FUTURE PERSPECTIVE IN PERIPHERAL NERVE RECONSTRUCTION

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Nerve injuries induce severe disability and suffering for patients. Profound alterations in nerve trunks, neurons, and the central nervous system are induced rapidly after injury. This includes activation of intracellular signal transduction mechanisms aiming at the transfer of the cells into a regenerative state through the induction of the appropriate gene programs. The understanding of the neurobiological mechanisms that occur after injury can be used to design modern strategies for reconstruction after nerve injuries. Signal transduction mechanisms for instance may be targets for pharmacological intervention to stimulate nerve regeneration. Nerve injuries, particularly where there is a defect between the severed nerve trunks like in brachial plexus lesions, remain a challenge for the surgeon. Reconstruction of nerve injuries with a defect requires utilization of graft material, which can be of various designs. Application of autologous nerve grafts and use of nerve transfers are the most common clinical solutions to overcome problems with nerve defects. In this chapter we discuss the future perspective of nerve reconstruction with focus on signal transduction mechanisms and new avenues to bridge nerve defects using nanomodified graft surfaces.

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I. Introduction

In spite of extensive research on nerve regeneration, with focus on both clarifications of the delicate molecular and cellular mechanisms, as well as direct clinically applied projects, results after nerve injury and repair are generally still insufficient. The outcome is particularly troublesome when larger nerve defects have to be reconstructed. A variety of factors influence the results after nerve injury and repair and reconstruction (Dahlin, 2008a,b,c, 2009). A poor result may often be observed when a nerve injury occurs in an adult, when a mixed nerve is injured proximally, such as the median and ulnar nerves, or when a nerve trunk or a spinal nerve root is severely lacerated, such as in the brachial plexus. Hence, such nerve injuries cause frustration not only for the patient, but also for the surgeon that has to deal with poor outcome. In contrast, injuries in young children, injuries to a pure motor nerve, such as the posterior interosseous nerve, and injuries where there is a short distance to the target, may sometimes have a favorable outcome, particularly in recovery of motor function. The present issue of *The International Review of Neurobiology* focuses on repair and reconstruction of nerve injuries today as well as future possibilities. Here we focus on future perspectives of nerve repair and in particular the possibility of targeting signal transduction to improve regeneration and secondly the possibility to use nanomodified surfaces for nerve reconstruction.

II. Intracellular Signaling

The reactions of neurons and nonneuronal cells after nerve injury are very complex processes that consist of temporally and spatially orchestrated mechanisms aimed at cellular repair. After an injury in larger and mature organisms, the axons have to grow over long distances, usually along basal lamina tubes. Sometimes the basal lamina tubes have to be recreated. An important question is if these pathways are optimal and if the Schwann cells along the paths are not optimally receptive for the outgrowing axons. A possible strategy to explore for improvements would be to try to mimic some of the developmental mechanisms of axonal growth. In such conditions there is a growth of axons over a limited distance with very receptive cells in a perfect environment with appropriate tropic and trophic signals to guide the axons to their target. Finally, the brain is very well adaptive to the new signals received from the periphery in young subjects and during development. Intensive research on mechanisms of nerve regeneration is necessary to clarify the events induced in neurons, Schwann cells, and other cells, like endothelial and inflammatory cells, in the regeneration process (Dahlin, 2008b).

It can be anticipated that stimulation or inhibition of these events with proper timing and positioning can promote both pathfinding and axonal outgrowth. In analogy with a symphony orchestra, a large number of molecular instruments are played in the cells, each with their own specific and optimal function, but in contrast to an orchestra, in the cellular context, the conductor—the surgeon—may have very limited influence on the cellular orchestra in a mature subject. New findings have been presented on the subcellular mechanisms, particularly when it comes to intracellular signaling—signal transduction, which are initiated rapidly after a cellular injury and then continuous during the entire regeneration process through autocrine and paracrine cellular signaling. Such injury-induced signal transduction will be one of the topics discussed in the present review.

III. Development of Nerve Repair and Reconstruction

Although nerve repair and reconstruction is problematic, some success has yet been achieved with the aim to improve outcome for nerve repair following injury. This progress was initiated by researchers in the beginning of the last century, such as by Ramon y Cajal (1928), who described, in meticulous studies, the biology after nerve injuries. With focus on clinical nerve repair and reconstruction, Bunnell (1944) presented an impressive description of the problem of nerve injury and repair. He described factors that influenced the outcome, techniques for how to repair nerves after injury, and also results of repair in individual patients. Other important contributions to the understanding of nerve injuries have been made by Sunderland, Seddon, Moberg, Narakas, Gilbert, Birch and many others. During the last 50 years, new strategies for nerve repair and reconstruction have evolved (Lundborg, 2000). Nerve graft techniques have been introduced by the pioneering work of particularly Millesi *et al.* (1972), and such procedures are now routine in the clinic. Thus, extensive reconstruction of brachial plexus lesions is possible by the use of nerve grafts performed at specific centers in the world, where also various nerve transfers are utilized to improve function after particularly nerve root avulsions. Recently, nerve transfers were described, which were made more distally in the arm and hand, and this technique is now frequently used (Brown and Mackinnon, 2008). For shorter nerve defects, various conduits have been, or will be, introduced in the clinic based on extensive experimental research in our laboratories (Dahlin *et al.*, 2007; Lundborg *et al.*, 2004; Nilsson *et al.*, 2005a; Scherman *et al.*, 2001, 2004). Specific rehabilitation strategies with sensory re-education are routine in the clinic. Finally, better tools to treat pain and allodynia in the injured patients are also available.

