Increased FGF-2 secretion and ability to support neurite outgrowth by astrocytes cultured on polyamide nanofibrillar matrices

Roberto Delgado-Rivera a,b,1, Suzan L. Harris a,1, Ijaz Ahmed a,1, Ashwin N. Babu a, Ripal P. Patel a, Virginia Ayres b,1, Dexter Flowers d, Sally Meiners a,⁎

a Department of Pharmacology, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ 08854, United States
b Department of Chemistry and Chemical Biology, Rutgers University, Piscataway, NJ 08854, United States
c Department of Electrical and Computer Engineering, Michigan State University, East Lansing, MI 48824, United States
d Department of Electrical and Computer Engineering, Wayne State University, Detroit, MI 48202, United States

A R T I C L E   I N F O

Article history:
Received 17 October 2008
Received in revised form 11 February 2009
Accepted 11 February 2009

Keywords:
- Nanofibrillar matrix
- Basement membrane
- Astrocyte
- Neuron
- Astrocyte growth factor-2
- Blood-brain barrier
- Fibroblast growth factor-2

A B S T R A C T

An electrospun nonwoven matrix of polyamide nanofibrils was employed as a new model for the capillary basement membrane at the blood-brain barrier (BBB). The basement membrane separates astrocytes from endothelial cells and is associated with growth factors, such as fibroblast growth factor-2 (FGF-2). FGF-2 is produced by astrocytes and induces specialized functions in endothelial cells, but also has actions on astrocytes. To investigate potential autocrine actions of FGF-2 at the BBB, astrocytes were cultured on unmodified nanofibers or nanofibers covalently modified with FGF-2. The former assumed an in vivo-like stellate morphology that was enhanced in the presence of cross-linked FGF-2. Furthermore, astrocyte monolayers established on unmodified nanofibers were more permissive for neurite outgrowth when cultured with an overlay of neurons than similar monolayers established on standard tissue culture surfaces, while astrocytes cultured on FGF-2-modified nanofibers were yet more permissive. The observed differences were due in part to progressively increasing amounts of FGF-2 secreted by the astrocytes into the medium; hence FGF-2 increases its own expression in astrocytes to modulate astrocyte–neuron interactions. Soluble FGF-2 was unable to replicate the effects of cross-linked FGF-2. Nanofibers alone up-regulated FGF-2, albeit to a lesser extent than nanofibers covalently modified with FGF-2. These results underscore the importance of both surface topology and growth factor presentation on cellular function. Moreover, these results indicate that FGF-2-modified nanofibrillar scaffolds may demonstrate utility in tissue engineering applications for replacement and regeneration of lost tissue following central nervous system (CNS) injury or disease.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

The blood-brain barrier (BBB) is comprised in large part of capillary-forming endothelial cells and surrounding astrocytes. An ultra-dense basement membrane (a sheet-like deposition of extracellular matrix) surrounds each capillary and contributes to the interstitial fluid-tight barrier. Astrocyte endfeet are closely associated with capillaries and further decrease their permeability. The nanofibrillar basement membrane also allows for an exchange of growth factors such as leukaemia inhibitory factor (LIF) and fibroblast growth factor-2 (FGF-2) between endothelial cells and astrocytes. Hence FGF-2 likely exists in both soluble and matrix-bound form at the BBB. FGF-2 is widely regarded as produced by astrocytes and is implicated in the induction of BBB properties in endothelial cells in vitro (e.g., tight junction formation) (reviewed by Abbott et al., 2006). FGF-2 also has autocrine effects on astrocytes (e.g., to promote proliferation, stellation) (Gómez-Pinilla et al., 1995, 1997), yet its influence on astrocytes in close apposition with the capillary basement membrane has received comparably little attention.

In addition to their role in formation of the BBB, astrocytes regulate neuronal growth during development of and following injury to the central nervous system (CNS). Astrocytes provide nutrients and remove wastes from neurons, provide mechanical support, and express interstitial extracellular matrix molecules (Meiners et al., 1993) and growth factors (Hatten et al., 1988; Le Roux and Esquenazi, 2002; Toyomoto et al., 2005) that can promote, inhibit, or direct
neurite outgrowth. Hence, as suggested by Abbott et al. (2006), astrocytes can be considered to be the cellular bridge between the capillary basement membrane and neurons. It is reasonable to suggest then that both the physical dimensionality and the chemical composition of the basement membrane at the BBB can strongly influence the net impact that astrocytes have on neuronal growth.

We investigated this hypothesis in vitro by employing a synthetic nanofibrillar matrix prepared by electrospinning. Electrospinning is a method that was initially developed to make nonwoven fabrics and has recently been adapted to produce nanofibrillar matrices that mimic the geometry and nanotopography of the extracellular matrix/basement membrane for cellular growth (Nur-E-Kamal et al., 2005; Schindler et al., 2005; Ahmed et al., 2006; Nur-E-Kamal et al., 2006). To include chemical elements of the capillary basement membrane and to evaluate putative autocrine contributions of FGF-2, nanofibers were left unmodified or were covalently cross-linked with FGF-2 as described (Nur-E-Kamal et al., 2008).

