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Development of a tissue-engineered composite implant for treating traumatic paraplegia in rats

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Abstract This study was designed to assess a new composite implant to induce regeneration of injured spinal cord in paraplegic rats following complete cord transection. Neuronal xenogeneic cells from biopsies of adult nasal olfactory mucosa (NOM) of human origin, or spinal cords of human embryos, were cultured in two consecutive stages: stationary cultures in a viscous semi-solid gel (NVR-N-Gel) and in suspension on positively charged microcarriers (MCs). A tissue-engineered tubular scaffold, containing bundles of parallel nanofibers, was developed. Both the tube and the nanofibers were made of a biodegradable dextran sulphate–gelatin co-precipitate. The suturable scaffold anchored the implant at the site of injury and provided guidance for the regenerating axons. Implants of adult human NOM cells were implanted into eight rats, from which a 4 mm segment of the spinal cord had been completely removed. Another four rats whose spinal cords had also been transected were implanted with a composite implant

of cultured human embryonic spinal cord cells. Eight other cord-transected rats served as a control group. Physiological and behavioral analysis, performed 3 months after implantation, revealed partial recovery of function in one or two limbs in three out of eight animals of the NOM implanted group and in all the four rats that were implanted with cultured human embryonic spinal cord cells. Animals of the control group remained completely paralyzed and did not show transmission of stimuli to the brain. The utilization of an innovative composite implant to bridge a gap resulting from the transection and removal of a 4 mm spinal cord segment shows promise, suggesting the feasibility of this approach for partial reconstruction of spinal cord lesions. Such an implant may serve as a vital bridging station in acute and chronic cases of paraplegia.

Keywords Olfactory mucosa · Spinal cord · Transection · Transplantation

Introduction

Spinal cord injuries involving partial or complete transection, as with other lesions in the central nervous system, are unable to heal on their own. Complete

spinal cord injuries in humans and other mammals cause loss of sensory, motor and, reflex functions below the site of injury. Several different approaches have been used in attempting to reconstruct an injured spinal cord. The use of growth factors, either by exogenous

administration [8, 14, 36] or by introducing growth factor-treated implants [22, 23, 39] and genetically engineered cells [34, 46] have been attempted with limited success. Others have concentrated their efforts on the use of various tissue-engineered scaffolds [4, 54, 57]. Spinal cord reconstruction using implantation of cells from various sources has been gaining momentum in recent years [28, 41]. However, one of the major disadvantages of the implantation or injection of cells alone is the limited viable cell survival after the procedure, as cells tend to desert the injury site.

One feasible innovative way of repairing injured mammalian spinal cord is by creating a composite implant, which contains cultured cells from an autologous or allogeneic source. The implanted cells grow and serve as a vital bridge to connect the stumps of the severed spinal cords [44]. Such a composite spinal cord implant includes a combination of the various elements that support the healing process, such as a tissue-engineered scaffold to anchor the implanted cells, which are embedded in a viscous adhesive milieu, supplemented with a variety of factors [47].

The dream of using primordial cells from early human embryos for implantation therapy in spinal cord injuries is now a step closer to reality [27, 37]. Recent discoveries extend the potential for isolation and cultivation of human embryonic stem cells [49], and their differentiation in culture to neurons and other cell types [43, 49]. Furthermore, viable treatment of spinal cord injuries using such human embryonic stem cells would be considered as an allogeneic graft, and should overcome the risks of rejection, immunogenic reaction and possible neoplastic transformation.

An excellent autologous source of adult neuronal precursor cells is the nasal olfactory mucosa (NOM). The NOM tissue is easily biopsied and the neurons and the sustentacular cells of the NOM renew themselves constantly during life by proliferation of the basal global stem cells [9, 15, 45]. Moreover, the unique sensory characteristics of the NOM neurons render them suitable for receiving and transmitting signals as a "relay station" in the spinal cord.

The scaffolds produced by tissue-engineering technologies for spinal cord implantation are categorized into synthetic and naturally occurring polymeric macromolecules. The naturally occurring ones are commonly grouped into protein-peptides-based scaffolds and carbohydrates-based scaffolds [26, 29, 52]. There are several published examples where the scaffold imitates the extracellular matrix (ECM) present in connective tissues, such as collagens and proteoglycans [55, 59, 60]. The latter type best matches the ECM constituents of many tissues, including that of the spinal cord.

Other promising strategies focus on creating a receptive environment for regeneration by abolishing the myelin-associated inhibitors of axonal growth and the

physical and biochemical barrier, the so-called "glial scar" [7, 17, 40, 48, 53].

Last, but not the least important, is the intercellular viscous adhesive milieu and its ingredients for supporting the vitality of the neuronal bridge. The ECM ground substances of the spinal cord have the consistency of colloidal-gels, and indeed, several types of gels have been proposed for repairing injured spinal cord [34, 56, 57].

In the current study we report on the development of an innovative composite implant designed to serve as a vital bridging station in acute and chronic cases of spinal transection or injury.