However, in spite of these improvements the outcome of nerve repair is still poor, particularly when sensory functions are considered.

IV. Nerve Reconstruction: Technique and Alternatives

The clinical routine to bridge defects between injured nerve trunks is the use of autologous nerve grafts. Many problems remain after such a procedure, such as the likely discrepancy in caliber between the graft and repaired nerve. In addition, there is probably a limit of how long a graft can be, which will still permit the growth of axons to reinnervate the target. Furthermore, there may also be a lack of availability of graft material. Alternatives to nerve grafts have been experimentally developed, but few of them are clinically applied. For short gaps, and in specific circumstances as an alternative to nerve repair, various conduits are available (Lundborg *et al.*, 2004; Weber *et al.*, 2000). Other alternatives are the simple technique by the use of longitudinal sutures to bridge short defects, which is developed in our laboratories (Scherman *et al.*, 2001). Acellular nerve grafts have been developed experimentally and are becoming more popular in the clinic. The presumption is that acellular nerve grafts are less prone to be attacked by the immune system than cell-containing nerve grafts (Hudson *et al.*, 2004; Kvist *et al.*, 2008; Sondell *et al.*, 1997). Making grafts acellular may for this reason even allow xenografting making the problem of shortage of graft material and the sacrifice of healthy donor nerve void. Recently, acellular nerve allografts, additionally treated with chondrotinase A (Krekoski *et al.*, 2001), have been used to bridge short defects in digital nerves (Karabekmez *et al.*, 2009). By making nerve grafts acellular with different techniques (Hudson *et al.*, 2004; Krekoski *et al.*, 2001; Sondell *et al.*, 1999), three-dimensional structures are obtained which still contain growth-stimulating substances like laminin. To improve the regeneration process through such acellular nerve grafts and other matrices used for bridging (e.g., tendon autografts), Schwann cells from the recipient, cultured or acutely dissociated from the injured nerve segment (Brandt *et al.*, 2005; Nilsson *et al.*, 2005a), have been added to such structures. Although axonal outgrowth can be improved initially in such Schwann cell enriched structures (Nishiura *et al.*, 2004), long-term functional recovery may be disappointing (Arino *et al.*, 2008), although a "blow-through" effect in experimental studies has been suggested (Keune *et al.*, 2006). However, further clinical studies are required to elucidate long-term outcomes. In the future, we may expect that stem cells from the recipient may be used. These may have advantageous influence on axonal outgrowth through different structures, where the stem cells can get the same characteristics and function as Schwann cells after differentiation (see Terenghi *et al.*, Chapter 21, this issue).

V. Signal Transduction in Peripheral Nerve Regeneration

Some decades ago the research on effects of different growth factors showed promising results with hopes to apply these factors to improve peripheral nerve regeneration. However, the use of neurotrophic factors to improve regeneration in the peripheral nervous system has not come to clinical use, either since the mechanisms of nerve regeneration are much more complex than first anticipated or because the treatment has drawbacks, like the induction of allodynia by NGF treatment.

Harvey *et al.* (2006) cites the neurobiologist Larry Benowitz, who described the regenerative response in neurons in the central nervous system with the analogy of the "break and the gas pedal." Growth-inhibitory substrates are the breaks, whereas the growth and trophic factors that provide growth enhancing signals is the accelerator or the gas pedal. To continue the analogy of a moving vehicle, it is not enough to take the foot from the break or to push harder on the accelerator if the handbrake is still engaged. In addition, we also have to add the "steering wheel" that directs the extension of the growth cones with their filopodia. Finally, a vehicle also has a clutch, which may represent so-far unrecognized signaling pathways, or specific switches in the cell machinery that have to be engaged for an optimal and directed growth (Harvey *et al.*, 2006).

A. The Injury Signal and the Cell Body Reaction

Cells respond to signals in their environment by translating them into intracellular messengers, which through their actions induce the appropriate stimuli-specific response. The neural response to an axonal injury is not simply localized to the site of the damage, but profound changes also occur in the cell body, sometimes long distances away from the injury site. These changes are collectively known as the cell body reaction, and involves alterations in transcription, translation, and posttranslational processes. How these changes are induced and orchestrated, both spatially and temporally, and how the information of the injury is conveyed from the injury site to the cell body still remains an enigma, but this matter is the focus of intense research (Ambron and Walters, 1996; Ambron *et al.*, 1995, 1996; Befort *et al.*, 2003; Boeshore *et al.*, 2004; Brindle and Montminy, 1992; Bussmann and Sofroniew, 1999; Chen and Strickland, 2003; Chen *et al.*, 1996; Costigan *et al.*, 2002).

Microarray analysis of injured neurons has revealed injury-induced regulation of hundreds of genes (Curtis *et al.*, 1998; Drysdale *et al.*, 1996; Gunstream *et al.*, 1995; Gupta *et al.*, 1996), including those encoding neurotrophin receptors, transcription factors and cytoskeletal components. It is hypothesized that such transcriptional changes come about following injury as a response to both negative and positive signals, that is, lack of signals from target tissue and injury-induced

signals from the damaged axon, respectively. The cell body reaction can, for instance, be initiated by the disruption of retrograde trophic support from the target tissue. There is ample experimental support for such negative control of the cell body response in injured sensory neurons, and it has been shown that pharmacological inhibition of retrograde axonal transport mimics several aspects of the cell body reactions associated with axotomy (Hai *et al.*, 1989, 1999; Hibi *et al.*, 1993). Also, injury-induced downregulation of the neurotransmitters substance P and neuropeptide Y in sensory neurons can be mitigated by distal application of nerve growth factor (NGF) or acidic fibroblast growth factor (aFGF), respectively, to the nerve (Ji *et al.*, 1996; Kallunki *et al.*, 1996). However, the cell body may also be triggered by positive signaling, that is, retrograde transport of proteins from the site of injury. The mollusk *Aplysia californica* has been extensively used as a model system to illustrate such positive regulatory mechanisms (Ambron *et al.*, 1995; Costigan *et al.*, 2002; Karin, 1995; Leah *et al.*, 1991).

