation and tissue growth, as well as a way to control the release of drugs at rates matching the physiological need of the tissue. The surface of the scaffold provides a substrate for cell adhesion and migration, which can influence the survival of transplanted cells or the invasion of cells from the surrounding tissue[4].

Although many promising strategies have been developed for controlling the release of drugs from scaffolds, there are still challenges to be addressed for these scaffolds to serve as successful treatments: (a) precise placement of the cells into the scaffold to prevent migration from the scaffold before it has been repopulated; (b) ability of the scaffold to allow cells to migrate into it, in order to reconstitute the tissue from the surrounding area and (c) prevention of the acidic breakdown of the cell scaffold, which results in an adverse environment for cell growth[4].

Many types of scaffolds, utilizing a wide range of materials, have been used for the regeneration and repair of the nervous system. In treating TBI or SCI drug delivering scaffolds may need to be combined with cell transplantation to obtain functional recovery[4].

TYPES OF NANOSCAFFOLDS

Natural materials can impart intrinsic signals within the structure that can enhance tissue formation. They include alginate, chitosan, collagen, fibrin and hyaluron. They possess many properties that make them attractive for tissue applications. Many of these materials contain sites for cell adhesion, allowing for cell attachment. These materials also exhibit similar properties to the tissues they are replacing. Since these materials are obtained from

natural sources, they must be purified to ensure that no foreign body response occurs after implantation. Homogeneity of product between lots can be an issue with natural materials.

In addition: (a) chitosan can cause an allergic reaction; (b) fibrin, from blood products, and collagen, from animal products, have been known to cause an immune response; (c) some, but not all, natural materials allow for cell infiltration; (d) the modulus of these materials may be very different from that of the tissue they are implanted in. In a pulsatile environment the materials will shear away from the surrounding tissue, causing additional damage; and (e) by creating an environment that will not move in the same way as the surrounding tissues, the cells are subjected to an environment different from their native one; this can lead to a very different expression pattern of genes, one that produces an extracellular environment that is not conducive to repair, possibly leading to the development of tissue unable to function like the original tissue[4].

Synthetic materials have known compositions and can be custom designed with specific properties. They include PEG, PLA/PGA/PLGA acid and pHEMA-MMA. PLAs, PLGAs and MMAs have recently been used to form scaffolds pre-impregnated with cells; the entire scaffold is then implanted in the damaged tissue area. Synthetic materials have many advantages for use as scaffolds. These polymers can be tailored to produce a wide range of mechanical properties and degradation rates. They also have known compositions and can be designed to minimize the immune response. Finally, synthetic polymers can be reacted together to combine the properties that are unique to each. A drawback is that degradation by-products can be absorbed by the body and may cause pH changes around the implantation site, leading to necrosis and delayed apoptosis[4].

The next generation of cell scaffolds to emerge are **synthetic biological materials**, or designed self-assembling peptides, that spontaneously form nanofibers creating a scaffold-like tissue-bridging structure that provides a framework for axonal regeneration. They are formed through the assembly of ionic self-complementary peptides and are designed by using alternating positive and negative L-amino acids that form highly hydrated scaffolds in the presence of physiological-concentration salts, i.e., saline, tissue culture media, physiological solutions, or human body fluids such as cerebrospinal fluid. Because these scaffolds assemble using weak ionic bonds and van der Waalís forces the scaffolds are motile and able to disassemble and reassemble in the nanodomain, depending on the charge change[4].

They allow for high cell implantation densities and enables cells to migrate freely in and out of the scaffold and the surrounding tissue. The material can also be designed to match the modulus of the surrounding tissue. There appears to be no immune response when the material is either (a) pre-buffered or (b) allows buffering by the surrounding tissue and liquid in the injury site. During breakdown there is no decrease of pH in the local environment. A drawback is that making pure nanomaterials in large quantities can be difficult because the process involves putting molecules together one at a time. To do that with high fidelity, in bulk, is a technological challenge[4].

EXPERIMENT: CREATING A MORE PERMISSIVE ENVIRONMENT

Behavioral testing of animals drives plasticity and rewiring of the brain in the superior colliculus. Neurons in the adult mammalian (CNS) have limited capability to regenerate their axons after injury. It is generally accepted that the lower capacity of CNS axons to regenerate is partly due to the local environment of the injured CNS axons. Traumatic injury in CNS is often followed by robust glial reaction, the failure of CNS axons to regenerate is partly attributed to the inhibitory surface of the glial scar and extracellular matrix (ECM) produced by oligodendrocyte and astrocyte. The glycosaminoglycan side chains of proteoglycans, such as chondroitin sulfate (CS) are putative components of the ECM contributing to the non-permissive properties of the injured CNS. Furthermore, a tissue gap formed after traumatic injury would completely block the re-innervation of the CNS axons.