Materials and methods

The composite implant elements

The scaffold is based on NVRs tissue-engineered technology (patent pending), consisting of two naturally occurring biological cross-linked polymers. It has a tubular format with a customized diameter of 2 mm and a wall thickness of 0.4 mm. In addition, the tubular scaffold contains a bundle of parallel nanofibers 50–100 μm in diameter, made of the same material as the scaffold (Fig. 1). In a series of preliminary experiments the scaffold was found to be biocompatible, non-toxic, and non-inflammatory (data not shown). The tube is transparent, suturable, and it can last for a period of more than 3 months until biodegradation takes place (data not shown).

NVR-N-Gel, which is a proprietary product of NVR Labs (patent pending), is composed of a cross-linked hyaluronic acid with the adhesive molecule laminin, and the following mixture of ingredients: antioxidants, neuronal growth factor; neuro-protective

Fig. 1 A tubular scaffold containing nanofibers. Original magnification 25 times

factors; EGF, bFGF, BDNF and NGF—20–50 ng/ml, IGF-I—50 ng/ml, LIF—0.5 u/ml, NAC (*n*-acetyl cysteine)—10 μ M, pifithrin α , cyclic—200 nM, and retinoic acid—1–5 μ M.

The NVR-N-Gel is transparent, highly hydrated with polar and non-polar (hydrophobic) residues, all bio-compatible and biodegradable. For cell cultivation, the gel is used at a concentration of 0.7–1% hyaluronic acid, however, for implantation a more viscous gel (1.2–1.5%) is used.

Cultivation of adult human NOM and embryonic spinal cord cells

Biopsies of adult human olfactory nasal mucosa and embryonic spinal cords of aborted fetuses (16–23 weeks of gestation) were collected for cell isolation and the establishment of cultures. The study with human derived samples was approved by the Helsinki committee of Assaf-Harofeh Medical Center (no. 13/03). Informed consent was obtained from each patient.

An innovative culture technique was introduced as outlined in the scheme (Fig. 2). It combines stationary cultures in gel, alternating with cells grown in suspension on an anion exchange, positively charged cylindrical, DEAE-cellulose (DE-53) microcarriers (MCs) (Whatman, England). The MCs are equilibrated with phosphate buffered saline (PBS) pH 7.4 and autoclaved in batches of 15 g in 100 ml PBS [19, 51].

The dissociated cells were grown in suspension attached to the MCs for periods of 1–4 weeks. At various times during their growth period in suspension, cell-MC aggregates were collected and reseeded in NVR-N-Gel as stationary cultures. The following growth media were used: modified NEP medium based on the Bottenstein and Sato [6] N2 medium or M-21 medium. The NEP medium contains DMEM-F12 (Invitrogen, UK), N2 additives (progesterone, putresine, selenium, insulin and transferrin) plus complex B27 supplements (Invitrogen) and 1% BSA. The M-21 medium is based on NEP medium, except that B27 is omitted and 100 μ M non-essential amino acids, 30 ng/ml triiodothyronine, and 1 mM sodium pyruvate are added. Both media were supplemented with the same factors used to enrich the gel. For cell expansion, the media were enriched with 10% fetal calf serum (FCS); for growth differentiation and implantation the serum was omitted from the media. Cells were usually cultured for 3–4 weeks prior to implantation.

Surgical and transplantation procedure

The study was authorized by the local ethical committee for experiments in laboratory animals. Animal treatment and maintenance were in accordance with the "Guide for the care and use of animals", Institute of laboratory animal resources commission on life sciences, National Research Council, National Academy Press, Washington, DC, 1966. The DHEW publication no. 80 (NIH)

Fig. 2 Preparation of adult human NOM and human embryonic SC cultures

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Twenty Sprague-Dawley rats, 3-months old, each weighing approximately 250 g, were used. All rats were anesthetized by intraperitoneal injections. For the operations, anesthesia consisted of Ketamine HCL (50501 USA) 125–130 mg/kg and Xylosine (B2370 Belgium) 4.8 mg/kg. For electrophysiological tests, rats were anesthetized for a short period (30 min) with Ketamine 75–85 mg/kg and Xylosine 3 mg/kg. For removal of stitches, light anesthesia was used: 50 mg/kg Ketamine and 1.8 mg/kg Xylosine. All the treatments and the follow up tests were performed in a double blind randomized manner.

All surgical procedures were performed on anesthetized rats, using a high magnification navigator micro-

scope (Zeiss NC-4) in a class 100 animal operating room. The spinal cord was exposed via a dorsal approach. The overlying muscles were retracted, T7–T8 laminae removed, the spinal cord was completely transected using micro-scissors and a 4 mm segment of the cord was removed. A 4 mm gap was chosen to match the gap size formed after removal of the scar in chronic spinal cord injuries.

Eight of the 20 rats who underwent spinal cord transection and the removal of a 4 mm spinal cord segment, were closed with no further treatment (sham treated control group). The remaining 12 rats underwent implantation of one of the composite implants. Eight rats were treated with implants containing NOM cells (Fig. 3) and four rats received human embryonal spinal cord-derived cells.