Currently, there is no established treatment other than surgery for peripheral nerve injuries. However, the molecular mechanisms that regulate the neuronal injury response could in the future be used as the basis for developing new clinical therapies. Ultimately, the goal would be to modify how a peripheral axonal lesion activates the intrinsic growth capacity of the injured neuron, which in turn would be aimed to promote the speed and accuracy of regeneration.

The intrinsic growth capacity of peripheral neurons has been suggested to be mediated through the actions of cyclic adenosine monophosphate (cAMP) (Liang *et al.*, 1996; Lindwall and Kanje, 2005; Lindwall *et al.*, 2004), which ultimately regulates organization of the cytoskeleton (Snider *et al.*, 2002). Also, among others, two transcription factors that have been demonstrated to be rapidly induced by peripheral nerve injury are c-Jun (Raivich *et al.*, 2004) and activating transcription factor 3 (ATF3) (Lindwall *et al.*, 2004) (Fig. 1). c-Jun is the fundamental component of the activating protein 1 (AP-1) complex (Karin, 1995), and is one of the targets of the stress activated c-Jun *N*-terminal kinase (JNK), which catalyzes its phosphorylation (Neumann *et al.*, 2002; Nilsson *et al.*, 2005b; Perlson *et al.*, 2004a). Conditional knockout of c-Jun (Raivich *et al.*, 2004) as well as pharmacological inhibition of JNK (Lindwall *et al.*, 2004) has been demonstrated to inhibit nerve regeneration. Thus, the JNK family of kinases is required for successful regeneration of peripheral sensory neurons (Middlemas *et al.*, 2003; Perlson *et al.*, 2004b; Qiu *et al.*, 2002a), and as such represents a target for future clinical therapies. ATF3 is a member of the ATF/CREB transcription factor family (Qiu *et al.*, 2002b; Raivich *et al.*, 2004), and is rapidly induced by a variety of signals, including agents that induce the JNK signaling pathway (Seijffers *et al.*, 2006, 2007; Snider *et al.*, 2002; Sung *et al.*, 2001; Tanabe *et al.*, 2003). Inhibition of JNK reduces ATF3 protein levels (Lindwall *et al.*, 2004), which in turn hamper regeneration. Importantly, ectopic expression of ATF3 can actually promote neurite outgrowth of peripheral neurons, possibly through an increase in the intrinsic

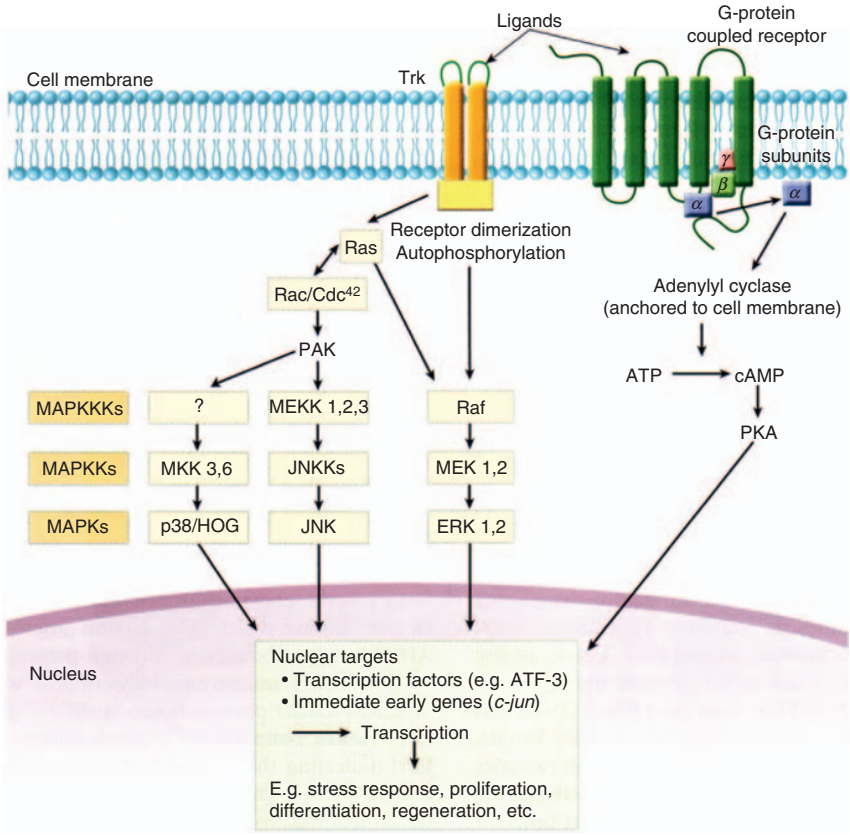

FIG. 1. Signal transduction steps in neurons and nonneuronal cells that occur after nerve injury. The schematic drawing shows the various steps that are needed to activate cells in response to a trauma. Different intracellular steps include phosphorylation steps by the MAPK modules. (Reproduced by kind permission of Elsevier.)

growth state of the neurons (Wong and Oblinger, 1991; Woolf *et al.*, 1990). The presence of these factors over time does probably influence the efficiency of axonal outgrowth; knowledge of outmost importance when considering timing of nerve repair (see below). Thus, if the levels of injury induced molecules, such as JNK, c-Jun, and ATF3, can be selectively modified following a peripheral nerve injury, augmentation of neuritogenesis can be obtained. Such treatment strategies for severe nerve injuries must, however, await a better understanding of the intrinsic molecular mechanisms initiating, and underlying, the regeneration process.