We now demonstrate that nanofibrillar scaffolds allow astrocytes to assume morphological forms and biologically relevant functions (e.g., promotion of neurite outgrowth) that more accurately recapitulate their physiology within the developing CNS than standard planar tissue culture surfaces. These physiologically relevant actions are augmented by the presence of covalently bound FGF-2. Soluble FGF-2 did not recapitulate the effects of substrate-bound FGF-2 in parallel experiments. This is highly significant in that binding of FGF-2 to heparan sulfate proteoglycans and related molecules in the extracellular matrix/basement membrane has been suggested to increase its half-life (Benoit and Anseth, 2005) and potentiate its autocrine actions on astrocytes (Gómez-Pinilla et al., 1995, 1997). Hence polyamide nanofibers provide a new model for the capillary basement membrane that can similarly potentiate the actions of FGF-2 even in the absence of the glycosaminoglycan link. Moreover, in contrast to the previous studies which suggest that autocrine actions of FGF-2 on astrocytes promote gliotic scarring (Gómez-Pinilla et al., 1995, 1997), our results provide evidence that FGF-2 may instead promote a permissive phenotype.

Fig. 1. (A) Epi-fluorescent images of astrocytes at the BBB in the cerebral cortex of developing rat brain. Immunolabeling was performed with antibodies against GFAP to detect astrocytes (green) and FGF-2 (red). Nuclei (blue) were detected using Hoechst dye. A capillary decorated with FGF-2 was observed running diagonally through the image. An astrocyte endfoot is marked with a single arrow. Scale bar, 10 μm. (B) Epi-fluorescent images of basement membrane proteins at the BBB. Immunolabeling was performed with antibodies against GFAP (green) and either fibronectin or laminin-1 (red). Antibodies against both matrix proteins intensely stained capillaries that were in contact with astrocyte endfeet (single arrow) and astrocyte cell bodies (double arrow). Scale bar, 25 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Tapping mode AFM images of electrospun unmodified polyamide nanofibers (− X-linked FGF-2) and polyamide nanofibers with covalently cross-linked FGF-2 (+ X-linked FGF-2). Height (top) and amplitude (bottom) images are shown for each. The difference in the surface roughness is apparent in the amplitude images. The dimensions of the images are shown at the bottom of each (+ X-linked FGF-2) image and apply to both the (− X-linked FGF-2) and (+ X-linked FGF-2) images.
2. Results and discussion

2.1. Localization of FGF-2 in developing rat cortex

To evaluate the localization of FGF-2 at the BBB in early postnatal cortex, cerebral cortical sections were immunolabeled with antibodies against glial fibrillary acidic protein (GFAP) and FGF-2 to stain astrocytes and growth factor, respectively. Cellular nuclei were stained with Hoechst dye. Astrocyte endfeet (single arrow) were observed to delineate structures that had the typical tube-like structures of capillaries (1A). Clusters of FGF-2 were observed at the capillary wall (Fig. 1A). Immunolabeling with antibodies against fibronectin and laminin-1 (Fig. 1B) was employed to stain the BBB basement membrane and provided further evidence that the tube-like structures were, in fact, capillaries. Single and double arrows point to astrocyte endfeet and astrocyte cell bodies, respectively, that both appear to be in contact with capillaries.

2.2. Atomic Force Microscopy (AFM) analysis of unmodified and FGF-2-modified polyamide nanofibers

Polyamide nanofibers were used as a model for the ultra-dense basement membrane at the capillary wall of the BBB. However, the contact angle measurements. Water drops were deposited on top of plastic, unmodified nanofibrillar, and FGF-2-modified nanofibrillar surfaces, and contact angles were determined. Bars represent the mean +/−the SEM (n=18). The unmodified and FGF-2-modified nanofibrillar matrices were slightly, albeit significantly, less polar than the plastic surface (asterisks, p<0.05, one-way ANOVA followed by Tukey–Kramer multiple comparisons test).

Fig. 3. Contact angle measurements. Water drops were deposited on top of plastic, unmodified nanofibrillar, and FGF-2-modified nanofibrillar surfaces, and contact angles were determined. Bars represent the mean ± the SEM (n = 18). The unmodified and FGF-2-modified nanofibrillar matrices were slightly, albeit significantly, less polar than the plastic surface (asterisks, p < 0.05, one-way ANOVA followed by Tukey–Kramer multiple comparisons test).

