Spinal motor neurite outgrowth over glial scar inhibitors is enhanced by coculture with bone marrow stromal cells

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Abstract

BACKGROUND CONTEXT: Transplantation of bone marrow cells into spinal cord lesions promotes functional recovery in animal models, and recent clinical trials suggest possible recovery also in humans. The mechanisms responsible for these improvements are still unclear.

PURPOSE: To characterize spinal cord motor neurite interactions with human bone marrow stromal cells (MSCs) in an in vitro model of spinal cord injury (SCI).

STUDY DESIGN/SETTING: Previously, we have reported that human MSCs promote the growth of extending sensory neurites from dorsal root ganglia (DRG), in the presence of some of the molecules present in the glial scar, which are attributed with inhibiting axonal regeneration after SCI. We have adapted and optimized this system replacing the DRG with a spinal cord culture to produce a central nervous system (CNS) model, which is more relevant to the SCI situation.

METHODS: We have developed and characterized a novel spinal cord culture system. Human MSCs were cocultured with spinal motor neurites in substrate choice assays containing glial scar–associated inhibitors of nerve growth. In separate experiments, MSC-conditioned media were analyzed and added to spinal motor neurites in substrate choice assays.

RESULTS: As has been reported previously with DRG, substrate-bound neurocan and Nogo-A repelled spinal neuronal adhesion and neurite outgrowth, but these inhibitory effects were abrogated in MSC/spinal cord cocultures. However, unlike DRG, spinal neuronal bodies and neurites showed no inhibition to substrates of myelin-associated glycoprotein. In addition, the MSC secretome contained numerous neurotrophic factors that stimulated spinal neurite outgrowth, but these were not sufficient stimuli to promote spinal neurite extension over inhibitory concentrations of neurocan or Nogo-A.

CONCLUSIONS: These findings provide novel insight into how MSC transplantation may promote regeneration and functional recovery in animal models of SCI and in the clinic, especially in the chronic situation in which glial scars (and associated neural inhibitors) are well established. In addition, we have confirmed that this CNS model predominantly comprises motor neurons via immunocytochemical characterization. We hope that this model may be used in future research to test various other potential interventions for spinal injury or disease states. © 2014 Elsevier Inc. All rights reserved.
Introduction

Injury to the central nervous system (CNS) usually initiates a poor intrinsic regenerative response for a number of reasons. Immune reactions, which in other tissues may help to recruit reparative cells, often have a devastating effect on CNS tissue function. Inflammation and ensuing secondary cascades can cause extensive neuronal and glial cell death, as well as glial cell activation and hypertrophy [1]. In an effort to restore the blood-brain barrier, astrocytes at the site of injury become reactive and synthesize a proteoglycan-rich matrix [2]. Myelin debris–associated molecules, including Nogo-A and myelin-associated glycoprotein (MAG), are also released from damaged neural tissues [3]. These events combine to produce a hostile environment for nerve regrowth [2–6].

There has been extensive interest worldwide in the development of cell transplantation strategies for the treatment of CNS damage, in particular spinal cord injury (SCI). Many diverse potential cell therapies have been tested, each targeting different distinct stages of SCI and mechanisms of spinal cord repair [7–10]. Allogeneic embryonic stem cells and umbilical cord–derived cells, as well as possible autologous cell sources, including adult neural stem cells, Schwann cells, and olfactory ensheathing cells, have been shown to promote axonal regeneration and restore function in animal models of SCI [11–17]. These types of cell are thought to act in a number of ways depending on the cell type transplanted, including replacing dead or damaged neurons and glia, reestablishing neural networks, remyelinating demyelinated axons, and reducing the hostile nature of the SCI lesion.

Autologous cell therapies derived from bone marrow have also been shown to enhance functional recovery in animal models of SCI and possibly in the clinic [10], but the repair mechanisms responsible are still largely unclear. Some controversial evidence exists which suggests that bone marrow cells, including marrow stromal cell (MSC) and hematopoietic stem cell fractions, may transdifferentiate to replace lost neurons and glia, in a manner similar to that proposed for embryonic stem cells and neural stem cells [18–22]. However, the consensus of opinion seems to be that for MSC transplantation at least, the most likely mode of activity is an induction of a diverse myriad of paracrine anti-inflammatory pathways and directly restorative cell-matrix and cell-cell interactions [23–29].