Fig. 3 Cultured adult human NOM neurons. **a–d** Sprouting of nerve fibers concomitantly with migration of nerve cells from M-cell aggregates in NVR-N-Gel. **a–c**—200 times, **d**—100 times. **a–d** Phase contrast microscopy. Immunofluorescent staining of NOM neurons with antibodies specific to MAP 2 (**e**) and olfactory mucosa protein (OMP) (**f**). Original magnification **e**—400 times, **f**—200 times, respectively

Embryonic human spinal cord cells were grown as long term cultures to the stage of myelin formation (Fig. 4). About $1\text{--}2 \times 10^6$ NOM or spinal cord cells, embedded in NVR-N-Gel, and encapsulated in NVR scaffold, were implanted into the site of the excised spinal cord segment.

Four-millimeter long composite implants were placed in the transected area of the spinal cord, in direct contact with the margins of the two stumps. The entire area of the lesion containing the implant was covered with a thin biodegradable membrane composed of the biological copolymer, attached by a few interstitial sutures for fixation of the implants at the desired sites. Finally, the muscular and cutaneous planes were closed and sutured.

Post-operative animal maintenance

In the post-operative management of the animals care was taken to minimize discomfort and pain. Following implantation the rats were assisted in urination and defecation with the help of a veterinarian, twice daily. Animals were maintained in ventilated cages, containing sterile sawdust and sterile food. The paraplegic rats were kept solitary in cages, but were gathered in groups for 1 h every day, in a large facility. At the termination of the experiments, the animals were sacrificed under general anesthesia.

Electrophysiological measurements

Somatosensory evoked potentials (SSEP) were recorded in the experimental and control groups in a blinded manner, immediately postoperatively and 3 months later. Conductivity of the spinal cord was studied by stimulation of the sciatic nerve and recording from two disc-recording electrodes, active and reference, placed on the rats' scalps. These electrodes, with conductive jelly, were attached to the scalp—active over the somatosensory cortex in the midline and reference electrode be-

tween the two eyes. The earth electrode was placed on the thigh, on the side of the stimulation. The sciatic nerve was stimulated by a bipolar stimulating electrode. Two hundred and fifty-six stimulation pulses of 0.1 ms in duration were generated at a rate of 3 s^{-1} . The stimulus intensity was increased gradually, until slight twitching of the limb appeared. The appearance of evoked potentials, as a response to stimulation in two consecutive tests, was considered positive.

Latency and amplitude (positive—P wave peak) were measured

The rats were anesthetized intra-operatively with diluted Nembutal 15 mg/kg weight.

The test was performed using the Medelec/Teca Sapphire 4 ME electromyography apparatus (20 Hz–2 KHz band pass filter and calibration sensitivity 10–20 mcV/div and time base 5 ms/div).

Locomotor rating scale

The BBB scale [5] describes the locomotor rating scale, graded from 0 (absent performance) to 21 (complete normal gait performance). This grading scale was used to assess behavioral recovery and gait performance weekly for 2–10 months after spinal cord injury.

Magnetic resonance imaging (MRI) analysis

Sample preparation: spinal cords were excised and fixed with formalin. The spinal cords were inserted into 5 mm NMR tubes with their long axis parallel to the z -direction (the B_0 direction) of the magnet and immersed in Fluorinert (Sigma Chemical Co., St. Louis, MO, USA). The temperature in the magnet was maintained at $25.0 \pm 0.1^\circ\text{C}$ for the duration of the experiments.

Fig. 4 Phase contrast microscopy of mature motor neuron in **a** and myelinated axons in **b** (arrows) in long-term cultures of human embryonic spinal cord cells. Original magnification 400 times

The MRI diffusion experiments were performed on a wide-bore 8.4T NMR spectrometer (Bruker, Karlsruhe, Germany) equipped with a micro5 imaging gradient probe capable of producing pulse gradients of up to 190 G cm^{-1} in each of the three directions. Diffusion weighted MR images were collected using the stimulated echo diffusion imaging pulse sequence with the following parameters: TR = 2000 ms, TE = 35 ms, $\delta = 3$ ms, and $\Delta = 50$ ms. The diffusion gradient strength, G , was incremented from 0 to 60 G cm^{-1} in 16 steps giving a maximal b value of $1.12 \times 10^6 \text{ s cm}^{-2}$ and $q_{\max}$ of 766 cm^{-1} . Diffusion was measured perpendicular and parallel to the long axis of the spine. The MR images were collected in a blind mode and the trauma site was placed at the center of the imaged region.

The signal decay of water was analyzed using the q-space approach [12] using the Matlab program.

Histological analyses

Prior to cultivation, a small segment of the NOM biopsy was taken for histological staining after fixation in 10% formalin at pH 7.4.

