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'Strategic Sequences' in Adipose Derived Stem Cell Nerve Regeneration

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Abstract

Background—Peripheral nerve injuries (PNI) are a major source of morbidity worldwide. The development of cellular regenerative therapies has the potential to improve outcomes of nerve injuries. However, an ideal therapy has yet to be found. The purpose of this study is to examine the current literature key points of regenerative techniques utilizing human adipose derived stem cells (hADSCs) for nerve regeneration, and derive a comprehensive approach to hADSC therapy for PNI.

Methods—A literature review was conducted using the electronic database PubMed to search for current experimental approaches to repairing peripheral nerve injuries using hADSCs. Key search elements focused on specific components of nerve regeneration paradigms, including, 1) support cells, 2) scaffolds and 3) nerve conduits.

Results—Strategic sequences were developed by optimizing the components of different experimental regenerative therapies. These sequences focus on priming hADSCs within a specialized growth medium, a hydrogel matrix base, and a collagen nerve conduit to achieve neuromodulatory nerve regeneration. Human ADSCs may exert their neuroregenerative influence through paracrine effects on surrounding Schwann cells in addition to physical interactions with injured tissue.

Conclusions—hADSCs may play a key role in nerve regeneration by acting primarily as support for local neurotrophic mediation and modulation of nerve growth rather than that of a primary neuronal differentiation agent.

INTRODUCTION

The standard method of treatment of peripheral nerve injuries (PNI) with autologous nerve grafts has limitations including donor site morbidity and suboptimal functional recovery. Tissue engineering provides an interesting alternative to current treatments and several variations of therapies have been studied including nerve conduits, regenerative stimulants and cellular components. However, intervening to change the natural physiologic course at this level requires a proper understanding of the timing and mechanisms of the events that occur during degeneration and regrowth after PNI.

Nerve injury is accompanied by a sequence of events that precede the final outcome of nerve healing.[1] This outcome ranges from almost complete nerve regeneration, to degeneration,

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nerve loss, neuroma formation and incomplete or absent nerve regeneration. Each sequence within this nerve injury process is accompanied by biologic events that may influence the ongoing regenerative process. Thus, any strategy relating to nerve regeneration should consider these sequences individually and cumulatively as the process of regeneration unfolds.

From this standpoint, it is pertinent to deconstruct peripheral nerve injuries into sequences relating to degeneration, initial regeneration and possible intervention strategies that may limit, speed up or facilitate such sequences. This approach enables us to examine the process at different time frames and to plan combined approaches that consider most of these components when designing a comprehensive regenerative device.

Human adipose tissue-derived stem cells (hADSCs) are a heterogeneous group of multipotent progenitor cells that can be harvested autogenously in high numbers with low donor site morbidity, and have been used in several studies to promote nerve regeneration.[2–6] This review focuses on the utilization of hADSCs as neurotrophic mediators, stimulating nerve regeneration from environmental cues rather than promoting the *in vitro* neuronal differentiation of hADSCs. The goal of this approach is to create an effective method of promoting nerve regeneration while minimizing the manipulation of autologous cells to move towards a transnational therapy.

METHODS

The electronic database PubMed was used to search for articles in the current literature focusing on the promotion of peripheral nerve regeneration to repair peripheral nerve defects. In particular, key aspects of current experimental approaches to peripheral nerve injuries using hADSCs were reviewed. Different paradigms for promoting nerve regeneration were categorized into three core components: 1) the support cells (hADSCs), 2) scaffold or matrix base and 3) nerve conduit. Based on published advances in these categories, a set of strategic sequences was created to address each particular regenerative component.

RESULTS

Strategic sequences were developed to create a comprehensive cell-based regenerative approach to PNI. These sequences rely on the coordinated interaction among different components of current experimental paradigms including, 1) the use of supporting cells (hADSCs), 2) the utilization of structural and directional support with conduits and 3) the development of an appropriate environment to help cells promote nerve regeneration using a matrix base. This nerve regeneration may be a result of neurotrophic factors produced by undifferentiated hADSCs at the site of nerve injury, or through a direct physical interaction between the stem cell and injured neural tissue.[7]

Supporting Cells: hADSC Utilization

hADSC Benefits—hADSCs have been widely demonstrated to be as efficient as mesenchymal derived stem cells in terms of differentiation ability and yield.[8,9] These cells are multipotent progenitors that have the ability to differentiate into several cell types such as neuron-like, endothelial, epithelial, hematopoietic and pancreatic cells. In addition, hADSCs can be harvested in large numbers: adipose tissue yields roughly 4500 colony-forming unit-fibroblasts (CFU-F) of stem cells per milliliter of original tissue sample (in contrast, bone marrow yields as little as 100 CFU-F per milliliter).[8,9]