Fig. 4. A comparison of astrocyte morphology cultured on plastic and nanofibrillar surfaces with astrocytes in neonatal rat cerebral cortex. Immunolabeling was performed with an antibody against GFAP. Astrocytes plated on nanofibers (B) had a stellate morphology that was reminiscent of astrocytes in the cerebral cortex (E), whereas astrocytes plated on plastic (A) were flat and well spread. Note that the in vivo-like appearance of the astrocytes was enhanced for cells plated on FGF-2-modified nanofibers (+ X-linked FGF-2) (D), but not for cells plated on unmodified nanofibers and cultured in the presence of soluble FGF-2 (C). Scale bar, 25 μm.
nanofibers are only an approximation, because we are unaware of any studies characterizing the nanoscale properties of the native BBB basement membrane. Tapping mode AFM (Braga and Ricci, 2003) Height images (5 × 5 μm²) of the unmodified nanofibers (top left) and nanofibers with modified covalently cross-linked FGF-2 (top right) are shown in Fig. 2. In Tapping mode AFM, the root mean square (rms) average height of the oscillating tip above the sample surface is held at a constant value by a feedback loop and is plotted as a function of the x–y position. Both images demonstrated a high packing fraction of randomly oriented nanofibers, ~10 nanofibers/μm² over 3–4 surface layers. The majority of the nanofibers ranged in diameter from 100–300 nm, as reported previously (Schindler et al., 2005). However, the cylindrical curvature and the random orientation of the individual nanofibers relative to the tip can both introduce substantial tip dilation artifacts, and as such, the apparent diameters may appear larger than the actual diameters (Fan et al., 2007).

Tapping mode AFM was also used to acquire 400 × 400 nm² close-up images of the surfaces of individual nanofibers. Amplitude images are shown, in which changes in the rms amplitude of the oscillating tip are plotted as a function of the x–y position. An amplitude image is therefore the derivative of a height image and is sensitive to even small changes in the rms average height. Comparison of the unmodified (bottom left) and FGF-2-modified (bottom right) nanofibers clearly indicated a difference in surface characteristics, with an enhanced surface roughness observed for the FGF-2-modified nanofibers. This enhanced roughness may be due to changes in both topographical surface geometry and surface chemistry of the modified nanofibrillar matrices. Similar effects have been observed by Karakecili et al. (2007) for unmodified vs. RGD-modified poly(caprolactone) membranes and by our group (Meiners et al., 2007) for unmodified vs. tenasin-C-modified polyamide nanofibrillar surfaces. To our knowledge, these are the first AFM images of FGF-2-derivatized nanofibrillar surfaces. Covalently cross-linked FGF-2 on the nanofibrillar surface (Fig. 2B) had an appearance of plaques or patches that was reminiscent of the pattern of FGF-2 at the capillary wall.

2.3. Polarity of nanofibrillar surfaces

To further characterize the biomaterial properties of the nanofibrillar surfaces, the surface energy was evaluated for unmodified and FGF-2-modified nanofibrillar matrices in comparison to a flat plastic (Aclar) surface (Fig. 3). This was done by measuring the contact angle that a drop of water makes with the dry surface. Three drops of water were placed on top of each substrate, and 6 independent contact angle measurements were made per drop. The nanofibrillar surfaces were slightly, but significantly, more hydrophobic than the plastic surfaces, with a contact angle of 110° ± 2 for unmodified nanofibers and 97° ± 8 for FGF-2-modified nanofibers vs. 88° ± 3 for the plastic surface (Fig. 3).

2.4. Morphology of astrocytes on nanofibrillar matrices

To investigate the hypothesis that astrocytes will adopt a more physiologically relevant phenotype on nanofibrillar matrices, cerebral cortical astrocytes were plated onto plastic coverslips, unmodified nanofibrillar matrices, or FGF-2-modified nanofibrillar matrices at a density of 50,000 cells/well. They were maintained for 24 h and were then fixed with paraformaldehyde and immunolabeled with an antibody against GFAP (Fig. 4). Fig. 4A shows that astrocytes plated on the plastic surface were well spread with fine GFAP filaments arranged in a delicate criss-cross pattern, whereas cells on nanofibrillar matrices (Fig. 4B) were fibrous with GFAP filaments that appeared to be more condensed. The latter is more typical of the appearance of astrocytes in vivo. This result may stem from both chemical and topological differences between the two surfaces, as could the small difference in surface free energy measured in Fig. 3.
arrow) and an extensive network of axonal processes. To model these relationships in vitro, cultured cerebral cortical astrocytes were plated onto plastic, nanofibrillar, or FGF-2-modified nanofibrillar surfaces at a density of 200,000 cells/well. This was done to ensure that the cells plated on nanofibrillar matrices formed confluent monolayers following 24 h and that the neurons in the co-cultures (below) came in contact with the surface of the astrocytes and not the surface of the nanofibers. The lower plating density employed for GFAP staining allowed for ready visualization of individual cells, particularly on FGF-2-modified nanofibers (Fig. 4D), but the astrocytes did not completely cover the nanofibrillar surface due to their stellate morphology (Compare to the more uniform cellular coverage of the well spread astrocytes plated on the plastic surface (Fig. 4A)).