The spinal cord area, containing the implant region with both the proximal and the distal normal healthy stumps, was fixed as a whole mount in 10% formalin for several days. Subsequently, samples were placed in ethylenediaminetetra-acetic acid (12.5%) at pH 7.0 for decalcification. The softening of the calcified bony spine enabled the smooth release of the whole region of the spinal cord, which could be inserted into the tube probe of the MRI system (described previously). For histological analysis, the spinal cord was dissected into seven sections of 3–4 mm each, in parallel with the MRI analysis. The pieces were rinsed thoroughly in running tap water. The samples were dehydrated in sequential alcohols, xylol and embedded in paraffin. Sections of 5–6 μm were re-hydrated and stained with Meyer's hematoxylin-eosin, Masson's trichrome, and Bodian silver methods [42].

Immunofluorescence

Cultures were fixed with 4% paraformaldehyde and incubated with antibodies against epitopes of Olfactory Marker Protein (OMP was kindly provided by Prof. F. Margolis, University of Maryland, Baltimore, MD, USA) and microtubulin associated protein-2 (MAP-2). Cells were then washed and incubated with CY2 or Texas Red conjugated goat anti-mouse or rabbit IgG (Jackson, diluted 1:100). The stained cultures were inspected under fluorescent microscope (Olympus, NY, USA) with suitable filters and photographed using Magnafire SP digital camera (Optronix, Mansfield NG, UK). For positive control, rat neuronal cultures were stained under the same conditions.

Results

When adult human NOM cells were seeded directly in NVR-N-Gel as primary stationary cultures, growth of morphologically different cell types was observed: epithelial cells organized in large laminae and tapered cells forming islands of confluent cells, giving rise to large numbers of round, unattached floating cell masses.

A large number of neuronal cells were observed among the epithelial laminae and the tapered cells in the cultures that were reseeded in NVR-N-Gel, following a growth period of 1–2 weeks in suspension on positively charged MCs (Fig. 4).

Post-operative observation

Seven animals out of eight in the control group exhibited complete paraplegic characteristics on physical examination and in their gait performance analysis (BBB = 0: no observable hind limb movement). One rat of the control group showed slight movement of the hip joints (BBB = 1) (Table 1).

Table 1 BBB locomotor rating scale of the three groups of animals, two experimental and one control

Control			Human nasal olfactory mucosa			Human embryonic spinal cord		
Rat No.	Onset of movement (days)	BBB scale	Rat No.	Start of movement (days)	BBB scale	Rat No.	Onset of movement (days)	BBB scale
1	–	0	1	15	10	1	15	13
2	–	0	2	–	0	2	15	6
3	–	0	3	13	13	3	48	10
4	–	0	4	92	3	4	12	3
5	–	0	5	–	0			
6	–	0	6	–	0			
7	30	1	7	–	0			
8	–	0	8	–	0			

Three of the eight rats in the group treated by a composite implant containing cultured human NOM showed varying degrees of active movements. One of the three rats showed BBB=3 (extensive movement of hip and knee joints), one rat showed BBB=10 (occasional weight-supported plantar steps; no FL–HL coordination) and one rat showed BBB=13 (frequent consistent weight-supported plantar steps and FL–HL coordination) (Table 1).

All four rats in the group treated by human embryonic spinal cord cells implantation showed varying degrees of leg movements: one rat showed BBB=3 (extensive movement of hip and knee joints), one rat showed BBB=6 (extensive movement of two joints and slight movement of the third), one rat showed BBB=10 (occasional weight-supported plantar steps; no FL–HL coordination), and one rat showed BBB=13 (frequent consistent weight-supported plantar steps and FL–HL coordination) (see Table 1, Fig. 5).

Statistical analyses of the three groups of animals graded by the BBB scale

The BBB scale does not follow a normal distribution (Shapiro-Wilk highly significant for two out of three groups). A non-parametric test is therefore used to compare the groups. The Kruskal–Wallis test shows a significant difference between the groups ($p=0.0117$).

To test which of the group pairs is significantly different, we used the Duncan multiple comparison test on the ranked data (as the raw data is non-normal). The mean rank of human embryonic spinal cord group (16.88) is significantly higher than both the human NOM and control groups (means of 10.5 and 7.31, respectively). No significant difference emerged when comparing the NOM and control groups.

Electrophysiological measurements

Spinal cord conductivities were measured immediately after spinal cord transection and again 3 months later in two groups—transection alone, or transection plus

implantation of composite NOM implants. In two out of the three NOM implanted-rats exhibiting legs movements, SSEPs were elicited (Fig. 6b). No SSEP response was elicited in the eight rats of the control group, nor in the five rats of the NOM implanted group that did not show leg movement (Fig. 6a).

MRI analysis

Representatives of the treatment groups were subjected to MRI analysis, which provided information on the state of the spinal cord tissue at the injury site. Magnetic resonance (MR) q-space displacement maps [1, 2], which were computed for three different spinal cords, revealed that fiber-like tissue with an amount of water-restricted diffusion was present only in the treated spinal cords and not in the controls (Fig. 7). Moreover, comparison of slices numbers 3–5, which are of the implantation sites, show small areas in which the mean displacement is less than $4\ \mu\text{m}$ (value consistent with normal white matter). Such areas are present only in slices of implanted rats, but not in the controls (Fig. 7).

