On day 10 after surgery, 5 animals from the laminectomy only and plain SCI groups were deeply anesthetized with barbiturate overdose and intracardially perfused with PBS. Their spinal cords were removed and immediately frozen in Tissue-Tek OCT medium (Sakura Finetek, Tokyo, Japan). The spinal cords were sectioned in the sagittal plane onto uncoated slides. A PixCell II LCM instrument (Arcturus, Mountain View, CA) was used to dissect the injury site, and RNA was extracted from the microdissected samples using the PicoPure RNA Isolation Kit (Arcturus), according to the manufacturer's instructions. Total RNA was pooled from the 5 animals from each group and amplified and labeled using the Amino Allyl MessageAmp aRNA Kit (Ambion,
Austin, TX). Briefly, after reverse transcription (2 mg total RNA/sample), double-stranded cDNA was transcribed in vitro using the amino allyl cRNA. The RNA was amplified twice, and the purified and concentrated cRNA (5 mg) was coupled with either Cy3 or Cy5 dyes (GE Healthcare). The dye-labeled aRNA was purified from uncoupled dye using Micro Bio-Spin P-30 Tris chromatography columns (Bio-Rad, Hercules, CA) and Microcon YM-30 centrifugal filter devices (Millipore, Billerica, MA). The cRNA was fragmented in a fragmentation buffer (40 mmol/L Tris acetate (pH 8.1), 100 mmol/L potassium acetate, and 30 ⌈mol/L magnesium acetate) at 94°C for 15 min and purified with Microcon YM-10 (Millipore). An oligonucleotide-based mouse DNA microarray, AceGene (mouse Oligo Chip 30K; DNA Chip Research, Yokohama, Japan) was preblocked with 1% bovine serum albumin (BSA) solution. The fragmented cRNA was added to the microarray in hybridization solution and subsequently hybridized at 42°C for 16 h. The arrays were then washed, scanned at a pixel size of 10 mm, gridded, and analyzed (GenePix 4000B; Axon Instruments, Union City, CA). The background was subtracted, and the medium sum intensity (CH1 and CH2) of <100 absorbance units was excluded. Data were normalized by the trimmed mean at 10% to account for the differences in the amounts of labeled RNA or labeling efficiencies.

Pathway analysis

We next attempted to identify the biological pathways involving novel gene networks that are activated in reactive astrocytes after SCI. For this purpose, we analyzed the genes whose expressions were upregulated in the lesion by at least a factor of 2.0, relative to their respective expressions in the normal spinal cord. The functional mapping tool MetaCore (GeneGO, St Joseph, MI) was used in this analysis. MetaCore is a web-based computational platform designed primarily for the analysis of high-throughput experimental data in the context of mouse regulatory networks and pathways. It includes a curated database of protein interactions, metabolism, and bioactive compounds. For a network of a particular size, MetaCore can be used to calculate statistical significance based on the probability of the network's assembly from a random set of nodes (genes) that is of the same size as the input list (p value).

In vivo migration of DiI-labeled NSCs

F3.LacZ cells were prelabeled by incubating for 20 min in culture medium containing Cell Tracker CM-DiI (Invitrogen). We evaluated whether DiI-labeled NSCs could migrate to the SCI site in adult mice after intravenous transplantation. Seven days after the induction of transection injury, the injured animals were injected via the tail vein with 2×10^6 DiI-labeled F3.LacZ NSCs in 100 µL of PBS. The control injured animals were injected with PBS alone. Seven days after NSC transplantation, the animals were euthanized and transcardially perfused with 4% paraformaldehyde (PFA).