Previously, we have used growth substrate choice assays to examine how human MSCs influence neurite outgrowths from explants of chick dorsal root ganglia (DRG). We have demonstrated that MSCs help neurites to overcome the effects of some of the major nerve-inhibitory molecules found in SCI lesions, including neural proteoglycans, Nogo-A, and MAG [30]. This established model of sensory nerve growth provided an excellent platform to examine in real-time possible cell-matrix and cell-cell interactions that may occur in the SCI milieu. In the present study, we have adapted and refined our system by replacing DRG explants with spinal cord cultures to provide a more relevant model of CNS nerve growth. We envisage that the establishment of a novel spinal nerve growth substrate assay, which comprises characterized motor neurons and relevant neural matrix molecules, will provide an invaluable research tool for testing SCI therapeutics, which will have further applications in the broader fields of CNS tissue engineering and repair.

Materials and methods

Ethics statement

All research involving human participants was completed with written informed consent and local research ethics committee approval: Shropshire & Staffordshire Strategic Health Authority (reference number: 04/02/RJH). Ethical approval and a Home Office project license for the study were not required under the United Kingdom Animal (Scientific Procedures) Act of 1986 because chicks were killed by decapitation (which is an appropriate method under Schedule 1 of the Act).

Human bone MSC culture

Bone marrow aspirates or bone chips were harvested from the iliac crest of individuals undergoing spinal fusion in the treatment for lumbar degenerative disorders (n = 5; aged 29–53 years). Bone marrow aspirates and bone chips were kindly collected by spinal surgeons from the Centre for Spinal Disorders and sent to the spinal studies research laboratories for processing (both based at the Robert Jones and Agnes Hunt Orthopaedic Hospital Orthopaedic Hospital, Oswestry, UK).

Bone chips were perfused with Dulbecco Modified Eagle’s Medium (DMEM/F12) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (P/S) (Invitrogen Life Technologies, Paisley, UK). Mononuclear cells isolated by density gradient centrifugation at 900 g for 20 minutes over Lymphoprep (Fresenius Kabi Norge AS, Oslo, Norway) were plated out in DMEM/20% FBS+P/S medium (Invitrogen Life Technologies) at a seeding density of 20 x 10⁶ cells per flask. After 24 hours, nonadherent cells were removed, and the adherent cell populations were cultured in monolayer and were maintained in a humidified atmosphere of 5% CO₂ at 37°C through to Passages II and III in DMEM/10% FBS+P/S medium. Marrow stromal cell cultures used in this study were characterized according to the MSC CD immunoprofile criteria published by the International Society for Cellular Therapy [31].

Embryonic chick neuronal cultures

Spinal cords were dissected from Day 4.5 hybrid brown chicks as described previously [32] and cut into 10 to 20
cells were seeded (at a density of 5 × 10³ cells/cm²) in DMEM/10% FBS+P/S into plates coated with nerve-permissive and nerve-inhibitory substrata (see in the following sections). After 24 hours, any nonadherent cells were removed and wells washed repeatedly before adding N2 and bFGF-supplemented NCM. Neuronal cultures were then immediately seeded into each well and the MSC/neuronal cocultures maintained in a humidified atmosphere of 5% CO₂ at 37°C for 72 hours.

Optimization of chick neuronal culture growth substrata

Briefly, some wells were precoated with a thin layer of protein-binding nitrocellulose (BA85; Schleicher & Schuell, Dassel, Germany). Precocated and uncoated plates were then further incubated with either PBS or 25 µg/mL of laminin (derived from Engelbreth-Holm-Swarm mouse tumor; BD Biosciences, Oxford, UK) in PBS. After coating, all wells were washed repeatedly with PBS before seeding with neuronal cultures. Control DRG plates for SC1 immunostaining were established using embryonic chick Day 10 DRG as described previously [30].

Mixed substrate preparation

Neurocan, isolated from embryonic chick brains and purified with a monoclonal antibody (Millipore, Billerica, MA, USA), was used to coat tissue culture plates in restricted localities, as described previously [30,33,34]. Briefly, wells were precoated with a thin layer of protein-binding nitrocellulose (see previous sections), which was then blotted with 350-µm wide strips of filter paper (Whatman No.1; GE Healthcare, Maidstone, Kent, UK) that had been soaked in neurocan at concentrations ranging from 1 to 50 µg/mL (in PBS). After the filter strips had dried and been removed, the plates were then washed with PBS. The restricted localization of the neurocan on the culture plates was visualized by inclusion of a marker dye (5% vol/vol rhodamine B; Sigma-Aldrich) in the neurocan solution. The same technique was used to prepare culture plates with substrates of 10 to 400 µg/mL of recombinant Nogo-A on nitrocellulose or 10 to 400 µg/mL of recombinant MAG on nitrocellulose (both from R&D Systems, Abbingdon, UK). After coating, all wells were washed repeatedly with PBS before seeding with neuronal cultures and/or MSCs.