Histological analysis

The dominant histological picture of the reparative tissue in the area of the excised cord tissue was the presence of fibrotic scar tissue, composed of glial cells and fibroblasts, together with the formation of new blood vessels. No inflammatory reaction was observed either in the histology of rats implanted with human cells or in the control rats.

In rats implanted with either human NOM or with embryonic spinal cord the H&E stained sections showed some areas of neurokeratin (shrunk axons surrounded by an empty space, residual of the myelin sheath that had been dissolved by the alcohol treatment). Furthermore, in one rat (that was walking on the right leg after NOM implantation) a number of large neuronal perikarya were observed in the implanted area (Fig. 8a, c). In silver stained sections of both composite implants

Fig. 5 **a** Complete paralysis of both legs, folded inward, of a control rat that underwent complete transection of the spinal cord and removal of a 4 mm segment. **b** Paraplegic rat showing restoration of partial gait performance (in the *right leg*) 3 weeks after implantation of a composite implant containing cultured adult human NOM cells into a 4 mm gap of transected spinal cord

Fig. 6 **a** Absence of spinal cord conductivity (SSEP) in a paraplegic control rat after complete transection of the spinal cord and removal of 4 mm segment. **b** Restoration of spinal cord conductivity after complete transection and implantation of composite implant containing NOM

several nerve fibers could be seen crossing the reparative tissue (Fig. 8b, d)

Discussion

Regeneration and repair of complete transection injuries of the spinal cord is still an unresolved clinical challenge. Various experimental approaches for reconstructive regeneration and renewal of damaged spinal cord are under intensive investigation in laboratory animals. The strategies include stimulation of positive autoimmune responses [16, 50], introduction of neurotrophic and neuroprotective agents [8, 14, 22, 23, 34, 36], or removal and elimination of scar inhibitory molecules [11, 21, 24, 30, 53]. The latest technologies for solving paraplegic conditions employ several kinds of cell therapy that are introduced into the damaged site of the spinal cord after removal of the accumulated scar [47]:

- (a) Implantation of tissue-engineered devices, without cells, for anchorage of the implant and for guiding axonal regeneration [59, 60].
- (b) Composite implants, containing cells of either autologous or allogeneic origin [18, 35, 37, 41]. The choice of cell resources involves implantation of either stem cells directed to differentiate toward mature neurogenic phenotypes or insertion of already mature neuronal committed cells.

In the current project, both cell sources of the composite implants are of human origin, serving in the rat spinal cord system as xenogeneic cell implants; they are meant in future clinical studies to simulate autologous (NOM cells) and allogeneic (embryonic spinal cord cells) sources, respectively. The innovative cell culture techniques described in the Methods section served the purpose of establishing high concentrations of neural cell cultures ideally [19, 51]. These cells are embedded in a milieu of a gel enriched with adhesive molecules [13, 38] and neurotrophic and neuroprotective agents as antioxidants, which are released slowly. In addition, the gel embeds the nanofilaments in the tubular scaffold. The gel and the scaffold are biocompatible, bioerodable, and biodegradable.

Other reports in the literature describe auxiliary tissue-engineered products that promote neuronal and axonal growth, such as nanofibrils [52, 59, 60], gels [34, 56, 57] and scaffolds [22, 54, 55]. Acellular devices yield

Fig. 7 q-Space displacement maps (x direction) sequential slices of MRI analyses

Fig. 8 Histological sections of implanted spinal cords 10 months (a–c) after adult NOM implantation and 3 months (d) after implantation of human embryonic spinal cord cells. Hematoxylin-eosin (H&E) staining demonstrates dispersed neuronal perikarya (a, arrows). Silver staining demonstrates nerve fibers—either single (b, arrows), or organized in parallel bundles (d, arrows). In addition, note areas of neurokeratin (c, arrows). Original magnification 400 times

ded poorer and limited results in comparison to cell-containing implants [47].

A variety of sources for implantation have been tested, including bone marrow stroma cells [25, 33, 58], skin, umbilical cord blood and embryonic and fetal stem cell lines [20, 37, 41, 43, 61]. However, those cell sources have proven to support only sporadic appearances of single cells or cell aggregates of neuronal cells, and failed to yield robust numbers of neuronal cells to create a successful implant that can replace massive segmental losses. In the current study, embryonic spinal cord and adult NOM cells showed vigorous vitality that established rich neurogenic cell cultures for implantation.

Both types of xenogeneic implants used in the current study bear the risk of inducing an immunogenic response. However, surprisingly, the results show that neither of them evoked a rejection response. On the other hand,

preliminary results of studies currently in progress show that when cultured NOM cells of adult dogs were implanted into paralytic rats, severe rejection led to the death of animals a few days after the implantation. It is important to note that the spinal cord and its surrounding fluids are considered an immunologically privileged site, with minimal immunologic responses.

The human embryonic spinal cord implants in our study showed significant advantages over the control animals, although repair of the tissue damage was by no means complete. The rats implanted with the adult human NOM cells showed statistically insignificant advantages over the control group of animals. In the course of further experiments being performed; we are gaining more insight into the growth of the human adult NOM biopsy, by further selecting and characterizing specific sub-populations of cells from the overall collected mixture. The outcome of these experiments may further clarify the contribution of the different adult human NOM cell-populations.