B. Extrinsic Properties Required for Axon Growth and Target Finding

The intrinsic growth capacity of peripheral nerve regeneration has to be combined with a proper environment to encourage axonal growth. Normally, peripheral axons are ensheathed and myelinated by Schwann cells. These cells also provide a basal lamina surrounding bundles of axons. Following an injury, Schwann cells de-differentiate and aid in the clearing of damaged debris, while during regeneration they act as guides for sprouting axons. During the regenerative process they upregulate several genes; the protein products of which may be involved in the guidance of axonal sprouts by Schwann cell-axon attachment (Martini *et al.*, 1994). For instance, the previously mentioned extracellular matrix (ECM) molecule laminin, which is produced by Schwann cells, plays a significant role during regeneration. Laminin receptors, such as integrins, are expressed on the growing axons, which supports regeneration. On the other hand, in mouse knockout models of laminin, axonal regeneration is significantly impaired (Zhang and Ambron, 2000). Thus, regeneration depends on a complex interplay and signals between several cell types within the nerve.

During regeneration the axonal sprouts grow down the distal nerve segment and, if successful, reinnervate their correct targets. Axonal outgrowth is, however, slow in humans, and occur at a rate of around 1 mm per day. Success of regeneration can only be judged following reinnervation of the target tissue, a process which, depending on where the damage was done, may take weeks or months after the initial insult, although the regeneration process can be followed by advancement of the Tinel sign. Unfortunately, at the time of reinnervation the window of successful regeneration may already have passed. Axons must also make correct discriminatory choices in order to reinnervate the correct target tissue, and during this process they are often misrouted. In order to develop therapeutic strategies to improve both rate and accuracy of target reinnervation we need to clarify the molecular events that influence the intrinsic growth capacity as well as axonal discrimination of the extrinsic cues, both substrate bound and diffusible, that is encountered by the axon along the regenerative pathway (Fig. 2). However, it should be stressed that these signal transduction mechanisms occur not only in neurons and its axons but also in all types of cells in a temporal and spatial resolution.

VI. Nanotechnology and Nerve Regeneration

The complex treatment of peripheral nerve injuries but also of the injured spinal cord, which involves a variety of strategies, has been emphasized by many authors (see, e.g., Garbossa *et al.*, 2006). To enhance axonal regeneration with a possible application in spinal cord repair a new generation of tissue compatible

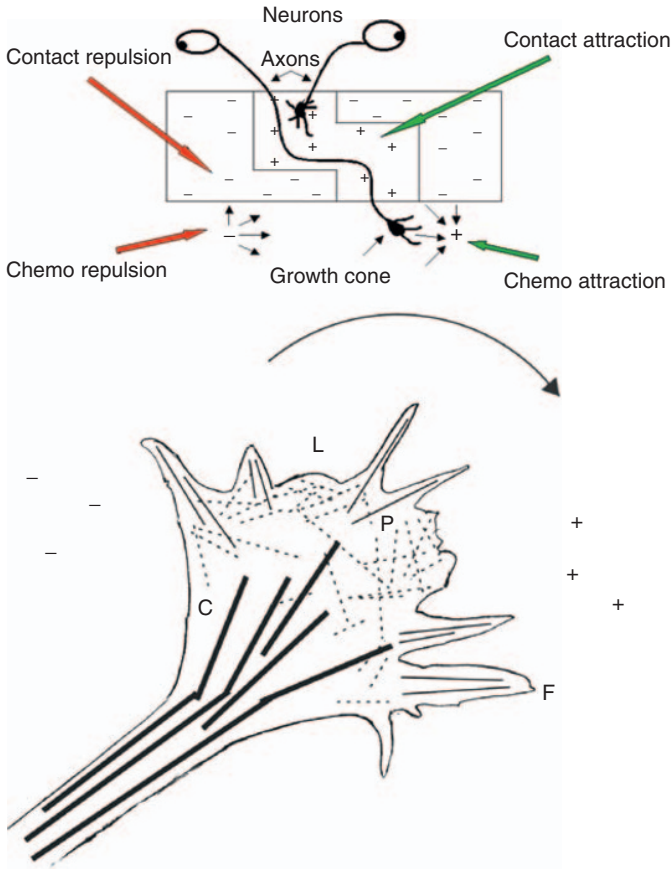

FIG. 2. Schematic drawing showing four different mechanisms that direct axonal outgrowth after nerve injury involving both attraction and repulsion of the growth cone (upper drawing). The lower drawing show details of a growth cone formed on the tip of each of numerous sprouts, which originate from the proximal end of the transected axon. C = domain where microtubules are located. P = domain where F-actin monomers and actin filaments are found. F = finger-like filopodia that palpates the surroundings directing growth. L = veil-like lamellipodia. (Reproduced by kind permission of American Society for Surgery of the Hand.)