SC1 and neurofilament 200-kD immunostaining

SC1 is a cell surface adhesion molecule expressed on motor neuron cell bodies and axons [35], which can be used to purify motor neurons from spinal cord tissues [36]. Neuronal cell cultures were immunolabeled with SC1 for motor neuron characterization, whereas neurofilament (NF) immunolabeling was used for neurite quantitation because the NF immunofluorescence was much stronger and hence better for the counting of fine neurite extensions.

Neuronal cell cultures were fixed by gently adding an equal volume of 4% (wt/vol) buffered paraformaldehyde (BDH Prolabo Chemicals, VWR, Lutterworth, Leicestershire, UK) to the culture medium in each well for 10 minutes. Wells were washed with PBS twice for 10 minutes. Cells were then incubated for 1 hour with a blocking buffer of 10% goat serum (Vector Laboratories, Burlingame, CA, USA) in PBS at room temperature. Mouse monoclonal anti-SC1 (neat) (kindly donated by Prof Hideaki Tanaka, Kumamoto University, Japan) or anti-NF (1:200) (clone NE14; Sigma-Aldrich) was used as the primary antibodies, and goat anti-mouse Alexa Fluor 488 (1:100) (Invitrogen Life Technologies) was used as a secondary antibody. Cells were incubated with the primary antibody for 1 hour and the secondary antibody for 40 minutes at room temperature to stain neuronal bodies and their neurites fluorescent green.

Fibronectin and laminin immunostaining

Marrow stromal cell cultures were fixed by gently adding an equal volume of 4% (wt/vol) buffered paraformaldehyde to the culture medium in each well for 10 minutes.
Wells were washed with PBS twice for 10 minutes. Cells were then incubated for 20 minutes with a blocking buffer of 15% horse serum (Vector Laboratories) in PBS at room temperature. Rabbit polyclonal antifibronectin (250 μg/mL) and antilaminin (25 μg/mL) (both, Sigma-Aldrich) were used as the primary antibodies, and biotinylated goat antirabbit (50 μg/mL; Vector Laboratories) was used as a secondary antibody followed by a fluorescein-streptavidin complex (20 μg/mL; Vector Laboratories). Parallel wells were incubated in the same blocking buffer as negative controls for polyclonal antibodies. Cells were incubated with the primary antibody or blocking buffer overnight, the secondary antibody for 40 minutes, and the fluorescein-streptavidin complex for 20 minutes at room temperature.

MSC-conditioned media neuronal culture assays

Marrow stromal cell-conditioned media (CM; n=6) were generated as described previously [30] and stored at −20°C before use. In brief, MSC cultures at 70% confluence were incubated in serum-free DMEM supplemented with antibiotics at 37°C, 5% (vol/vol) CO2 for 48 hours. The MSC-CM generated from these cultures were passed through a 0.2-μm filter (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA) to remove any cell debris and stored at −20°C before use, which was within 1 week of collection. Neuronal cell cultures were seeded in MSC-CM in culture plates that had been uniformly either coated in nitrocellulose or coated with nitrocellulose and strips of neurocan (50 μg/mL) or Nogo-A (400 μg/mL), as described.
previously. Control neuronal cultures were maintained in non-CM under the same conditions. Neurite outgrowth was measured after 72 hours in culture.