After 24 h, astrocytes were rinsed in Hank’s buffered saline solution (HBSS), and cerebellar granule neurons were suspended in neuron medium and plated at a density of 50,000 cells/well on top of the astrocyte monolayers. Cerebellar granule neurons were used for these experiments because they are a homogenous population and yield relatively small population variances, aiding in the interpretation of results. Co-cultures were then fixed after an additional 24 h, and neurons were immunolabeled with an antibody against beta III tubulin. Images of the astrocyte–neuron co-cultures are shown in Fig. 6A–C. Neurites were visibly longer for neurons plated on top of astrocytes cultured on nanofibers (Fig. 6B) in comparison to astrocytes cultured on plastic (Fig. 6A) and longer yet for neurons plated on top of astrocytes cultured on FGF-2-modified nanofibers (Fig. 6C). The processes of the latter also demonstrated more branching. The astrocyte monolayers were faintly visible in these images due to background fluorescence. Sister cultures were immunolabeled with an antibody against GFAP instead of with the antibody against beta III tubulin. Immunolabeling with an antibody against beta III tubulin was performed to visualize process extension from neurons plated on top of astrocyte monolayers that were established on plastic (A), nanofibrillar matrices (B), and FGF-2-modified nanofibrillar matrices. Sister cultures were immunolabeled with an antibody against GFAP to visualize the monolayers established on FGF-2-modified nanofibers (D). Scale bar, 20 μm.

Fig. 6. Astrocyte–neuron co-cultures. Immunolabeling with an antibody against beta III tubulin was performed to visualize process extension from neurons plated on top of astrocyte monolayers that were established on plastic (A), nanofibrillar matrices (B), and FGF-2-modified nanofibrillar matrices. Sister cultures were immunolabeled with an antibody against GFAP to visualize the monolayers established on FGF-2-modified nanofibers (D). Scale bar, 20 μm.
experiment of 5 is shown. Neurites were significantly more permissive than the media conditioned for 24 h. By comparison, median neurite outgrowth was only about 75 μm for neurons cultured on Nano brushes alone; in this case, median neurite outgrowth was 148 μm for the latter (not shown) (see also Fig. 8C), respectively.

The mechanism whereby astrocytes exposed to FGF-2 facilitate neuronal process extension was not explored in the Di Prospero et al. (1997) study. To begin to address this question, neurite outgrowth experiments were also performed using conditioned media collected from the astrocytes (Fig. 7B, C) to evaluate the contribution of secreted factors. Monolayers were established as above, and then the astrocyte medium was changed to neuron medium to reproduce the conditions of the astrocyte-neuron co-cultures. Astrocytes on the different surfaces were allowed to condition the medium for 24 h (Fig. 7B) or 48 h (Fig. 7C). Conditioned media samples were collected and cerebellar granule neurons were suspended in the media and plated at a density of 50,000 cells/well onto poly-L-lysine (PLL)-coated plastic coverslips, our standard control surface for neuronal culture (Ahmed et al., 2006). Neurons were then allowed to extend neurites for an additional 24 h and were fixed, stained, and analyzed as above.

Fig. 7B, C demonstrates that the ability of the conditioned media to support neurite outgrowth mirrored that of the intact astrocytes. Media collected from astrocytes cultured on FGF-2-modified nanofibers was the most permissive for process extension, and media collected from astrocytes cultured on plastic was the least permissive. Furthermore, media fractions conditioned for 48 h (Fig. 7C) promoted more neurite outgrowth than did corresponding media fractions conditioned for 24 h (Fig. 7B). In fact, media conditioned for 24 h by astrocytes plated on plastic was not especially permissive for neurite outgrowth in comparison to control, unconditioned neuron medium (median neurite lengths were 48 μm for the former condition (Fig. 7B) and 42 μm for the latter (not shown) (see also Fig. 8C), respectively.

Table 1
Neurite outgrowth in co-culture from cerebral cortical neurons.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Astrocyte culture surface</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plastic</td>
</tr>
<tr>
<td>None</td>
<td>144 μm</td>
</tr>
<tr>
<td>Soluble FGF-2</td>
<td>152 μm</td>
</tr>
<tr>
<td>Cross-linked FGF-2</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Median total neurite length from 1 representative experiment of 3 is shown. Neurite outgrowth was significantly enhanced on astrocytes plated on unmodified nanofibers or unmodified nanofibers in the presence of soluble FGF-2 in comparison to astrocytes plated on plastic (single asterisks, p<0.05, Kolmogorov-Smirnov test). Neurite outgrowth was further enhanced on astrocytes cultured on FGF-2-modified nanofibers in comparison to unmodified nanofibers; the difference was significant (double asterisk).
The differences were not due to the number of astrocytes present (e.g., more astrocytes on the FGF-2-modified nanofibers at the end of the experiment) since whole cell extracts yielded similar amounts of protein for astrocytes cultured on each substrate at each time point (not shown). Since FGF-2 is a mitogen for astrocytes (Gómez-Pinilla et al., 1995), the most likely explanation for this observation is that the astrocytes were contact inhibited due to the high plating density employed.