Over the recent years MRI has developed into the most important imaging modality for the central nervous system (CNS). Water diffusion is an important contrast mechanism in MRI of the CNS, diffusion weighted imaging (DWI) and diffusion tensor imaging (DTI) are

used extensively for studying CNS pathologies and structures [31, 32]. The DTI is currently also used to study fiber orientations [31]. Recently, we have demonstrated that high *b*-value *q*-space diffusion MRI is extremely sensitive to the structural and the pathophysiological state of axons and fibers of the CNS [1–3, 10], and thus can be used to study CNS maturation and degeneration [3]. Interestingly, in the current study the first two rats transplanted with cell-containing composite implants showed some regeneration of motor function and axons, consistent with the electrophysiological measurements, MRI, and some histological evidence of formation of cells and axonal fibers. It is clear that more MRI experiments are needed to verify if these preliminary results imply that axonal connection was indeed established across the transplanted zone. Such MRI experiments are underway.

The MRI analyses are supported by both the electrophysiological tests and the histological findings of neuronal cells and crossing axons along sections of the composite implanted transplants, versus their absence in the sections of the control animals.

In summary, in the current study we describe the combined efforts of cell cultivation, differentiation and integration into a tissue-engineered scaffold, as a composite implant for repairing spinal cord injury. Reliable tests to evaluate the final outcomes of the spinal cord injured regions yielded results that brought us closer to the goal of treatment of damaged spinal cord. Nevertheless, we did not face the problem of elimination of scar tissue, which still needs to be addressed. These results are encouraging, but we have still a long way to go before complete motor function and full gait performance are restored.

References

- Assaf Y, Cohen Y (2000) Assignment of the water slow-diffusing component in the central nervous system using *q*-space diffusion MRS: implications for fiber tract imaging. *Magn Reson Med* 43:191–199
- Assaf Y, Mayk A, Cohen Y (2000) Displacement imaging of spinal cord using *q*-space diffusion-weighted MRI. *Magn Reson Med* 44:713–722
- Assaf Y, Mayk A, Eliash S, Speiser Z, Cohen Y (2003) Hypertension and neuronal degeneration in excised rat spinal cord studied by high-*b* value *q*-space diffusion magnetic resonance imaging. *Exp Neurol* 184:726–736
- Bakshi A, Fisher O, Dagci T, Himes BT, Fischer I, Lowman A (2004) Mechanically engineered hydrogel scaffolds for axonal growth and angiogenesis after transplantation in spinal cord injury. *J Neurosurg Spine* 1:322–329
- Basso DM, Beattie MS, Bresnahan JC (1995) A sensitive and reliable locomotor rating scale for open field testing in rats. *J Neurotrauma* 12:1–21
- Bottenstein JE, Sato GH (1979) Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proc Natl Acad Sci USA* 76:514–517
- Bradbury EJ, Moon LD, Popat RJ, King VR, Bennett GS, Patel PN, Fawcett JW, McMahon SB (2002) Chondroitinase ABC promotes functional recovery after spinal cord injury. *Nature* 416:636–640
- Bregman BS, McAtee M, Dai HN, Kuhn PL (1997) Neurotrophic factors increase axonal growth after spinal cord injury and transplantation in the adult rat. *Exp Neurol* 148:475–494
- Calof AL, Bonnin A, Crocker C, Kawachi S, Murray RC, Shou J, Wu HH (2002) Progenitor cells of the olfactory receptor neuron lineage. *Microsc Res Tech* 58:176–188
- Cohen Y, Assaf Y (2002) High *b*-value *q*-space analyzed diffusion-weighted MRS and MRI in neuronal tissues—a technical review. *NMR Biomed* 15:516–542
- Condic ML, Lemons ML (2002) Extracellular matrix in spinal cord regeneration: getting beyond attraction and inhibition. *Neuroreport* 13:A37–A48
- Cory DG, Garroway AN (1990) Measurement of translational displacement probabilities by NMR: an indicator of compartmentation. *Magn Reson Med* 14:435–444
- Costa S, Planchenault T, Charriere-Bertrand C, Mouchel Y, Fages C, Juliano S, Lefrancois T, Barlovatz-Meimon G, Tardy M (2002) Astroglial permissivity for neuritic outgrowth in neuron-astrocyte cocultures depends on regulation of laminin bioavailability. *Glia* 37:105–113
- Coumans JV, Lin TT, Dai HN, MacArthur L, McAtee M, Nash C, Bregman BS (2001) Axonal regeneration and functional recovery after complete spinal cord transection in rats by delayed treatment with transplants and neurotrophins. *J Neurosci* 21:9334–9344
- Cunningham AM, Manis PB, Reed RR, Ronnett GV (1999) Olfactory receptor neurons exist as distinct subclasses of immature and mature cells in primary culture. *Neuroscience* 93:1301–1312
- David S, Ousman SS (2002) Recruiting the immune response to promote axon regeneration in the injured spinal cord. *Neuroscientist* 8:33–41
- Fouad K, Dietz V, Schwab ME (2001) Improving axonal growth and functional recovery after experimental spinal cord injury by neutralizing myelin associated inhibitors. *Brain Res Brain Res Rev* 36:204–212
- Galvin KA, Jones DG (2002) Adult human neural stem cells for cell-replacement therapies in the central nervous system. *Med J Aust* 177:316–318
- Goldman SA, Nedergaard M, Crystal RG, Fraser RA, Goodman R, Harrison-Restelli C, Jiang J, Keyoung HM, Leventhal C, Pincus DW, Shahar A, Wang S (1997) Neural precursors and neuronal production in the adult mammalian forebrain. *Ann NY Acad Sci* 835:30–55