**MSC-CM neurotrophic protein arrays**

Marrow stromal cell-CM were screened for a panel of 23 neurotrophic proteins using custom-designed antibody arrays (RayBiotech Inc, Norcross, GA, USA) according to the manufacturer’s instructions. In brief, array membranes with protein antibodies spotted in duplicate were incubated with blocking buffer for 30 minutes at room temperature. Marrow stromal cell-CM were thawed and incubated with the membranes overnight at 4°C. Membranes were washed and then incubated with a biotin-conjugated antibody for 1 hour. Wash steps were repeated as before and membranes incubated with horseradish peroxidase–conjugated streptavidin for 2 hours. Following another series of wash steps, membranes were incubated with a chemiluminescent detection reagent provided in the kit for 2 minutes. Positive signals were visualized with a chemiluminescence imaging system (ChemiDoc EQ; Bio-Rad Laboratories Segré, Italy). Array data were semiquantified by measuring the sum of the intensities of the pixels within each spot boundary × pixel area, with image analysis software (Quantity One version 4.6.3; Bio-Rad Laboratories). A signal from a clear part of the array was subtracted from all data to account for background signal. A mean was taken from the two duplicate spots for each factor. Levels of neurotrophic factors were normalized to positive controls (provided in the kit) and to the number of MSCs that had generated a standard volume of CM.

**Microscopy, image capture, and analysis**

Cultures were viewed using phase contrast and fluorescence microscopy (Nikon Eclipse TS100; Nikon, Kingston-upon-Thames, UK). Digitized images were captured with a black and white Hamamatsu digital camera (C4742-95) and examined using IPLab software (version 3.6; Nikon). For determination of the optimal substrate for growth of embryonic chick spinal neuronal cultures, cell aggregates and neuronal adhesion and neurite outgrowth were counted using phase contrast and fluorescence images. A cell aggregate was determined as a cluster of more than one adhered cell visible under phase microscopy. Neurofilament immunolabeling, visible under fluorescence microscopy, was used to stain neurites and determine those cell aggregates that were of a neuronal phenotype. Hence, those cell aggregates that were immunopositive for NF and possessed neurites (ie, if a neurite ≥ 25 μm in length were in contact with a neuronal cell aggregate) were then described as “neuronal bodies.” For substrate choice assays, the number of neuronal bodies with neurites that had adhered onto substrates of plastic, nitrocellulose, laminin, neurocan, Nogo-A, or MAG were quantified using fluorescent images. For all analysis, adhered neuronal bodies with neurites were counted after 72 hours in culture. The number of red fluorescent MSCs that were present on the nitrocellulose, neurocan, Nogo-A, or MAG substrates in each digitized image was also scored. For substrate choice assay quantitation, results from at least five separate cultures and five separate images per culture were pooled and combined and examined using IPLab software (version 3.6; Nikon).
Marrow stromal cell-CM neurite outgrowth assays were viewed and quantified using phase contrast microscopy and digitized images captured and examined using the Cell-IQ Imagen system and Analyser software (Chip-Man Technologies, Tampere, Finland). In brief, phase contrast images of cultures (n=12 controls and n=24 MSC-CM) were captured using a fully automated system every 2 to 3 hours over a period of 72 hours. From these images, the Cell-IQ Analyser software automated search tool “neurite finder” generated temporal neurite length data for each culture condition.

**Statistical analysis**

The Mann-Whitney U test was used to assess significant differences: between the frequency of neuronal bodies adhered with extending neurites onto uniform substrates of plastic, nitrocellulose, and laminin; between the frequency of neuronal bodies adhered with neurites on plastic, nitrocellulose, and laminin; and between the frequency of neuronal bodies adhered with neurites on nitrocellulose and laminin.

Fig. 3. Neurocan, Nogo-A, and MAG spinal neuronal adhesion and neurite outgrowth assays. (Top, Middle, and Bottom) Digitized images of fluorescence microscopy show NF immunolabeled neuronal bodies and neurites. (Dotted lines illustrate the location of the neurocan, Nogo-A, or MAG borders; calibration bars, 100 μm.) (Top) Neurocan substrates repelled neuronal adhesion and neurite outgrowth in a dose-dependent manner. The difference in the frequency of neuronal bodies with neurites which had adhered to neurocan substrates compared with nitrocellulose was significant at concentrations of 1, 5, 10, and 50 μg/mL (**p<0.059 and ***p<0.0001 Mann-Whitney U test). (Middle) Nogo-A substrates repelled neuronal adhesion and neurite outgrowth in a dose-dependent manner. The difference in the frequency of neuronal bodies with neurites which had adhered to Nogo-A substrates compared with nitrocellulose was significant at concentrations of 50, 100, 200, and 400 μg/mL (***p<0.0001 Mann-Whitney U test). (Bottom) There was no difference in the frequency of neuronal bodies with neurites, which had adhered to MAG substrates compared with nitrocellulose at any of the concentrations tested (10, 50, 100, 200, or 400 μg/mL). Data shown are from at least five separate cultures and five separate images per culture combined ± standard error of the mean. MAG, myelin-associated glycoprotein; NF, neurofilament.
of neuronal bodies adhered with extending neurites onto each of the adjacent substrates in substrate choice assays of nitrocellulose versus neurocan, Nogo-A, or MAG in neuronal and MSC cocultures compared with control neuronal cultures alone; and between the frequency of MSCs adhered onto each of the adjacent substrates in substrate choice assays of nitrocellulose versus neurocan, Nogo-A, or MAG. The relationship between the relative amounts

