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Nanomedicine: Nanotechnology, Biology, and Medicine
xx (2016) xxx–xxx
nanomedicine
Nanotechnology, Biology, and Medicine
nanomedjournal.com

Schwann cells and neurite outgrowth from embryonic dorsal root ganglions are highly mechanosensitive

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Received 10 March 2016; accepted 24 June 2016

Abstract

Biochemical interactions between Schwann cells (SCs) and their substrate are crucial for the peripheral nervous systems (PNS). They are among the major parameters used in the design of nerve grafts for nerve injuries treatment, yet with unsatisfactory success despite pressing need worldwide. Mounting evidence demonstrates the fundamental physiological importance of mechanical cell–substrate interactions. Substrate stiffness modulates cell differentiation, development, maintenance and regeneration. Mechanosensitivity may therefore be a key parameter to advancing nerve graft research. However, very little is known about PNS mechanosensitivity. Here, we explore mechanosensitivity of SCs and embryonic dorsal root ganglions (DRGs) under constant biochemical conditions but varying substrate stiffness adjusted to their physiological–developmental nature. We found SC stiffness, morphology, adhesion, motility, and neurite outgrowth from DRGs to be strongly substrate stiffness-dependent. These initial observations refine our knowledge of PNS physiology, development and regeneration, and demonstrate promise for advancing nerve grafts.

Key words: Atomic force microscopy; Mechanosensitivity; Peripheral nervous system; Schwann cells; Nerve regeneration

Peripheral nerve injuries have a high incidence worldwide.[1,2] They may result from compression, stretch, inflammation, car accidents, cancer, diabetes and combat among others.[1] On the one hand, the peripheral nervous system (PNS) has the intrinsic ability to regenerate on its own after certain types of injuries. On the other hand, regeneration is generally unsatisfactory and functional recovery is almost never complete.[1,3,4] Besides, injuries frequently have severe consequences for the afflicted individuals with devastating impact on their life quality.[1] Surgical intervention then becomes the only step that may alleviate the patients' suffering. In certain cases, the surgical intervention may bring some relief. However, the general outcome is far from being satisfactory with many individuals acquiring a life-long disability.[1,4] Hence, significant advance in this field is worldwide a pressing medical need, which presents a particular clinical challenge.[1,3] After a nerve is transected following a severe injury, the multi-step degeneration process, termed Wallerian degeneration,[2,5,6] sets off eventually resulting in disintegration of the proximal and distal stumps of the nerve. Consequently, Schwann cells, the principal glial cells of the PNS, are recruited in an attempt to re-integrate the two ends of the nerve.[2,4] Their recruitment is based on their profound importance. They produce an extracellular matrix, high levels of growth factors and myelin.[7,8] Besides, they are capable of clearing the debris resulting from nerve degeneration.[7,8] Surgical repair of transected nerves requires a reconnection of their proximal and distal ends and relies very strongly on the diverse physiological roles of Schwann cells in the PNS.[1,4] The gold standard for surgical nerve repair is the autologous nerve grafting. However, its success remains far from satisfactory due to frequently disappointing outcome, formidable restrictions and serious drawbacks.[1,4] These facts have evoked the development of artificial nerve grafts over the past years. Meantime, some advance has been made and a wide range of nerve graft strategies are available.[1,4] However, like the autologous grafts the success of artificial grafts is not satisfactory.[1–3] One common principle of artificial nerve grafts is to use Schwann cells as the main

We confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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http://dx.doi.org/10.1016/j.nano.2016.06.011
1549-9634/

player to mediate nerve repair.[1] Schwann cells are placed on the inner surface of a bioengineered neural scaffold which may be made of natural, artificial or diverse other materials.[9–11] The scaffold is basically a hollow tube meant to provide physical guidance for growing axons. It typically contains cellular and molecular components which should enable the regeneration process.[1,4] To date, the main concern was dedicated to the biochemical properties of the neural scaffolds.[1] Obviously, the biochemical aspects must be complemented powerfully with others in the design of neural scaffolds, and mechanical aspects suggest themselves. Diverse cell types have been shown to be highly sensitive to the mechanical cues, in particular stiffness, of their surroundings.[12] The evidence is overwhelming that mechanosensitivity modulates physiological functions, development, maintenance and regeneration of cells and tissues.[13–18] Moreover, diverse diseases including cancer,[19] fibrosis[20] and atherosclerosis[21–23] are closely associated with changes in the stiffness of cells and tissues.[24] Hence, consideration of mechanical aspects in the design of neural scaffolds alongside the biochemical aspects holds the promise to advance the field significantly. Unlike many other cell types, mechanosensitivity of cells in the nervous system is just beginning to be explored. Recent observations point out the relevance of substrate stiffness for bioengineering of neural grafts.[25,26] The present study combines atomic force microscopy with confocal microscopy to study the impact of substrate stiffness on Schwann cells' mechanics, structure and function, as well as neurite outgrowth from embryonic dorsal root ganglions.