20. Gorio A, Torrente Y, Madaschi L, Di Stefano AB, Pisati F, Marchesi C, Belicchi M, Di Giulio AM, Bresolin N (2004) Fate of autologous dermal stem cells transplanted into the spinal cord after traumatic injury (TSCI). *Neuroscience* 125:179–189
21. Hermanns S, Klapka N, Muller HW (2001) The collagenous lesion scar—an obstacle for axonal regeneration in brain and spinal cord injury. *Restor Neurol Neurosci* 19:139–148
22. Houle JD, Ziegler MK (1994) Bridging a complete transection lesion of adult rat spinal cord with growth factor-treated nitrocellulose implants. *J Neural Transplant Plast* 5:115–124
23. Houweling DA, Lankhorst AJ, Gispens WH, Bar PR, Joosten EA (1998) Collagen containing neurotrophin-3 (NT-3) attracts regrowing injured corticospinal axons in the adult rat spinal cord and promotes partial functional recovery. *Exp Neurol* 153:49–59
24. Jones LL, Yamaguchi Y, Stallcup WB, Tuszynski MH (2002) NG2 is a major chondroitin sulfate proteoglycan produced after spinal cord injury and is expressed by macrophages and oligodendrocyte progenitors. *J Neurosci* 22:2792–2803
25. Kabos P, Ehteshami M, Kabosova A, Black KL, Yu JS (2002) Generation of neural progenitor cells from whole adult bone marrow. *Exp Neurol* 178:288–293
26. Kataoka K, Suzuki Y, Kitada M, Hashimoto T, Chou H, Bai H, Ohta M, Wu S, Suzuki K, Ide C (2004) Alginate enhances elongation of early regenerating axons in spinal cord of young rats. *Tissue Eng* 10:493–504
27. Keirstead HS (2001) Stem cell transplantation into the central nervous system and the control of differentiation. *J Neurosci Res* 63:233–236
28. Keyvan-Fouladi N, Raisman G, Li Y (2003) Functional repair of the corticospinal tract by delayed transplantation of olfactory ensheathing cells in adult rats. *J Neurosci* 23:9428–9434
29. Kisiday J, Jin M, Kurz B, Hung H, Semino C, Zhang S, Grodzinsky AJ (2002) Self-assembling peptide hydrogel fosters chondrocyte extracellular matrix production and cell division: implications for cartilage tissue repair. *Proc Natl Acad Sci USA* 99:9996–10001
30. Krautstrunk M, Scholtes F, Martin D, Schoenen J, Schmitt AB, Plate D, Nacimiento W, Noth J, Brook GA (2002) Increased expression of the putative axon growth-repulsive extracellular matrix molecule, keratan sulphate proteoglycan, following traumatic injury of the adult rat spinal cord. *Acta Neuropathol (Berl)* 104:592–600
31. Le Bihan D (2003) Looking into the functional architecture of the brain with diffusion MRI. *Nat Rev Neurosci* 4:469–480
32. Le Bihan D, van Zijl P (2002) From the diffusion coefficient to the diffusion tensor. *NMR Biomed* 15:431–434
33. Lee JB, Kuroda S, Shichinohe H, Yano S, Kobayashi H, Hida K, Iwasaki Y (2004) A pre-clinical assessment model of rat autogeneic bone marrow stromal cell transplantation into the central nervous system. *Brain Res Brain Res Protoc* 14:37–44
34. Loh NK, Woerly S, Bunt SM, Wilton SD, Harvey AR (2001) The regrowth of axons within tissue defects in the CNS is promoted by implanted hydrogel matrices that contain BDNF and CNTF producing fibroblasts. *Exp Neurol* 170:72–84
35. Lu J, Feron F, Mackay-Sim A, Waite PM (2002) Olfactory ensheathing cells promote locomotor recovery after delayed transplantation into transected spinal cord. *Brain* 125:14–21
36. Lu P, Yang H, Jones LL, Filbin MT, Tuszynski MH (2004) Combinatorial therapy with neurotrophins and cAMP promotes axonal regeneration beyond sites of spinal cord injury. *J Neurosci* 24:6402–6409
37. McDonald JW, Liu XZ, Qu Y, Liu S, Mickey SK, Turetsky D, Gottlieb DI, Choi DW (1999) Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. *Nat Med* 5:1410–1412
38. McKeon RJ, Hoke A, Silver J (1995) Injury-induced proteoglycans inhibit the potential for laminin-mediated axon growth on astrocytic scars. *Exp Neurol* 136:32–43
39. Meijs MF, Timmers L, Pearse DD, Tresco PA, Bates ML, Joosten EA, Bunge MB, Oudega M (2004) Basic fibroblast growth factor promotes neuronal survival but not behavioral recovery in the transected and Schwann cell implanted rat thoracic spinal cord. *J Neurotrauma* 21:1415–1430
40. Moon LD, Asher RA, Rhodes KE, Fawcett JW (2001) Regeneration of CNS axons back to their target following treatment of adult rat brain with chondroitinase ABC. *Nat Neurosci* 4:465–466
41. Ogawa Y, Sawamoto K, Miyata T, Miyao S, Watanabe M, Nakamura M, Bregman BS, Koike M, Uchiyama Y, Toyama Y, Okano H (2002) Transplantation of in vitro-expanded fetal neural progenitor cells results in neurogenesis and functional recovery after spinal cord contusion injury in adult rats. *J Neurosci Res* 69:925–933
42. Pearse A (1972) *Histochemistry theoretical and applied*, vols 1&2, 3rd edn. Little Brown and Co., Boston, MA
43. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A (2000) Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat Biotechnol* 18:399–404
44. Rochkind S, Shahar A, Amon M, Nevo Z (2002) Transplantation of embryonic spinal cord nerve cells cultured on biodegradable microcarriers followed by low power laser irradiation for the treatment of traumatic paraplegia in rats. *Neurol Res* 24:355–360
45. Roisen FJ, Klueber KM, Lu CL, Hatcher LM, Dozier A, Shields CB, Maguire S (2001) Adult human olfactory stem cells. *Brain Res* 890:11–22
46. Ruitenberg MJ, Levison DB, Lee SV, Verhaagen J, Harvey AR, Plant GW (2005) NT-3 expression from engineered olfactory ensheathing glia promotes spinal sparing and regeneration. *Brain* 128:839–853
47. Schmidt CE, Leach JB (2003) Neural tissue-engineering: strategies for repair and regeneration. *Annu Rev Biomed Eng* 5:293–347
48. Schnell L, Schwab ME (1990) Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. *Nature* 343:269–272
49. Schuldiner M, Eiges R, Eden A, Yanuka O, Itskovitz-Eldor J, Goldstein RS, Benvenisty N (2001) Induced neuronal differentiation of human embryonic stem cells. *Brain Res* 913:201–205
50. Schwartz M (2003) Sell memorial lecture. Helping the body to cure itself: immune modulation by therapeutic vaccination for spinal cord injury. *J Spinal Cord Med* 26(Suppl 1):S6–S10
51. Shahar A (1990) Cultivation of nerve and muscle cells on microcarriers. *Methods Neurosci* 2:195–209
52. Silva GA, Czeisler C, Niece KL, Benish E, Harrington DA, Kessler JA, Stupp SI (2004) Selective differentiation of neural progenitor cells by high-epitope density nanofibers. *Science* 303:1352–1355
53. Silver J, Miller JH (2004) Regeneration beyond the glial scar. *Nat Rev Neurosci* 5:146–156
54. Stokols S, Tuszynski MH (2004) The fabrication and characterization of linearly oriented nerve guidance scaffolds for spinal cord injury. *Biomaterials* 25:5839–5846