Fig. 4. In MSC cocultures, the inhibitory effects of neurocan and Nogo-A substrates on neuronal adhesion and neurite outgrowth were reduced. MSC cocultures also enhanced neuronal adhesion and neurite outgrowth over MAG. (A–C) Digitized images of identical fields are shown from left to right under fluorescence microscopy. (Left panels illustrate the location of the neurocan, Nogo-A, or MAG and fluorescently labeled MSC; dotted lines illustrate the location of substrate borders; and right panels show NF immunolabeled neuronal bodies and neurites; calibration bars, 100 μm.). (A) MSC adhesion was reduced on high concentrations of neurocan (10 and 50 μg/mL) compared with nitrocellulose (*p=0.0217 and ***p=0.0001 Mann-Whitney U test). Neuronal adhesion and neurite extension were only inhibited at the highest concentration of neurocan (50 μg/mL) in MSC cocultures (***p=0.0001 Mann-Whitney U test). (B) MSC adhesion was reduced on 400 μg/mL of Nogo-A substrates compared with nitrocellulose (**p=0.0001 Mann-Whitney U test). Neuronal adhesion and neurite extension were only inhibited at the highest concentration of Nogo-A (400 μg/mL) in MSC cocultures (**p=0.0001 Mann-Whitney U test). (C) There was no difference in the frequency of MSC or neuronal bodies with neurites which had adhered to MAG substrates compared with nitrocellulose at any of the concentrations tested (10, 50, 100, 200, or 400 μg/mL). Black arrows indicate colocalization of MSC and spinal neurites and white arrows indicate independent binding of neurites to inhibitory substrates. Data shown are from at least five separate cultures and five separate images per culture combined; ± standard error of the mean. (D) Merged digitized phase and fluorescence images are shown (illustrating the location of MSC-associated laminin or fibronectin, dotted lines illustrate the location of substrate borders; calibration bars, 100 μm). MSCs shown bridging nerve-inhibitory substrata were immunopositive for laminin (right panel) and fibronectin (middle panel). Left panel illustrates negative control staining for polyclonal antibodies. MSC, marrow stromal cell; MAG, myelin-associated glycoprotein; NF, neurofilament.
Fig. 5. Marrow stromal cell-conditioned media (MSC-CM) stimulates spinal neurite outgrowth but not over inhibitory neurocan or Nogo-A substrata. (Top) Representative digitized images of neurite outgrowth over nitrocellulose in control media (top panel) and MSC-CM (bottom panel) under phase contrast microscopy are shown with digitized Cell-IQ "neurite finder" overlays, (calibration bars, 100 μm). Analyzing pooled data (n=6; MSC-CM) demonstrated a marked and significant increase in neurite length after culture in MSC-CM compared with control medium (*p<0.0384, Mann-Whitney U test). (Middle) MSC-CM contained several neurotrophic proteins that were detected using custom-designed antibody arrays. Arbitrary signal intensity readings were normalized to MSC number, data shown are from MSC-CM combined±SEM. (Bottom) MSC-CM was not sufficient stimuli to promote neurite extension over inhibitory substrata of neurocan (top panel) or Nogo-A (bottom panel). Digitized images are shown under fluorescence microscopy show NF-immunolabeled neuronal bodies and neurites. (Dotted lines illustrate the location of the neurocan or Nogo-A; calibration bars, 100 μm.) There was no difference in the frequency of neuronal bodies with neurites which had adhered to nitrocellulose or inhibitory neurocan or Nogo-A substrata in neuronal growth media compared with MSC-CM. Data shown are from at least five separate cultures and five separate images per culture combined±SEM. SEM, standard error of the mean; NF, neurofilament.
of each neurotrophic protein and the total neurite outgrowth in each MSC-CM was determined using the Spearman ranked correlation coefficient $r_s$.