matrices are currently developed using recent developments in nanotechnology. Nanostructures can be a suitable environment for outgrowing axons in different situations; not only to create bridges for nerve defects but also for other reasons with focus on brain machine interface (BMI) issues. Such reasons are directionality of axonal growth and sorting of nerve fibers (Johansson *et al.*, 2006; Prinz *et al.*, 2008). Nanotechnology and nerve regeneration is a future exciting field which

also will be covered in the present chapter. Development of biologically compatible scaffolds that can serve as permissive substrates for growth of neurons, migration of Schwann cells, influence differentiation, and minimize scar and inhibitory environment is a challenge where specific criteria have been outlined for application in the central nervous system (Ellis-Behnke *et al.*, 2007). In the CNS, the four important P's of regeneration as a framework has been stressed (Ellis-Behnke *et al.*, 2007): *Preservation* of neurons (no cell death); Growth-*permissive* environment; *Promotion* of growth through the permissive environment of preserved neurons, axons and their sprouts and growth cones, and, finally, utilized and improved *plasticity* after reconnection. Nanotechnology may offer solutions to several of these criteria. In combination with genomic and proteomic revolution this nanomic one will help to understand pathophysiological events and to improve results as has been suggested for the visual system (Harvey *et al.*, 2006).

A. Nanostructures for Neurite Regeneration

For the development of a new generation of artificial scaffold implants, which are tissue compatible, which smoothly integrate with the host, and which also enhance axonal regeneration, the influence of the implant surface, that is, the topography and chemophysical properties on which the cells/neurites will grow, is of paramount importance. Such tissue engineering principles can also be adopted for the study of cellular behavior associated with regenerating nerve tissue *in vitro*.

Nanotechnology has provided us with new tools that allow the design of structures with dimensions of only a few nanometers that may interact with cells and subcellular processes on a suitable cellular scale (Yim *et al.*, 2005). Extensive research on cell reactions to nanostructures *in vitro*, as well as on cell and cell extensions—neurites—has been performed during the last decade. It is conceivable that nanostructured implant surfaces can be tuned to interact smoothly with the tissue on the implant site and evoke less of an immune response than would nonstructured surfaces. Furthermore, such surfaces can be modified for the organization of the attached cells and thereby tissue formation, resulting in enhanced regeneration. We are presently pursuing these ideas for the repair and reconstruction of peripheral nerves.

B. Neurites and Topography: From Micro to Nano

During nerve regeneration the outgrowth of axons is influenced by a variety of factors, local or distant, and by the cues in the surrounding (Fig. 2). Almost a hundred years ago, R. G. Harrison reported that cells and neurites grown on threads from a spider's web followed the fibers (Harrison, 1911). In 1945,

the American developmental neurobiologist Paul Weiss named this behavior "contact guidance" (Weiss, 1945), but it was not until the early 1970s that biologists seriously tested the idea of contact guidance again, starting with growing cells on grooved substrata and on spheres (Maroudas, 1972; Rovensky *et al.*, 1971). Since then, the cellular responses to topographical cues of many different kinds have been tested, including curved surfaces, single steps, angled planes, pillars, pits, pores, cylinders, spheres, and last, but not least, the most studied structure, parallel grooves and ridges (Flemming *et al.*, 1999). The explosion of research on such artificial topographical cues was mainly due to the rapid development of techniques in the computer industry. Hence, structures with micrometer, and in the last 10–15 years even submicrometer, sized objects are possible to produce and have become available for biomedical research.

Although the exact cell reaction to a specific topography may not easily be predicted, since it is cell type dependent, a great pool of structures and cell types have been tested. Today, it is clear that structures as small as 5–10 nm can change the morphology of some cells, that is, macrophages (Wojciak-Stothard *et al.*, 1996) and that axons may follow grooves and fibers with widths of around 100 nm (Johansson *et al.*, 2006). The latter is perhaps not too surprising considering the fasciculation (minifasciculation) that occurs once a pioneering axon (that in mice may be as thin as 100 nm) has found a path during embryogenesis or regeneration. A simple, although elegant, model for neurite guidance on fibers with different diameters has been presented (Smeal *et al.*, 2005), and shows an enhanced neurite alignment along thin fibers as compared with thicker ones. The basic idea for this guidance phenomenon appears to be the stiffness of the cytoskeleton of the extending neurites. These extensions can simply not curve around a fiber with too small a curvature radius and therefore extend along the fiber in an aligned manner.

For tissue engineering applications in general, and nerve grafts in particular, ordered outgrowth/morphology is often requested. Even though 100 years have passed, the old finding of Harrison is still applicable, not only in the micrometer domain, but also in the nanometer range: parallel structures of grooves with ridges and fibers will orient cells and cell extensions, for example, axons along the structures. Such guided axons often display a simplified growth cone and a higher outgrowth rate as compared to a similar smooth or irregular surface (Corey *et al.*, 2007), a desirable feature in clinical applications. We have found excellent axonal guidance on substrates with rows of 2.5 μm long, vertically standing nanowires separated by 400 nm. Axons from dorsal root ganglia (mouse) were found to be unable to cross between, or climb the nanowires when the distance between two standing wires was small enough (sufficient with 400 nm separation but not with 1 μm) (Prinz *et al.*, 2008). Again, the explanation of this behavior is probably due to the rigidity of the cytoskeleton, a model that may explain several contact guidance phenomena.