Among other possibilities, hADSCs can be converted into neurospheres capable of forming Schwann cell (SC)-like cells with neurites that can produce myelin structures.[10] A neurosphere is a culture system composed of free-floating clusters of neural stem cells. Since neural stem cells cannot be studied in vivo, neurospheres provide a method to investigate neural precursor cells in vitro. Using a procedure similar to the one used for propagation of neural stem cells, researchers have converted rat adipose-derived stem cells (ADSC) into floating neurospheres. In addition to being able to differentiate into neuronal- and glial-like cells, neurospheres could be induced to differentiate into SC-like cells.[10] These cells may be an ideal alternative to SCs and these functional benefits make them ideal candidates for aiding peripheral nerve repair. This is especially relevant to large nerve gaps where the prospect of autogenous nerve grafting has multiple disadvantages including donor nerve fallout, secondary operative procedures and poor outcomes in many cases.

Differentiation capacity vs. paracrine function of hADSCs?—hADSCs have excellent potential for transformation or differentiation into nerve cells phenotypes cells that elute nerve growth factors. The question that remains unanswered is: how important is the in vitro differentiation process? More specifically if relatively undifferentiated hADSCs are placed in the right wound milieu, will these cells influence surrounding host cells to transform or elute nerve growth factors (or both) to promote nerve regeneration?[11] This paracrine *in vivo* situation may be preferable to the *in vitro* situation where full nerve cell differentiation is sought.

In *in vitro* experimentation in our laboratory in the past, we have produced nerve factor delivery by hADSCs via production of induced pluripotent cells (iPS) initially using viral vectors followed by the use of circular non-viral DNA vectors (mini-circle).[12] Transfection techniques have improved with micro/electroporation offering a new rapid and efficient method of transfecting cells with improving efficacy and cell survival being reported.[13] For neural induction and neurosphere production by hADSCs, growth medium containing basic fibroblast growth factor (bFGF) and forskolin appear to be extremely effective in inducing transformation.[14,15]

In contrast to this targeted approach at producing nerve-like cells, other approaches rely on intrinsic paracrine capabilities of hADSCs to initiate transformation and growth factor (GF) elution *in vivo*. It would appear that hADSCs, under the orchestration of resident SCs, promote the further production of SCs when instilled in a matrix and placed in the *in vivo* environment. This may be a result of neurotrophic factors produced at the site of nerve injury or through a direct physical interaction between stem cell and injured neural tissue.[7] Thus, it may not be necessary to fully differentiate hADSCs into nerve-like structures when working *in vivo*.

Priming hADSCs for in vivo placement—It is anticipated that the potential for hADSC function *in vivo* may be promoted by prior *in vitro* ‘priming’ of hADSCs prior to implantation. This priming is aimed at maximizing the secretome function (elution of growth factors) of hADSCs to prepare the cells for their *in vivo* tissue milieu. Priming techniques that our laboratory are employing include exposure of hADSCs to specialized conditioned media or hypoxic environments.[16,17] In addition, degradation products of the conduit and nerve guidance entities within the core are all factors that may influence hADSC nerve regeneration. Thus selecting the correct matrix/scaffold to envelope these cells is important in promoting their sustainability and performance.

Scaffold: hADSC and enveloping matrix constituents

To promote nerve regeneration *in vivo*, hADSCs need an appropriate surrounding microenvironment within the nerve conduit. The non-cellular components inside this conduit must, at the most basic level, allow for hADSC survival, proliferation, communication and growth factor elution (paracrine functions). Several scaffolds have been utilized as matrices for cells in previous studies including hydrogels, matrigel, and fibrin (Table 1).

Hydrogels have been widely used in biomedicine and are made up of different variations of 3-dimensional networks of cross-linked molecules.[18] They are particularly useful materials for scaffolds due to their structural and compositional similarities to the extracellular matrix (ECM), which allow for efficient nutrient transfer to cells and provide a framework that supports cell survival and proliferation. In addition, hydrogels have good biocompatibility as they cause minimal inflammatory responses and tissue damage upon implantation.[18] Hydrogels can be constructed from many different materials, several of which have shown promise for culturing hADSCs. Sukarto et al. utilize N-Methacrylate glycol chitosan (MGC) to create gels for hADSC mediated chondral repair.[19] MGC is conveniently soluble in culture media and can be injected into the desired site to then be cross-linked by photopolymerization with visible or ultraviolet (UV) light.[20] hADSC viability after 14 days in photo-crosslinked MGC constructs was fairly low. However, RGD (Arg-Gly-Asp) modification of the MGC scaffolds yielded much higher values with viability maintained over 85% over the 14 days. The MGC gels show promise for facilitating hADSCs implantation *in vivo*. However, the modifications needed to adapt this scaffold to optimize hADSC viability may detract from its use.