Results

**Nitrocellulose substrates promote optimal growth of embryonic chick spinal neuronal cultures**

Embryonic chick spinal cells formed aggregates that adhered to both plastic and nitrocellulose substrates with or without laminin coating to varying degrees (Fig. 1A). A number of fibroblastic cells adhered to substrates of plastic alone; a small proportion of these cells extended neurites, but these were difficult to distinguish from neighboring aggregates in close proximately. Cells seeded onto substrates of plastic coated with laminin or nitrocellulose formed discrete cell aggregates, and the majority of these aggregates possessed neurites. The frequency of neurites was increased on substrates of plastic coated with nitrocellulose compared with plastic (with or without laminin) (Fig. 1B). For substrates of nitrocellulose-coated plastic with laminin, fibroblastic cells were so confluent they could not be reliably separated for quantitation.

Embryonic chick spinal cell aggregates and neurites were then fixed and immunostained for NF (Fig. 1C). A large proportion of these cell aggregates that had adhered to plastic alone were lost after fixation and immunostaining. The discrete cell aggregates that had adhered to the laminin or nitrocellulose-coated plastic were identified as NF immunoreactive. These NF-immunoreactive cell aggregates that had extended NF-immunoreactive neurites were identified as neuronal bodies. The frequency of neuronal bodies was increased on substrates of nitrocellulose-coated plastic compared with plastic (with or without laminin) (Fig. 1D). For substrates of nitrocellulose-coated plastic with laminin, there was no clear aggregation of NF-immunoreactive cells to form discrete neuronal bodies, with a confluence of cells growing across the substrate instead; hence, the distribution of discrete NF-immunoreactive neuronal bodies with neurites could not be measured.

Embryonic chick spinal neuronal cultures are immunopositive for the motor neuron marker SC1

Embryonic chick DRG cultures were negative for SC1 immunocytochemical staining as were isotype-matched control wells (Fig. 2A and B). In contrast, a large proportion (more than 99%, data not included) of spinal neuronal cultures were immunopositive for SC1 (with the corresponding negative staining of isotype-matched control wells) (Fig. 2C and D). Uniform SC1 staining of spinal neuronal cultures was visible over large regions of interest, demonstrating that almost all spinal neurites visible under phase microscopy were SC1 immunopositive (Fig. 2E). Under high magnification, the numerous cell bodies that contribute to the formation of neuronal bodies were clearly visible. SC1 staining appeared to uniform throughout the main neuronal cell cluster and along each individual neurite (Fig. 2F).

**Embryonic chick spinal neuronal cultures are inhibited by neurocan and Nogo-A but not MAG**

Neuronal bodies with neurites were repelled by neurocan and Nogo-A in a concentration-dependent manner (Fig. 3, Top and Middle). At high neurocan and Nogo-A concentrations (50 and 400 $\mu$g/mL, respectively), neuronal adhesion and neurite outgrowth were almost completely inhibited (less than one neuronal cell aggregate with at least one neurite per image). At lower neurocan and Nogo-A concentrations (1–10 and 10–200 $\mu$g/mL, respectively), increasing numbers of neuronal bodies and neurites adhered to neurocan and Nogo-A substrates and extended neurites. In contrast, neuronal cultures seeded onto nitrocellulose: MAG substrate assays showed no preference for either substrate, that is, neuronal bodies and neurites were not inhibited by MAG at any concentration (5–400 $\mu$g/mL) (Fig. 3, Bottom). No evidence of neuronal cell death (as delineated by cell detachment or ethidium bromide nuclear localization, data not included) was observed in any of the cultures tested.

MSCs promote embryonic chick spinal neuronal adhesion and neurite extension over substrata of neurocan, Nogo-A, and MAG