On nonordered substrates, such as porous silicon, which has a sponge-like appearance, attachment and proliferation have been shown to be dependent on pore size *in vitro* (Bayliss *et al.*, 1999a,b, 2000; Sapelkin *et al.*, 2006). In this way, we have demonstrated ordered axonal outgrowth from mouse dorsal root ganglia on porous stripes in otherwise smooth silicon (Johansson *et al.*, 2005, 2008). Such porous silicon has also been shown to induce less encapsulation than smooth silicon *in vivo*, indicating a more biocompatible structure (Rosengren *et al.*, 2002). On random meshes of polymer fibers produced by electrospinning (see below), axonal outgrowth is hampered as compared to aligned fibers of the same material, probably due to irregular contact guidance cues (Corey *et al.*, 2007; Wang *et al.*, 2008). For clinical applications, such as nerve grafts where fast Schwann cell migration and axonal regeneration is crucial, ordered linear structures are obviously of essence.

Some of the intracellular molecular components of the guiding system have been identified (Nobes and Hall, 1999; Patel and Van Vactor, 2002), although still many pieces in the puzzle are missing. All cell reactions to the topographies described above depend on highly coordinated assembly and disassembly of the cytoskeleton and in particular microfilaments. The intracellular signaling pathways arising from the extracellular cues, and leading to the rearrangement of the cytoskeleton, involve signal transduction described previously.

For migrating cells the small GTPases, Rac, Cdc42, Ras, and Rho have been shown to be important for organizing the cytoskeleton during migration. Rac is essential for the protrusion of lamellipodia and thereby forward movement, Cdc42 is necessary for maintaining cell polarity, while Ras regulates focal adhesions and associated actin fibers (Nobes and Hall, 1999). The last one, Rho, has been reported as necessary for cell adhesion during movement and thereby contact guidance (Nobes and Hall, 1999; Rajnicek *et al.*, 2008). The guidance of axons thus depends on the same molecules, Cdc42 and Rac, that mediate growth cone attraction and elongation, while Rho mediates repulsion and growth cone collapse (Patel and Van Vactor, 2002). The alternating activation GTPases of by the external cues via the membrane receptors can thus guide the axon in a stop and go fashion. These mechanisms are examples of the intracellular signal transduction pathways.

C. Why Nanostructures?

So, if the results of an experiment performed 100 years ago on spider silk gave the same result as the most advanced structures today, why is there still an interest in nanotechnology in tissue engineering? Artificial nanopatterns can be controlled with respect to size, chemical composition, and physical properties. From an engineering point of view, the spatial resolution is extremely high using

nanotechnology enabling influence and guidance on single axons (Figs. 3 and 4). This may be very important for high-resolution neural interfaces (BMI) that may support axons bypassing injuries, controlling artificial limbs or restore other functions including hearing and vision (Donoghue, 2002). From a more biological/clinical point of view, the ECM is composed of fibers and fibrils

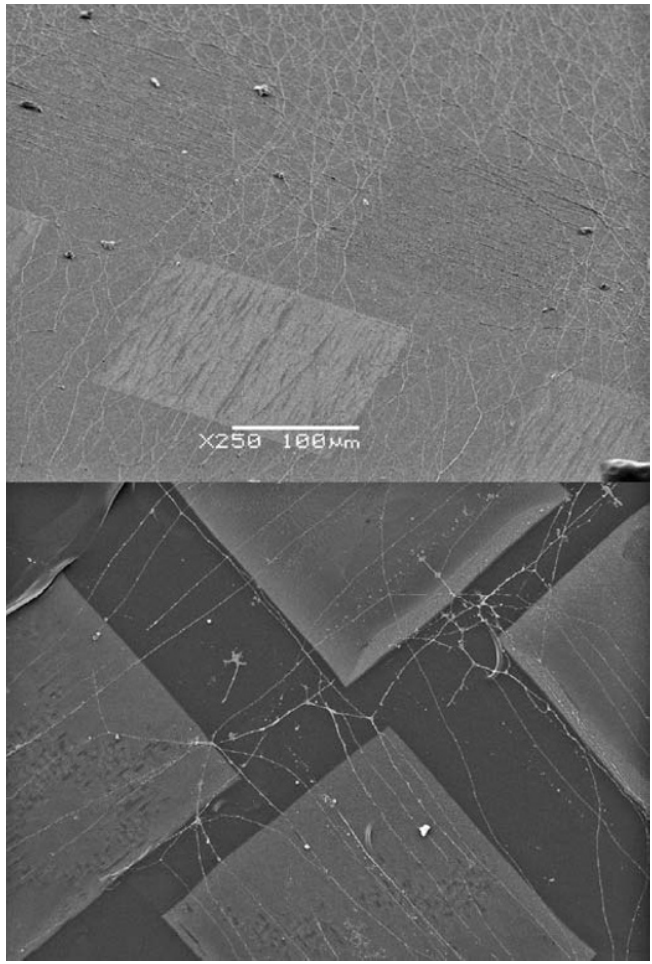

FIG. 3. Upper and lower: Axons grown *in vitro* are highly sensitive for topographies such as grooves and ridges. Here, DRG axons grown on grooves and ridges as small as 100 nm wide, display contact guidance and follow the patterned areas (squares with orthogonal grooves/ridges in an otherwise plane polymer surface produced with Nanoimprint Lithography). (Scanning electron microscope images.)