Alginate gels are another type of hydrogel that have been widely used in tissue engineering.[21,22] Alginate is a natural polysaccharide that rapidly crosslinks in the presence of divalent cations such as calcium (Ca^2) and has been FDA-approved for use in wound dressings.[23] It is a favorable biomaterial as it is biodegradable, injectable and porous; however, it is very rapidly cross-linked by Ca^2, which makes it impossible to obtain a homogenous gel by simply crosslinking with $CaCl_2$. Galateanu et al. circumvent this problem by not directly mixing Ca^2 with an alginate solution but instead placing filter paper soaked with the crosslinking agent on top of the suspension.[24] They demonstrate that these matrices allow for the viability and proliferation of hADSCs and do not alter cell morphology.[24] However the technique may be cumbersome particularly in relation to nerve conduit usage.

hADSCs have also been successfully cultured in hyaluronic acid (HA)-based hydrogels.[25,26] Espander et al. examine hADSCs survival and proliferation after 14 days in 3 different HA-derived scaffolds, HyStem-HP, HyStem-CSS and Extracel-SS, (Glycosan Biosystem Inc.) and demonstrate cell counts that approach those of hADSCs cultured in tissue culture flasks.[25] The Extracel scaffold may be particularly useful as it allows for the controlled release of growth factors that can be added during preparation due to thiolated heparin contained within the scaffold.[27,28] Furthermore, Espander et al. alter some of their preparations by modifying the crosslinking agents to slow down the gelling process for several hours and create a less stiff and more viscoelastic gel.[25] This construct allows for the injection of a “fluid” gel into a compartment, such as a nerve conduit, after which the gel would solidify to conform to the shape of its container. It should be noted that Extracel contains glycosan, which does not have a Device Master File with the FDA and may therefore only currently be used for research. In our laboratory, we prefer this readily available commercial product that can be mixed with conditioned media and cells prior to injection. Hystem C (Biotime Inc., Alameda CA) can be modified to be collagenase/hyaluronidase sensitive for *ex vivo* digestion and has been used successfully as a scaffold incorporating hADSCs for corneal reconstruction.[25] Thus hyaluronic acid/hydrogel

combinations with proven track records with hADSC use, are an attractive candidate for enveloping the primed hADSCs.

Other hydrogel constructs have also shown promise for tissue engineering applications.[29,30] Studies have shown that Poly(ethylene) glycol (PEG) hydrogels are compatible with adipocyte culture and are efficient scaffolds as they allow for diffusion of nutrients and metabolites and also are biocompatible due to their similarity to the ECM.[29–31] Brandl et al. report using modified PEG hydrogels that are sensitive to proteases to promote biodegradability, an important characteristic of scaffolds, and demonstrate the viability and differentiation of preadipocytes within the construct though the *in vivo* applicability of the design still remains to be tested.[29]

Fibrin is another biopolymer that has been used in a variety of tissue engineering applications.[32] Copolymerizing fibrin with PEG has helped overcome the drawbacks associated with pure fibrin such as mechanical stiffness and rapid degradation, and has rendered it a more biocompatible compound.[32–34] Cho et al. demonstrate that fibrin matrices can be used as injectable carriers of human preadipocytes to enhance adipose tissue formation *in vivo*.[35] Natesan et al. similarly show that adipose-derived mesenchymal stem cells can survive and proliferate in a PEGylated fibrin matrix.[32] In addition, Gardin et al. show no significant difference between proliferation of hADSCs implanted in hyaluronan versus fibrin matrices.[36] It should be noted that fibrin hydrogels have been shown to promote angiogenesis. Indeed in the study by Natesan et al., ADSCs differentiated into vascular cell types including endothelial-like and pericyte-like cells without growth factor stimulation.[32] Furthermore, the same cells that resided in a collagen matrix instead of a PEG-fibrin exhibited a different morphology, emphasizing the importance of the matrix microenvironment in determining cell phenotype.[32] This may be a potential limitation of fibrin for use in nerve regeneration.