In MSC/neuronal cocultures, neuronal bodies were able to adhere and extend neurites over high neurocan and Nogo-A concentrations, such that approximately five neuronal bodies with neurites per image were present on 50 $\mu$g/mL of neurocan and approximately three neuronal bodies with neurites per image were present on 400 $\mu$g/mL of Nogo-A. However, the inhibitory effects of neurocan and Nogo-A on neuronal adhesion and neurite outgrowth were only partially abrogated compared with substrates of nitrocellulose. Some of the preseeded MSCs appeared to align at the borders of nitrocellulose with neurocan or Nogo-A, suggesting that these cells were also inhibited by the nerve-inhibitory matrix molecules. However, it was apparent that even at high neurocan and Nogo-A concentrations, some MSCs were still able to adhere to the neurocan and Nogo-A substrates and it was to these MSCs that the adherent neuronal bodies and neurites were often colocalized (Fig. 4A and B). Marrow stromal cells, neuronal bodies, and neurites were not inhibited by MAG at any concentration (5–400 $\mu$g/mL) (Fig. 4C). Nonetheless, neuronal adhesion and neurite extension were increased on all substrates, including neurocan, Nogo-A, MAG, and nitrocellulose when in coculture with MSCs in comparison with the absence of MSCs. In addition, MSCs traversing inhibitory...
substrata were immunopositive for the nerve-permissive matrix molecules laminin and fibronectin (Fig. 4D).

**MSC-CM promotes spinal neurite outgrowth over nitrocellulose but not neurocan or Nogo-A inhibitory substrata**

Marrow stromal cell-CM significantly increased spinal neurite extension over nitrocellulose substrates compared with control cultures in non-CM (Fig. 5, Top). We have detected several neurotrophic proteins in MSC-CM, which may be important in stimulating spinal neurite outgrowth (Fig. 5, Middle). Of the neurotrophic factors identified, the levels of granulocyte colony–stimulating factor, fibroblast growth factor 4, and matrix metalloproteinase (MMP) 8 correlated significantly to the quantity of neurite outgrowth detected (Spearman rank rs, 0.57; *p = .014; rs, 0.57, ***p < .0001; and rs, 0.66, **p = .0032, respectively). However, MSC-CM alone were not sufficient stimulus to promote neurite outgrowth over inhibitory concentrations of neurocan or Nogo-A (Fig. 5, Bottom).

**Discussion**

Marrow stromal cell transplantation for the treatment of SCI has proven efficacious in terms of promoting axonal regeneration and functional recovery in animal models and possibly in the clinic [10]. However, few definitive experiments have addressed the mechanisms involved in this process. We have developed a substrate choice assay to examine how spinal nerves interact in coculture with MSCs, specifically in the context of molecules that are present at the site of SCI and that are considered to form major inhibitors to axonal regeneration. Using this model, we have shown that spinal neuronal bodies and neurites are inhibited by neurocan and Nogo-A in a concentration-dependent manner, akin to DRG sensory neurites, which we have reported previously [30]. Increased concentrations of these extracellular inhibitors, however, were required to observe a similarly “complete” inhibition, for example, 50 μg/mL of neurocan and 400 μg/mL of Nogo-A completely inhibited spinal neurites compared with 10 μg/mL of neural proteoglycans (which includes neurocan) and 200 μg/mL of Nogo-A for the complete inhibition of DRG neurite outgrowth. However, unlike DRG sensory neurites, spinal cultures were not inhibited by MAG substrates at any of the concentrations tested (up to 400 μg/mL). Hence, using this CNS model, we have shown that one of the proposed inhibitors in the glial scar (MAG) may not be as potent in CNS as it is in DRG systems, which may have important implications for our understanding of nerve growth inhibition in the SCI setting. There is some supportive evidence in the literature for these findings, which suggest that MAG may not be a crucial inhibitor of axonal regeneration in the CNS. For example, Bartsch et al. [37] have shown that MAG-deficient mice exhibit poor axonal regrowth after either optic nerve or corticospinal tract transection in vivo, although MAG has been shown by others to repel both peripheral nervous system (PNS) and CNS nerve growth [30,38,39].