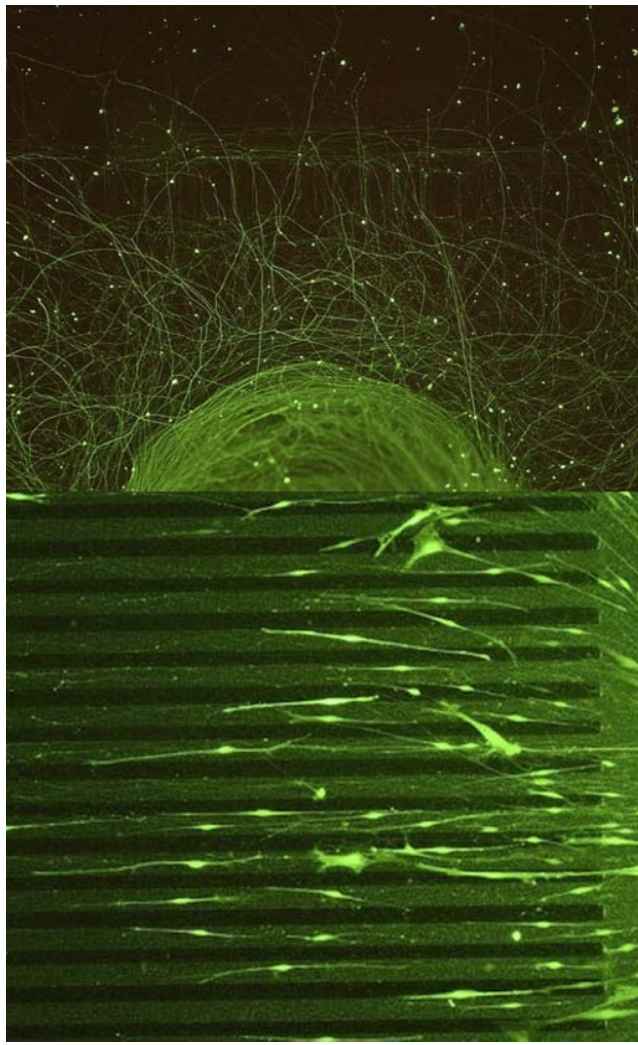

FIG. 4. *Top*: A DRG mounted in Matrigel sends out axons in a random fashion on a flat polymer surface. Once entering squares with grooves and ridges, most axons are guided along the imprinted topography. The top row holds horizontal grooves, while the lower row holds vertical grooves. Note that the thicker axons appears to be less guided than the thinner on those nanometer sized grooves. (Flourescence microscope image.) *Bottom*: On stripes (light green) of porous silicon, pore sizes of 500 nm, in an otherwise flat substrate (black), both axons and Schwann cells from an explanted DRG *in vitro*, prefer to grow and elongate on the porous stripes rather than on the flat areas. This behavior could be utilized for guidance of such cells and extensions in many different applications. (Flourescence microscope image.)

ranging from nanometers to micrometers. Hence, the addition of nanostructures for tissue engineered implants can mimic the ECM structure—the natural environment of cells and growing axons *in vivo* (Ma *et al.*, 2005).

In many other bio-nano applications, such as quantum dots, the effect of quantum physics is taken advantage of. These quantum effects and the potential for such nanostructures are so far not included in tissue engineering, although the extreme area to volume ratio can be employed for built-in drug delivery systems. The substance that should be delivered can either be adsorbed on the artificial substrate, and then the area-to-volume ratio is critical for how much substance an implant can hold. For biodegradable implants (usually polymers), where substances can be incorporated in the implant material, the delivery rate depends on the area rather than the volume when the substrate is degraded. Nanostructured/porous substrates will therefore represent faster delivery systems than bulk substrates of the same volume. The possibility to include substances which promotes survival of neurons in nanostructured nerve implants should be explored.

D. From Cell Reactions to Nanostructures *In Vitro* to Nerve Regeneration Applications

Most *in vitro* studies on nanostructures have been made on flat (rather 2.5-D than true 3-D), hard substrates such as silicon, glass, and plastics. The reason is the limitation of the patterning techniques inherited from the computer industry, that is, photolithography and electron beam lithography that usually must be used at some stage in processing a structured surface. "Soft lithography" is an overall description of many techniques where rubber molds from templates, created by the techniques mentioned above, can produce new topographical or chemical patterns. The use of such techniques can transit such structured surfaces onto irregular shapes to some degree, but only within certain limits. Although very different from an *in vivo* situation, such flat test structures have supplied us with most of the basic knowledge concerning cell adhesion, migration, and alignment etc.

From the clinical perspective, a technique called electrospinning may be better suited for nerve repair. In short, this technique is based on a polymer that is pushed out of a thin syringe. At the syringe tip, the polymer is surface charged and forms a jet stream toward an electrically grounded target, where the polymer is collected when the solvent evaporates. The thickness of such polymer fibers can be tuned from some nanometers to micrometers and the fiber alignment can be manipulated by rapid movement of the target. In this way, fabrics of

nanometer polymer fibers resembling the ECM have been produced (Kumbar *et al.*, 2008; Ma *et al.*, 2005; Murugan and Ramakrishna, 2006) and aligned such fibers guide extending neurites and migrating Schwann cells (Kim *et al.*, 2008; Schnell *et al.*, 2007; Yang *et al.*, 2005). The layers of ECM-like, biodegradable polymers, for example, poly-L-lactic acid (PLLA), can be added onto many macroscopic surfaces as a way to enhance biofunctionality, or work on its own as an artificial ECM scaffold. Besides the obvious resemblance of the ECM structure, the polymer itself can be blended with axon promoting factors, such as laminin (Koh *et al.*, 2008). By using highly aligned structures, contact guidance may also help to enhance axonal outgrowth and nerve regeneration *in vitro* and *in vivo* (Kim *et al.*, 2008; Wang *et al.*, 2008). The use of biodegradable polymers, such as PLLA with the opportunity to blend in other substances, together with the porous structure of the fabric that enables diffusion of nutrition and oxygen prior to vascularization, fulfills many clinical requirements of a nerve graft.