Methods

Preparation of laminin-coated polyacrylamide substrates with varying stiffness

Compliant polyacrylamide (PA) precursor gels were produced from 40% (w/v) acrylamide (Sigma-Aldrich, USA), 2% (w/v) bis-acrylamide (Bio-Rad Laboratories) and 97% (w/v) *N*-hydroxyethyl acrylamide (Sigma-Aldrich, USA) stock solutions to generate three types of gels of different stiffnesses. The final concentration of each component produced PA gels with elastic moduli of 1 kP, 10 kPa and 20 kPa, which were corroborated by indentation measurements using atomic force microscopy. First, 25 mm glass coverslips were cleaned with 70% ethanol and treated with 0.1% sodium hydroxide (NaOH) to make the surface hydrophobic. Then coverslips were incubated for 3 min with (3-aminopropyl) trimethoxysilane (APTMS, Sigma-Aldrich, USA) to functionalize the glass surface. Subsequently, pretreated coverslips were coated for 30 min with 0.5% glutaraldehyde. PA precursor mix containing 500 μl 40% acrylamide (Sigma-Aldrich, USA), 65 μl 100% hydroxyacrylamide (Sigma-Aldrich, USA) and 250 μl 2% bis-acrylamide (Bio-Rad) was shortly degassed in vacuum desiccator. Gel polymerization was initiated by adding 0.1% w/v Ammonium-Persulfate (Sigma-Aldrich, USA) and 0.1% w/v tetramethylethylenediamine (TEMED, Bio-Rad Laboratories) to the PA precursor mix. A drop of polymerizing PA mix was added to the 25 mm coverslips and immediately covered with water-repellent solution RainX® (RainX Co, USA) pre-treated (20 mm) glass coverslips in order to produce approximately 30 μm thick gel substrates (Supplementary Figure 2). After PA polymerization (approx. 30 min) the gels were placed in 6-well culture plate containing phosphate buffer solution (PBS) and the 20 mm coverslips were removed. Gels were rinsed with PBS several times to remove the non-polymerized acrylamide. PA gels sterilization was carried out exposing the gels to UV light for ~10 min. Surface coating to promote Schwann cell adhesion was induced incubating PA gels with poly-D-lysine (Sigma-Aldrich, USA) overnight at 4 °C. The next day gels were washed and incubated for 2 h at room temperature with 10 μg/ml laminin (Sigma-Aldrich, USA). PA gels were covered with cell culture medium and allowed to equilibrate inside incubator at 37 °C for 1 h before seeding the cells.

Isolation and purification of primary Schwann cells

Animal studies and procedures were conducted in accordance with the European Convention for Animal Care and Ethical Use of Laboratory Animals and have been approved by the local governmental authorities (State Office for Nature, Environment and Consumer Protection, North Rhine-Westphalia, Germany; File reference 84-02.05.20.12.146). Sprague–Dawley adult rats were euthanized by isofluoran overdose followed by guillotine decapitation. Both sciatic nerves were carefully dissected using sterilized surgical material and nerves were immediately immersed on ice-cold Dulbecco's modified Eagle's medium (Life technologies, Germany) containing 5% fetal bovine serum (FBS HyClone, GE Healthcare Life Sciences, USA), 100 μg/ml penicillin/streptomycin and 100 μg/ml glucose. Under binocular microscope the perineurium was completely removed in order to reduce contamination with fibroblasts. Nerves were cut into small pieces (3-4 mm) and transferred to a sterile culture dish containing culture medium and placed in the incubator at 37 °C 5% CO_2 for 7 days. Nerve tissue was dissociated by digestion with collagenase and dispase (Sigma-Aldrich, USA) followed by mechanical trituration and plating. After day 8 the nerve tissue was vigorously mixed and cells were centrifuged 3 min at 250*g* and suspended in medium containing 10% FBS (HyClone), 2 μM Forskolin (Sigma-Aldrich, USA), 10 ng/ml Neuregulin (Peprotech, Hamburg Germany), 10 μg/ml bovine pituitary extract (Sigma-Aldrich, USA), and 100 μg/ml glucose. Schwann cells were plated on poly-L-lysine coated culture flasks and incubated at 37 °C 5% CO_2 for 48 h. At day 10, cells were harvested using trypsin and centrifuged 3 min at 250*g* and kept in culture flasks until reaching confluence. Schwann cells were separated from fibroblasts applying the cold-jet technique previously described by Jirsova et al.[27] Cells used in the experiments were between passages 4 and 7.

Isolation and culture of dorsal root ganglia explants

Dorsal root ganglia explants were isolated from embryonic day 12.5 (E12.5) to 13.5 C57BL6/J mouse pups and plated on PA gels previously coated with poly-D-lysine (Sigma-Aldrich, USA) and 10 μg/ml laminin (Sigma-Aldrich, USA). The explants were maintained in Neurobasal medium (Invitrogen, USA) containing 20 μl/ml B27 supplement 50X (Gibco), 10 μl/ml antibiotic–antimycotic, 2 mM Glutamax (Invitrogen, USA) and 0.01 μg/ml nerve growth factor (NGF, Invitrogen, USA). After 4 days in