The extent of neurite outgrowth afforded by the astrocytes in coculture (Fig. 7A) was in each case greater than that afforded by the corresponding medium alone (Fig. 7B, C). This indicates that both soluble and non-soluble components contributed to the facilitation of neuronal process extension and that the differential neurite outgrowth-promoting abilities of the astrocytes resulted in part from the profiles of expressed extracellular matrix and adhesion proteins. We therefore evaluated tenasin-C production by the astrocytes cultured on the various surfaces. Tenasin-C (but not laminin-1 or fibronectin) is up-regulated in astrocytes by soluble FGF-2 (Meiners et al., 1993; Di Prospero et al., 1997) and promotes neurite outgrowth when bound to the surface of cells (Meiners and Geller, 1997; Meiners et al., 1999b). However, expression of the largest splice variant of tenasin-C, which is the most stimulatory for neuronal process extension (Meiners and Geller, 1997; Meiners et al., 1999a,b), did not vary appreciably for astrocytes cultured on plastic, nanofibers, and FGF-2-modified nanofibers in our early experiments (not shown).

2.6. Effect of culture surface on FGF-2 secretion by astrocytes

We next evaluated conditioned media fractions for the presence of neurite outgrowth-promoting molecules. FGF-2 was a key candidate given that an additional autocrine action of FGF-2 is to up-regulate its own expression in astrocytes (Gómez-Pinilla et al., 1995, 1997) and fibroblasts (Arose et al., 1999). Moreover, astrocyte-derived FGF-2 encourages process extension from cerebellar granule (Hatten et al., 1988; and cerebral cortical neurons (Le Roux and Esquenazi, 2002), and FGF-2 is also involved in neurite branching (Szbenyi et al., 2001). Aliquots of media conditioned for 48 h were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970), transferred to nitrocellulose paper (Towbin et al., 1979), and immunoblotted with an antibody against FGF-2. FGF-2 was detected in all of the samples (Fig. 8A), with the highest levels in conditioned media collected from astrocytes cultured on nanofibers with covalently cross-linked FGF-2 (Fig. 8A, B). However, nanofibers alone were able to up-regulate FGF-2, again demonstrating the significance of both topography and chemistry on cellular function.

Paralleling results from the morphology studies and neurite outgrowth assays, soluble FGF-2 (5 ng/ml) added to astrocytes cultured on unmodified nanofibers for 48 h did not further up-regulate FGF-2 (Fig. 8A, B). This is similar to the work of Gómez-Pinilla et al. (1997), where treatment of cerebral cortical astrocytes with 5 ng/ml of FGF-2 for 48 h up-regulated expression of FGF-2 mRNA by only 35%.

2.7. Contribution of secreted FGF-2 to neurite outgrowth promotion

To evaluate the contribution of secreted FGF-2 to promotion of neurite outgrowth, cerebellar granule neurons were plated onto PLL-coated plastic coverslips in unconditioned neuron medium or...
this study, exogenously administered FGF-2 promoted significant functional recovery, albeit through an undetermined mechanism. Moreover, results from do Carmo Cunha et al. (2006) suggested that endogenous FGF-2 up-regulated at the injury site in the contused thoracic spinal cord and soluble FGF-2 or bovine serum albumin administered via an osmotic minipump. As in our study, exogenously administered FGF-2 promoted significant functional recovery, albeit through an undetermined mechanism. Moreover, results from do Carmo Cunha et al. (2006) suggested that endogenous FGF-2 up-regulated at the injury site in the contused spinal cord can induce its own expression in reactive astrocytes, contributing to neuronal protection and plasticity processes as well as wounding events. It therefore remains unclear whether reactive astrocytes have a permissive or an inhibitory phenotype, and whether the growth factor, like FGF-2, is secreted by cerebral cortical astrocytes (Toyomoto et al., 2005). Both FGF-2 and NGF significantly promoted neurite outgrowth in comparison to neuron medium alone (Fig. 9). The antibody had no effect on the extent of neuronal process extension afforded by either neuron medium alone, or by medium supplemented with NGF. However, it abolished neurite outgrowth promoted by FGF-2. These results indicated that the neutralizing actions of the antibody are selective for FGF-2 and further, that the antibody did not have a direct effect on the neurons.