Matrigel (BD Biosciences, Mississauga, Canada) is a widely used matrix scaffold that consists of ECM proteins including laminin, collagen IV and enactin, extracted from Engelbreth-Holm-Swarm tumors in mice.[37] Stillaert et al. use Matrigel matrices to deliver stem cells from the stromal vascular fraction (SVF) of adipose tissue to induce adipogenesis in mice.[26,38] Of note, Matrigel has been shown to maintain the self-renewal and pluripotency of stem cells, keeping them in an undifferentiated state.[38] This quality is optimal for stem cell culture but may hinder efforts to obtain more differentiated lineages from stem cells for particular applications.

Other options for cell matrix conduits can utilize techniques that allow for the use of autologous tissue as scaffolds. Muscle-vein-combined grafts for example, have been used to repair nerve defects ranging from 0.5 to 4.0cm with recovery of sensory and motor function in most patients.[39] Such tissue alternatives should also be considered as they circumvent the cost of artificial conduits while avoiding the morbidity associated with nerve autografts.

Several favorable candidates that are widely used in tissue engineering experiments are available for developing a specialized matrix base. Deciding upon the best construct requires characterizing a medium for a particular application. As discussed above, priming of hADSCs is a possible strategy for in vivo nerve regeneration. Among other possibilities we are considering a purified culture medium obtained as a by-product from embryonic motor neuron stem cell lines (California Stem Cells Inc Irvine CA) to sustain hADSC cellular viability, promote adherence and elaborate growth factors. This growth medium is rich in a mix of growth factors such as bFGF, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), which are relevant to neurotrophic mediator release that suits nerve regeneration.

This contrasts with other media such as alginate matrices that may exhibit untoward effects on cellular differentiation during degradation and may provide physical barriers to cellular propagation.[24]

Nerve Conduits

Nerve conduits or guides are particularly important in the repair of peripheral nerve injuries involving nerve gaps. The technology may well be applicable to nerve injuries of all forms if it can improve to the extent that regeneration and protection of injured nerves is successfully accomplished by an interactive nerve conduit or one that accommodates an interactive core, The aim of the nerve conduit at this stage is to improve the results when regeneration distance is 20–25 mm (equivalent to 10–20mm in rat sciatic nerve or greater). Studies have demonstrated improved results when conduits incorporate coatings, cellular elements, peptides, differing porosities and smooth/rough internal surfaces.[40–42] It would appear that the best way to maximize nerve regeneration involves cell-containing devices that can improve regeneration distance and speed of repair, or cause a differentiational shift to SC morphology, growth factor production or both. Of paramount importance is the ability of this differentiation to provide guidance for progressing axons to complete the circle of nerve growth factor transmission described earlier.

Conduits may be natural or synthetic. The utilization of natural conduits derived from tissue sources is a promising technique to aid regeneration across a nerve gap. Autologous nerve conduits can be created from a variety of different tissue types. These are not limited tubular structures such as arteries and veins, but can also be formed others tissue types such as connective tissue, *in vivo*.[43] Autologous conduits can also be augmented with growth factors like VEGF to encourage neuron and Schwann cell regeneration.[44] Allogenic options are also available, and allografts such as processed nerve allografts have demonstrated comparable recovery rates to nerve autografts.[45]

With regards to artificial conduits, the bioactivity of natural products provides a distinct advantage - cell binding sites and molecular adhesion potential provide the possibility of elemental incorporation and intrinsic repair. They also tend to degenerate naturally with time without the potential toxic by-products and pH changes that may accompany the synthetic alternatives.[46] Although naturally derived products are more expensive and usually derive from animal sources, the risk of disease transmission is minimal and most have regulatory approval. Certain bioabsorbable synthetic conduits such as polyglycolic acid (PGA) conduits, however, have shown promising results in clinical studies.[47] These conduits should also be considered when deciding on peripheral nerve repair with conduits as they have several favorable characteristics such as porosity, permeability to oxygen, and biodegradability.[48]

In relation to the sequences described above, the need to incorporate cellular components in the form of hADSCs necessitates cellular binding sites and intrinsic interactions that would be more suited to natural nerve conduits. Clinical results and adaptable, compatible material profiles make type 1 collagen conduits and decellularized nerve conduits appropriate choices for use with hADSCs. The extensions to the basic design profiles involve coatings–fibronectin and laminin–which promote axonal guidance and SC proliferation, and are synergistic with bFGF, thus enhancing neurite outgrowth.[49] Although many studies have shown beneficial neurite growth when growth factors or SCs have been added to the mix, the rationale of using hADSCs is to produce the SCs and growth factors intrinsically–thus incorporation of these elements into the nerve conduit is not a priority if hADSCs are used.