The use of a tissue-engineered nerve graft that performs as good as, or even better, than a standard autologous graft may minimize costs and trauma after nerve injuries. Nanostructures assist in mimicking actual tissue, enable designs on a subcellular level, and may thus be used in future nerve grafts.

VII. Clinical Development: Future Perspectives

To improve nerve regeneration and the outcome after various injuries there is a requirement for the exploration of new research avenues. Such avenues can be signal transduction and nanotechnology as discussed above. There are several other aspects which require attention from the clinical perspective. One is the problem of comparing new with conventional repair and reconstruction techniques. Another is the timing of repair and reconstruction. A third is the problem of neuronal cell death which may be a target for pharmacological intervention. Finally, focus is now also directed towards brain plasticity and the patient's ability to utilize coping strategies to adjust to the impaired function.

In the short perspective, in clinical studies, and particularly multicenter studies, we can investigate the effectiveness of different nerve reconstruction techniques, such as the new alternatives to nerve grafts. However, in more extensive nerve injuries, like in brachial plexus injuries, there are difficulties to evaluate used repair and reconstruction techniques since no lesion is similar to the other, that is, there are differences in the individual extent of injury and thereby the need for different reconstruction procedures. Thus, it is difficult to collect an appropriate number of patients with similar injuries, where such injuries are reconstructed with well-defined techniques. Previous findings have revealed an impaired functional recovery if nerve reconstruction of the brachial plexus lesion

is done 6 months or later following the injury. The timing for nerve reconstruction has been emphasized based on neurobiological alterations in neurons and Schwann cells (Saito and Dahlin, 2008). Interestingly, Kay and co-workers (Jivan *et al.*, 2009) have recently presented data indicating that functional outcome after brachial plexus lesions involving C5-C6 is better if reconstruction is done within 2 weeks after injury. Such notion is supported by the neurobiological data indicating that cellular alterations in both neurons and Schwann cells are time dependent. In Schwann cells, signal transduction mechanisms are rapidly initiated, even within 30 min, which are important for the proliferation of the Schwann cells after a nerve injury (Martensson *et al.*, 2007) and thereby the outgrowth of axons. Schwann cells can also modify the growth environment in the distal nerve segment after injury (Danielsen *et al.*, 1995). Transcription factors, upregulated rapidly in Schwann cells and neurons, subside over time with a subsequent impaired activation of Schwann cell in the distal nerve segment and decreased axonal outgrowth (Saito and Dahlin, 2008). Similarly, a rapid upregulation of the transcription factor ATF3 in neurons is also deteriorated over time. The diminution of that ATF3 response in neurons seems to correlate to impaired nerve regeneration (Saito and Dahlin, 2008). Interestingly, the decline of ATF3-containing neurons is more rapid in motor neurons than in sensory neurons (Kataoka *et al.*, 2007; Saito and Dahlin, 2008). However, the cell death of neurons is more pronounced in sensory neurons than in motor neurons (Hart *et al.*, 2004; McKay Hart *et al.*, 2002). Neuronal cell death can also be diminished if nerve trunks are repaired early after injury (Ma *et al.*, 2003). To prevent or decrease neuronal cell death, particularly among motor neurons, a pharmacological intervention can be considered. Experimental data indicate that early treatment, perhaps within the first 24 h after injury, with *N*-acetylcystein (Hart *et al.*, 2002, 2004) can reduce the number of neurons that go through programmed cell death, apoptosis. However, such treatment has to be tested clinically, preferably in multicenter studies, utilizing the specific protocols for evaluation of function after nerve injury and repair.

A problem after repair and reconstruction of proximal nerve injuries is the extended time before reinnervation of the target can be expected. The Schwann cell response to injury deteriorates over time leading to impaired axonal regeneration after proximal nerve injuries. In this respect, the described nerve transfers in the hand and distal forearm (Brown and Mackinnon, 2008) is an alternative since the surgeon can transfer the nerve injury from a proximal to a distal one. The growing axons from the transferred nerve are thus allowed to grow into an environment that is still permissive in the originally injured distal nerve segment. Nerve transfers can also be applied for a distal nerve segment when there is a lack of a proximal nerve trunk as a source of axon—end-to-side nerve repair (see Bontioti and Dahlin; Chapter 12, this issue). In addition, there are also a large number of other clinically potentially exciting additional treatments as an adjunct

to conventional nerve repair and nerve grafting techniques, which can improve nerve regeneration covered in the present issue of *International Review of Neurobiology*.

Following nerve reconstruction the surgeon also has to plan and initiate the rehabilitation phase and focus on the central nervous system, where extensive reorganization in the cerebral cortex and other levels occur after injury. Early, before reinnervation (phase I), new concepts for rehabilitation have to be considered followed by novel rehabilitation techniques when reinnervation of the hand and arm has occurred (phase II). Several new strategies have been introduced in recent years including the use of EMLA® (local anesthetics) cream application to the forearm leading to improved sensibility in the hand after repaired median and ulnar nerve injuries (Lundborg *et al.*, 2007; Rosen *et al.*, 2006). Thus, brain plasticity is a central issue in nerve reconstruction. Furthermore, individual care of the patients is crucial to direct them along their inborn strategies to cope with such an injury (Cederlund *et al.*, 2008), strategies which they may not immediately be aware of.

Taken together, although conventional nerve reconstruction techniques are used frequently in clinical practice, the outcome is generally still insufficient. Thus, new treatment strategies have to be introduced based on new avenues of research. The utilization of knowledge of intracellular signal transduction mechanisms, and the use of nanotechnologies are exciting perspectives in nerve reconstruction in the future.

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