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55. Tatard VM, Menei P, Benoit JP, Montero-Menei CN (2005) Combining polymeric devices and stem cells for the treatment of neurological disorders: a promising therapeutic approach. *Curr Drug Targets* 6:81–96
 56. Woerly S, Doan VD, Evans-Martin F, Paramore CG, Peduzzi JD (2001) Spinal cord reconstruction using NeuroGel implants and functional recovery after chronic injury. *J Neurosci Res* 66:1187–1197
 57. Woerly S, Petrov P, Sykova E, Roitbak T, Simonova Z, Harvey AR (1999) Neural tissue formation within porous hydrogels implanted in brain and spinal cord lesions: ultrastructural, immunohistochemical, and diffusion studies. *Tissue Eng* 5:467–488
 58. Woodbury D, Schwarz EJ, Prockop DJ, Black IB (2000) Adult rat and human bone marrow stromal cells differentiate into neurons. *J Neurosci Res* 61:364–370
 59. Yoshii S, Oka M (2001) Collagen filaments as a scaffold for nerve regeneration. *J Biomed Mater Res* 56:400–405
 60. Yoshii S, Oka M, Shima M, Akagi M, Taniguchi A (2003) Bridging a spinal cord defect using collagen filament. *Spine* 28:2346–2351
 61. Zigova T, Song S, Willing AE, Hudson JE, Newman MB, Saporta S, Sanchez-Ramos J, Sanberg PR (2002) Human umbilical cord blood cells express neural antigens after transplantation into the developing rat brain. *Cell Transplant* 11:265–274