In this experiment, the brachium of the superior colliculus was completely transected in a group of 22 adult golden hamsters and 20 µl of 1% Self-Assembling Peptide (SAP) and/or 2.5 units/ml (final concentration) Chondroitinase ABC were injected into the lesion site. The progression of axonal regeneration and the re-innervations of the superior colliculus were monitored at 4, 6, and 12weeks following the lesion by intravitreal injection of CTB-FITC. We found that SAP nanofiber scaffold used in combination of Chondrointinase ABC could facilitate the retinal fibers to regenerate across the lesion site. The re-innervations of the superior colliculus were observed in the combination group as early as four weeks after the transection while controls showed no reinnervation at any time point. In behavioral study, the adult hamsters showed a functional return of vision in the SAP/Chondroitinase ABC treated cases beginning at 6 weeks post surgery sooner than those treated with either SAP or Chondroitinase ABC alone. Thus, the SAP/Chondroitinase ABC combination is shown to offer an effective new means of creating a more permissive environment for growth after traumatic injury to the CNS, allowing regrowth of axons into the denervated site[5].

EXPERIMENT: IMAGING REGENERATION

A tissue gap caused by deep transections of the optic tract (OT) in the midbrain can completely block the re-innervation of the superior colliculus (SC) by the retina, even when done at young ages when the axons have regenerative potential. Previously we demonstrated that the SAP facilitated the reconstruction of a tissue substrate that supports regeneration across the tissue disruption,

even if treated 3 months after the original lesion. We showed that by using a nano contrast agent (NCA) optic tract regeneration can be visualized *in vivo* in a mammalian chronic injury model.

In a group of young adult hamsters (8 wk), the OT at the brachium of the SC was completely severed with a deep knife wound, extending 1–2 mm below the surface from the midline to a point beyond the lateral margin of SC. Following the transection of the optic tract at the brachium of the SC, the eyes were injected with a NCA and imaged in a 7 Tesla fMRI. This was repeated 3 more times just before the second surgery and SAPNS treatment, then twice following the treatment. During the second OT surgery the animals had a partial scar resection and were injected with 100 µl of 1% SAPNS into the site of injury. The contralateral side of the same animal served as the control. Imaging revealed that the first transection was complete. Imaging after the second treatment revealed regenerated axons in the SC of the SAPNS–treated animals. A 7 Tesla fMRI is able to detect axons in the optic tract in hamsters before, during and after regeneration in a chronic injury treatment model[6].

EXPERIMENT: PLACING CELLS INTO A DEFINED 3-DIMENSIONAL ENVIRONMENT

Here we show the importance of placing cells into a defined system within a 3–dimensional (3D) environment, allowing better control over growth and differentiation in order to effectively mimic the extracellular matrix both *in vitro* and *in vivo*.

This paper will discuss: (a) the appropriate concentration of material based on the type of cells being implanted; (b) determining if the material needs to be pre–buffered, or if the cells need to be pre–cultured in the material before it is then injected into the lesion area; (c) controlling the physical environment to make it more permissive for cell migration while at the same time slowing, or even arresting, the proliferation, elongation, and differentiation of the implanted cells; and (d) modulation of the immune system with a unique physical environment around the implant site[4].

Within the emerging field of stem cells there is a need for an environment that can regulate cell activity, to slow down differentiation or proliferation, in vitro or in vivo while remaining invisible to the immune system. By creating a nano environment surrounding PC12 cells, Schwann cells and neural precursor cells (NPCs), we were able to control the proliferation, elongation, differentiation and maturation in vitro. We extended the method, using SAP, to living animals with implants in the brain and spinal cord. Here we show that when cells are placed in a defined system we can delay their proliferation, differentiation and maturation depending on the density of the cell population, density of the matrix, and the local environment. A combination of SAP and young cells can be implanted into the CNS, eliminating the need for immuno–suppressants[4].

SUMMARY

The ability to keep the cells forever young during implantation is very important to the reconstitution of tissue or organs being replaced. In the emerging field of stem cells there is a need for an environment that can be tuned to the needs of the cells to slow down the differentiation or proliferation in vitro or in vivo while remaining invisible to the immune system. We have shown that when cells are placed into a defined system we can delay their proliferation, differentiation and maturation depending on the density of the cell population, density of the matrix, and the local environment. This is done in a three dimensional environment that allows the benefits of cell therapy to be more fully realized.

CME ANSWERS

1. The optic tract can be regenerated in the brain and there is evidence that the optic nerve can be regenerated in mammals.
2. Time from injury to treatment will determine the type of treatment that is needed. If there is an extended time from injury to treatment, a scar will form and decrease the number and ability of axons to regenerate. The scar will have to be removed and the axon stimulated to grow before they can reconnect.
3. The material can create an environment that mimics early development, allowing cells to migrate and regrow.

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