2.8. In vivo considerations

We have recently shown that the FGF-2-modified nanofibrillar fabric employed in this work can be cut into strips and longitudinally bundled with low melting temperature agarose, yielding a multi-layered implant that correctly guided regenerating axons in a targeted fashion across a lesion gap in the over-hemisectioned thoracic spinal cord and significantly improved functional recovery (Meiners et al., in press). This nanofibrillar implant is similar to the one developed concurrently for the repair of tibial nerve injury (Kim et al., 2008). Surprisingly, astrocytes failed to re-populate the FGF-2-modified nanofibrillar scaffold in vivo. Similar results were obtained by Silva et al. (2004), who showed that a self-assembling nanofibrillar scaffold of laminin IKVAV peptide amphiphile molecules selectively induced differentiation of murine neural progenitor cells into neurons while discouraging development of astrocytes. The self-assembling nanofibrillar scaffold, with encapsulated neural progenitor cells and Schwann cells, was subsequently transplanted into the dorsal columns of the rat spinal cord, where it promoted migration of blood vessels and axons into the scaffold (Guo et al., 2007).

On one hand, the lack of astrocyte infiltration into these scaffolds, with their potential to form inhibitory glial scars, could be beneficial to neuronal regeneration and functional recovery. However, given that FGF-2–modified nanofibers promoted a permissive rather than an inhibitory astrocytic phenotype, we hypothesize that polyamide nanofibrillar scaffolds that include astrocytic cells will encourage more robust axonal regeneration and recovery of function in comparison to scaffolds with no astrocytes. It is possible that alteration of certain parameters of the polyamide nanofibrillar fabric, such as fiber diameter or density, would help to encourage astrocyte infiltration into the implant in vivo, or that pre-seeding of the nanofibers with astrocytic cells or stem cells would provide a viable alternative to introduce these supportive cells.

Finally, the role of FGF-2 in promoting reactive gliosis, and indeed, the very definition of reactive gliosis, remains controversial. For example, Eclancher et al. (1996) demonstrated that injection of soluble FGF-2 injected into the cortex, striatum, hippocampus, or corpus callosum of adult rats brain promoted reactive gliosis in comparison to a buffered saline injection, as assessed by an increase in astrocyte proliferation, stellation, and GFAP expression. However, functionality of the reactive astrocytes was not addressed.
the FGF-2-treated astrocytes in this work better fit the profile of reactive or quiescent astrocytes.

2.9. Conclusions

Astrocytes cultured on a synthetic polyamide nanofibrillar matrix that is structurally reminiscent of the basement membrane at the BBB are morphologically and functionally more similar to their in vivo counterparts than astrocytes cultured on a flat plastic surface in terms of stellation, ability to promote neurite outgrowth, and secretion of FGF-2. Covalent modification of the nanofibrillar matrix with FGF-2 increases all of these properties. Importantly, secretion of FGF-2 into the conditioned media was in part responsible for the enhancement of neurite outgrowth by astrocytes plated on FGF-2-modified nanofibers, supporting a role for autocrine actions of FGF-2 in promoting a supportive rather than an inhibitory astrocyte phenotype. These results in turn suggest that FGF-2-modified nanofibrillar implants could be used to replace lost tissue following CNS damage or disease by either encouraging the infiltration of resident astrocytes or by acting as a scaffold to graft stem cells committed to a glial lineage into the injury site, thereby encouraging regrowth of severed neuronal processes.

3. Experimental procedures

3.1. Immunohistochemistry

P8 Sprague Dawley rats (Hilltop Laboratories Animals, Inc., Scottsdale, PA) were transcardially perfused under deep terminal anesthesia (ketamine (100 mg/kg) + xylazine (10 mg/kg), IP) with 4% paraformaldehyde in 0.05 M sodium phosphate buffer. Brains were removed and post-fixed in 4% paraformaldehyde in 0.05 M sodium phosphate buffer overnight. All animal procedures were performed in strict accordance with institutional guidelines (Institutional Animal Care and Use Committee Approval # I07-049-5).

Following post-fixation, the brains were serially immersed for 24 h in each of 3 sucrose solutions (10%, 20%, and 30% sucrose in .05 M sodium phosphate buffer) to ensure cryoprotection. Cerebral cortices were removed and transversely sectioned on a cryostat at 20 μm.

Sections were mounted on plus (+) gold slides (Fisher Scientific, Inc.). They were stained using Hoescht 33258 nucleic acid stain (1:200 dilution for 15 min at room temperature) (Molecular Probes, Eugene, OR) to detect nuclei and double immunolabeled with 1) a mouse monoclonal antibody GFAP (Millipore Corporation, Billerica, MA) (1:300 dilution overnight at room temperature) followed by a CY2-conjugated goat anti-mouse secondary antibody (1:500 dilution for 1 h at room temperature) to detect astrocytes; and 2) a rabbit polyclonal antibody against FGF-2 (Millipore Corporation) (1:3000 dilution overnight at room temperature) followed by a CY3-conjugated goat anti-rabbit secondary antibody (1:500 dilution for 1 h at room temperature) to detect axons. All secondary antibodies were from Jackson Laboratories (West Grove, PA), and all antibodies and Hoescht stain were diluted in phosphate buffered saline (PBS) containing 0.3% Triton X-100. Images of the slides were captured using a Zeiss Axioplan microscope equipped with an epi-fluorescence illuminator.