There are a number of distinctions between these neuronal cultures, which might account for the differences we have observed in their response to substrate choice assays compared with those results previously reported. The most obvious is the developmental stage of each tissue source; in the present study, spinal cultures were isolated 4.5 days after fertilization compared with our previous work using DRG explants from Day 10 embryos [30]. The expression of axonal guidance ligands and receptors, including myelin receptors, is known to change throughout CNS and PNS development [40–44], which may explain why spinal and DRG cultures exhibit different sensitivities to MAG. In addition, both our current and previous methods of primary neuronal culture isolation included few (if any) purification steps, and hence, these cultures are composed of mixed cell populations. We are in the process of characterizing those “fibroblast-like” cells visible in CNS and PNS cultures, which are likely to have influenced the sensitivity of neuronal cultures in substrate “choice” assays. There is a possibility that other CNS cell types may have reduced the sensitivity of spinal neurites to MAG substrates, perhaps by physically masking or blocking inhibitory epitopes or by secreting growth factors that blocked the inhibitory effects of MAG, for example, brain-derived neurotrophic factor [45]. In contrast, Schwann cells, which may be present in mixed PNS cultures, could exacerbate sensory nerve reactivity to MAG via an additive effect as Schwann cells themselves express nerve-inhibitory MAG [46]. Furthermore, each culture environment varies greatly in media composition and growth factor supplementation, which may also impact directly on the sensitivity of neurites to inhibitory substrates, including MAG [45,47]. For example, the exposure to neurotrophins has been shown to upregulate chimaerin (one of the Rho-GTPase–activating proteins) in cerebellar neurons [48]. The expression of chimaerin in the cerebellum is correlated with abolishment of the inhibitory effects of MAG in development and ectopic expression of chimaerin in cerebellar neurons in vitro results in resistance to MAG-induced neurite inhibition [48].

We have demonstrated that MSC coculture reduces the inhibitory effects of neurocan and Nogo-A on spinal neuronal adhesion and neurite outgrowth and enhances spinal neurite outgrowth over all the substrates tested (neurocan, Nogo-A, MAG, and nitrocellulose). We have also shown that MSCs were repelled by high concentrations of neurocan and Nogo-A (but not MAG substrates). Hence, at high concentrations, MSCs could clearly be seen to align along inhibitory neurocan and Nogo-A borders, although MSCs were inhibited to a much lesser extent than spinal neuronal bodies and their associated neurites. This is not too surprising as we already know that MSCs may have an increased capacity to adhere to and migrate over neural
proteoglycans, Nogo-A, and MAG compared with other cell types [30]. The exact mechanisms responsible for the abrogation of spinal nerve inhibition to neurocan and Nogo-A in MSC cocultures may involve a number of complex paracrine, cell-matrix, and cell contact–mediated interactions. We and others have previously reported that MSC-CM promote neurite outgrowth from DRG explants and that MSCs synthesize a number of soluble cytokines and other growth factors that are known to stimulate nerve extension, including nerve growth factor, brain-derived neurotrophic factor, and vascular endothelial growth factor [26,30,49]. In this study, we have shown that MSC-CM promote spinal neurite outgrowth and contains several neurotrophic proteins, including granulocyte colony–stimulating factor, fibroblast growth factor 4, and MMP-8 which significantly correlated to the level of spinal neurite stimulation observed. However, we show that MSC-CM alone were insufficient stimuli to promote spinal neurite extension over inhibitory concentrations of neurocan or Nogo-A.

There are other explanations that might account for spinal neurites extending over inhibitory substrates in MSC cocultures. Marrow stromal cells are known to synthesize numerous extracellular matrices that support neuronal cells and provide an optimal surface for nerve growth [50]. We have shown using our model that migrating MSCs provide permissive matrix “bridges” of laminin and fibronectin over nerve-inhibitory substrates. In addition, in many sequences and on all inhibitory substrata tested, MSCs and spinal neurites colocalized, whereby MSCs appeared to act as adhesive “stepping stones” for neurite extension. Alternatively, nerve-inhibitory molecules, particularly neural proteoglycans such as neurocan, may have been degraded by MMPs, for example, MMP-1, MMP-2, and MMP-13, which MSCs are known to synthesize [51]. We have previously demonstrated that cell contact-mediated events, such as towing of neurites and bridging of inhibitory substrata, may play an important role in MSC abrogating the DRG nerve-inhibitory effects of neural proteoglycans, Nogo-A, and MAG [30]. Further experimentation using this system will aim to elucidate which of these mechanisms contribute to MSC stimulation of spinal neurite outgrowth over neurocan and Nogo-A and to what extent. This may help to identify molecular targets to further enhance nerve growth in SCI environments.

There are few primary motor neuron culture protocols available for scientists to examine new therapies for CNS repair, particularly in the context of the injured spinal cord. We have modified an existing protocol [32] to test embryonic motor neurons, as characterized by SC1 staining, to examine motor neuron and glial cell-matrix and cell-cell interactions, not readily achieved when using complex in vivo models. We anticipate that this novel system may help to further elucidate some of the mechanisms of increased axonal regeneration that has been noted after MSC transplantation for the treatment of SCI and having wider application in the field of spinal therapeutics.

References