As observed early on in this paper, nerve conduits used in large nerve gap injuries have yet to achieve predictable improved outcomes over autogenous nerve grafting. In this regard,

the application of denatured muscle and vein grafts has shown promising results only in relatively short defects.[50] Several important factors, as outlined by Meek et al., should be kept in mind when considering the use of nerve conduits, and include the availability of clinical data, price, length, composition and biodegradability of each conduit.[50] Though no ideal conduit exists, addressing these issues with regards to the clinical need for a nerve conduit can help optimize decision-making.

DISCUSSION

To achieve the ultimate goal of a device generated nerve repair matching or improving on autogenous nerve grafting, the newest advances in technology should be utilized. To this end, sequential strategies may be adopted using advances in techniques and technology at each level of nerve injury and regeneration to improve the overall outcome of nerve repair. An incorporation of discussed issues in the these strategies has resulted in a combined plan for paracrine nerve regeneration constituted as follows:

1. Use of natural collagen based nerve conduit[41,51] - although a host of synthetic conduits have shown promising results, we have favored the use of natural collagen based conduits due the bioactivity of natural products with potential cell binding sites (RGD) and molecular adhesion potential These provide the possibility of elemental incorporation and intrinsic repair. They also tend to degenerate naturally with time without the potential toxic by-products and pH changes that may accompany the synthetic alternatives.[46] It is also re-assuring to know that the conduit has been successfully used clinically, is FDA approved and standardized in manufacture. That reduces one element of uncertainty in a regenerative effort that incorporates cellular and matrix elements with a host of variables that need to be considered.
2. Comprehensive *in vitro* phase of core matrix construction–utilizing ‘priming’ of hADSCs with strategies such as limited hypoxic exposure, culture within a specialized growth medium derived as a by-product from embryonic cell line production; and then incorporating these primed hADSCs into a hydrogel/ hyaluronic acid base resulting in a ‘nerve-blast ‘ matrix (NBM).
3. Aiming to achieve neuromodulatory nerve regeneration relying on microenvironmental cues from resident SCs and ECM of residual nerve tissue; this modulation likely takes the form of elaboration of neurotrophic mediators that encourage attachment of axon sproutings, elaboration of nerve growth factors and ECM production. The hADSCs thus behave as a ‘trigger’ to neurotrophic nerve regeneration rather than as a primary nerve cellular differentiation agent.

The suggested overall strategy may be criticized on the basis that much of the data discussed above is derived from animal studies and there is no absolute quantitative data to back up the approach suggested. However, we believe that the approach of neuromodulation and stimulation of host tissue regeneration utilizing preprimed cells in an accepted matrix that allows 3-dimensional cross talk with host cells, implanted hADSCs and a natural based conduit, incorporates a comprehensive strategic plan for neuromodulatory nerve regeneration. We believe the successful nerve conduit filler will be the tipping point for nerve regeneration advancement with the conduit being of secondary importance in the overall scheme.

CONCLUSIONS

The quest for an ideal replacement for autogenous nerve grafting in larger nerve gaps is ongoing. Although newer technologies have brought us closer to working solutions there is

still no definitive device that fulfills all the criteria for use in nerve regeneration. By systematically reviewing technological aspects and breakthroughs related to different sequences within the nerve injury/regeneration paradigm, we analyzed ‘strategic sequences’ and optimize a workable model for an interactive nerve conduit. This device will essentially be made up of a natural collagen nerve conduit with an interactive core matrix that encourages hADSC survival and neuromodulatory nerve regeneration. It is anticipated that this milieu for nerve regeneration will stimulate intrinsic healing factors that will encourage axonal progression under SC guidance. The working model will be adapted and nuanced in relation to the ongoing findings of the research project.

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Table 1

Available scaffolds for cell matrices

Scaffold Category	Scaffold Types	Characteristics
Hydrogel	MGC*	Soluble in culture media Can be injected and then cross- linked by photopolymerization
	Alginate	Biodegradable, injectable and porous
	HA-based†	Release of growth factors (Extracel)
	PEG‡	Biocompatible due to similarity to ECM
Fibrin		Can be used as injectable carriers Can promote angiogenesis
Matrigel		Composed of extracellular matrix proteins Can maintain pluripotency of stem cells

*N-Methacrylate glycol chitosan,

†Hyaluronic acid-based,

‡Poly(ethylene) glycol