3.2. Polyamide nanofibers

Randomly oriented polyamide nanofibers (median diameter of ~180 nm) were electrospun from a blend of two polymers [(C28O4N4H47)n and (C27O4.4N4H50)n] onto plastic (Aclar) coverslips by Donaldson Co., Inc. (Minneapolis, MN). They were then cross-linked in the presence of an acid catalyst. The resulting nonwoven polymeric nanofibrillar matrix was approximately 2 μm thick and was interspersed with pores 100–800 nm in diameter (Schinder et al., 2005). The nanofibers were either left unmodified or were covalently coated with a proprietary polyamine polymer using PhotoLink® chemistry by Surmodics, Inc. (Eden Prairie, MN) to provide functional groups for further covalent modification of the nanofibers with bioactive molecules. The unmodified and amine modified nanofibers are commercially available as 96 well plates from Corning Life Sciences (Corning, NY).

A heterobifunctional cross-linker (Sulfo-LC-SDAP (Pierce Biotechnology, Inc., Rockford, IL) was then used to covalently bind the amines on the surface of the nanofibers to cysteines within the sequence of FGF-2 as we have previously described. The amount of FGF-2 bound to the nanofibrillar matrix by this method was found to be approximately 0.125 ng/mm² (Nur-E-Kamal et al., 2008). Importantly, the cysteines are not found within the active site of FGF-2, and the covalent modification procedure does not destroy the activity of the bound growth factor (Nur-E-Kamal et al., 2008).

3.3. AFM analysis

AFM was used to acquire survey and close-up images of the unmodified nanofibers and nanofibers with covalently cross-linked FGF-2. The AFM experiments were performed using a Veeco Instruments Nanoscope IIIa (Veeco Instruments, Woodbury, NY) operated in tapping mode in ambient air (Andrea and Paolo, 2005; Meiners et al., 2007). Other instrumental parameters included the use of an E scanner with a maximum of 13 × 13 μm x-y, 3.6 μm z scan range and etched silicon nitride tips with a nominal 20 nm tip radius of curvature.

3.4. Contact angle measurements

Contact angles were obtained by the sessile drop technique (Allen, 2003) with a Naval Research Lab contact angle goniometer (Rame-Hart, Netcong, NJ). For each condition (plastic, nanofibrillar matrix, and FGF-2-modified nanofibrillar matrix), 3 individual drops of dd H2O (5 μl each) were placed at room temperature on the sample surfaces, and 6 contact angle measurements were taken per drop. Measurements were taken within the first minute of placing the drops on the samples.

3.5. Astrocyte cell culture

Rat cerebral cortical astrocytes were prepared from postnatal day P1 (P1) Sprague Dawley rats and grown to confluence in astrocyte medium in 75 cm² tissue culture flasks as we have done previously (Meiners et al., 1993, 1995). Astrocyte medium was comprised of Dulbecco’s Modified Eagle’s Medium (DMEM, Invitrogen, Carlsbad, CA) + 10% calf serum (Invitrogen). After reaching confluence (~8–10 days), flasks were shaken overnight on a rotary shaker at 37 °C to remove any loosely adherent oligodendrocytes, neurons, or...
macrophages. Astrocytes were then subcultured onto 12 mm plastic coverslips or onto 12 mm plastic coverslips coated with nanofibers or FGF-2-modified nanofibers in 24 wells trays. The subcultured astrocytes were plated in astrocyte medium (0.5 ml) at a density of 50,000 cells/well. Other astrocytes were subcultured onto nanofiber-coated coverslips in astrocyte medium supplemented with soluble FGF-2 (5 ng/ml) instead of onto nanofiber-coated coverslips with covalently cross-linked FGF-2. The astrocytes were maintained for an additional 24 h, at which point they were used for GFAP staining. An additional set of astrocytes was subcultured in the same manner for neurite outgrowth assays, the only exception being that they were initially plated at a density of 200,000 cells/well instead of 50,000 cells/well.

3.6. Neuronal cell culture

Cerebellar granule neuronal cultures were prepared from P8 rat pups as described previously (Mercado et al., 2004), whereas cerebral cortical neurons were prepared from E17 rat pups (Meiners et al., 2001). The pellets containing cerebellar granule or cerebral cortical neurons were resuspended in neuron medium, a mixture of 100,000 cells/ml. Neuron medium for cerebellar granule neurons consisted of neurobasal medium (GIBCO BRL, Rockville, MD) supplemented with B27 (GIBCO BRL) and 25 mM KCl (neuron medium). Neuron medium for cerebral cortical neurons consisted of neurobasal medium supplemented with B27. Neurons were subsequently used for neurite outgrowth assays.

3.7. GFAP immunolabeling of astrocytes

Astrocytes were initially plated at 50,000 cells/well and were fixed with 4% paraformaldehyde in PBS. After fixation, they were immunolabeled with a polyclonal rabbit antibody against GFAP (1:300 dilution overnight at room temperature) followed by a CY3-conjugated goat anti-rabbit secondary antibody (1:500 dilution for 1 h at room temperature). Images of the cultures were captured using a Zeiss Axiosplan microscope equipped with an epi-fluorescence illuminator.

3.8. Astrocyte–neuron co-cultures

Astrocytes were initially plated at 200,000 cells/well. Confluent monolayers were rinsed after 24 h with HBSS (Mediatech, Inc., Herndon, VA). Dissociated cerebellar granule or cerebral cortical neurons (50,000 cells/well) were plated on top of the monolayers and allowed to extend processes for 24 h. Cultures were fixed in 4% paraformaldehyde in PBS, and neurons were immunolabeled with a monoclonal mouse antibody against beta III tubulin from Millipore Corporation (1:100 dilution overnight at room temperature) followed by a CY3-conjugated goat anti-mouse secondary antibody (1:500 dilution for 1 h at room temperature). Four wells were utilized per condition here and elsewhere for neurite outgrowth experiments. Images of the cultures were captured as above, and image analysis was performed as we have described previously (Ahmed et al., 2006) using NIH Image J Software (Abramoff et al., 2004) (available at http://rsb.info.nih.gov). For image analysis, only neurons with processes equal to or longer than the diameter of one cell soma were considered. The length of each primary process and its branches was measured for 50–100 neurons per condition, and the total neurite length for each neuron was calculated as the sum of the lengths of individual neurites.

3.9. Culture of neurons in astrocyte conditioned media

Confluent astrocyte monolayers initially plated at 200,000 cells/well in 24 well trays were rinsed with HBSS, and monolayers were incubated with 0.5 ml/well of neuron medium. Astrocytes on the different surfaces were allowed to condition the medium for 24 or 48 h. Conditioned media samples (0.5 ml) were collected and cerebellar granule neurons were suspended in the medium and plated onto PLL-coated plastic coverslips at a density of 50,000 cells/well. After 24 h neurons were fixed with paraformaldehyde (4%) and immunolabeled with an antibody against beta III tubulin, and neurite outgrowth was analyzed as described above.

4. Western blot analysis

Confluent monolayers of astrocytes established on plastic, nanofibrillar matrices, or FGF-2-modified nanofibrillar matrices were rinsed with HBSS. The monolayers were then incubated with neuron medium (0.5 ml/well) for 48 h. Other monolayers on nanofibrillar matrices were incubated for 48 h with neuron medium supplemented with 5 ng/ml FGF-2. Aliquots of conditioned media (5 μl of media dissolved in 5 μl of Laemmli sample buffer (Laemmli, 1970)) were resolved on SDS-PAGE gels (15%) (Laemmli, 1970) and transferred to nitrocellulose paper (Towbin et al., 1979). The presence of FGF-2 was detected using a monoclonal mouse antibody against FGF-2 (Millipore Corporation) and an enhanced chemiluminescence system, as described (Nur-E-Kamil et al., 2008).

To quantify the relative intensities of the FGF-2 bands, images of the immunoblots were analyzed using NIH Image J Software (Abramoff et al., 2004) according to methods that we have used previously (Meiners et al., 1993; Di Prospero et al., 1997). Within each blot, the level of FGF-2 secreted into conditioned media by astrocytes cultured on plastic was assigned a value of 1. Levels of FGF-2 secreted by astrocytes in the other conditions were then normalized to this value. The data from three independent experiments were pooled.

4.1. Experiments with neutralizing antibodies against FGF-2

Cerebellar granule neurons were plated onto PLL-coated plastic coverslips in neuron medium (0.5 ml/well) or neuron medium conditioned for 48 h by astrocyte monolayers at a density of 50,000 cells per well. They were then allowed to extend processes for 24 h. In some cases, the neurons were first allowed to adhere for 2 h, followed by the addition of a neutralizing polyclonal rabbit antibody against FGF-2 (Invitrogen) (1:1500 dilution) for another 22 h. In order to evaluate the specificity of the antibody against FGF-2, neurons were also cultured in neuron medium containing either FGF-2 (5 ng/ml) (Invitrogen) or NGF (25 ng/ml) (Invitrogen) in the absence or presence of the antibody. After 24 h, all neuronal cultures were fixed, stained, and analyzed for extension of processes as described above.

Acknowledgements

This work was supported by New Jersey Commission on Spinal Cord Research Grants 06A-007-SCR1 and 06-3058-SCR-E-O, and funds from Donaldson Co., Inc., to SM; and by National Science Foundation grant DMI-0400298 to V.A. R.D.-R. gratefully acknowledges the Rutgers-National Science Foundation Integrative Graduate Education and Research Traineeship (IGERT) program on Integratively Engineered Biointerfaces, and D.F. gratefully acknowledges the Michigan- and Research Traineeship (IGERT) program on Integratively Engi-

References