RT-PCR for IFN-β and TLR-2 and -4 expression at the SCI site

The total RNA extracted from the harvested tissues was subjected to DNase (Invitrogen) treatment prior to reverse transcription using a Transcriptor First-Strand cDNA Synthesis Kit (Roche, Mannheim, Germany), according to the manufacturer's protocol. PCR amplification

was performed using GoTaq DNA polymerase (Promega, Madison, WI) with the following primers: human IFN-β, 5′-GCCGCATTGACCATCTATGAGA-3′ (sense) and 5′-GAGATCTTCAGTTTCGGAGGTAAC-3′ (antisense); mouse TLR-2, 5'-ACAGCTACCTGTGTGACTCTCCGCC-3' (sense) and 5'-GGTCTTGGTGTTCATTATCTTGCGC-3' (antisense); mouse TLR-4, 5'-ACCTGGCTGGTTTACACGTC-3' (sense) and 5'- CAGGCTGTTTGTTCCCAAAT-3' (antisense); mouse β-actin, 5'-GACATGGAGAAGATCTGGCACCACA-3' (sense) and 5'-ATCTCCTGGTCGAAGTGTAGAGCAA-3' (antisense); human and mouse GAPDH (product length; 454 bp), GACCACAGTCCATCGCATCA (sense) and GTCCGCCACCCTGTTGCTGT (antisense).

Immunohistochemistry

The mice were deeply anesthetized with an overdose of barbiturate and intracardially perfused with PBS followed by 4% PFA in PBS. The lesioned spinal cord region was removed and postfixed overnight in the same fixative. The fixed tissues were embedded in paraffin, and 3-μm sections were prepared for immunohistochemistry. Immunohistochemical staining was performed as per the manufacturer's instructions. Briefly, sections were preblocked with the blocking reagent (DAKO REAL Peroxidase-Blocking Solution, DAKO), and antigen retrieval was performed for GFAP. After sections were stained with rabbit anti-GFAP (DAKO), mouse anti-neurofilament (NF) (Nichirei, Tokyo, Japan), and a polymer reagent (Chemomate Envision kit/HRP, DAKO), color was developed with diaminobenzidine (DAB). Finally, sections were

counterstained with hematoxylin. The corresponding tissue sections were routinely stained by hematoxylin and eosin for morphological evaluation. Five areas of immunostained sections were randomly selected, and the color intensity was measured using NIH image software as reported(44). Background intensity was subtracted from the mean intensity of the images, and the means were used for statistical analysis.

Basso Mouse Scale for locomotion

The recovery of hindlimb motor function in 7 mice in each treatment group was measured using the BMS for locomotion, as previously described(2). The behavior of each animal was videotaped for 5 min, and 2 investigators provided a score (on a scale of 0–9) for each hindlimb at 1, 2, 3, 4, 6, and 8 weeks after injury. The scores from the 2 hindlimbs were averaged to obtain a single value per animal for each time point.

Inclined plane test

We constructed an inclined plane as previously described(36), and tested the ability of each animal to maintain its position on the incline at 1, 2, 3, 4, 6, and 8 weeks after injury. The maximum inclination at which a mouse could maintain itself for 5 s was recorded; this value was considered to represent the functional ability of the mouse. In practice, the angle was either increased or decreased by 5° intervals until the mouse could maintain its position on the inclined plane for 5 s without falling.

Electrophysiology

To measure signal conduction in mouse motor pathways following SCI, transcranial electrical MEPs were measured at 4 and 8 weeks after injury. To record MEPs, mice were anesthetized with 1.5% halothane and maintained in 1.0% halothane in oxygen. All recordings were performed using standard clinical electromyographical analysis with a 3000-Hz hi-cut filter and a 30-Hz low-cut filter (Nihon Kohden, Tokyo, Japan). Electrical stimulation (1 Hz, 15 mA) was delivered over the cranium at an area 1 mm lateral to bregma. Evoked responses were recorded with needle electrodes from the contralateral femoral muscle. Response amplitudes were measured from base to peak.

In vivo MRI

To investigate the status of injured neural tissue at the SCI site in a noninvasive manner, MRI was performed using 7.0-T MRI (superconducting magnet: Kobelco and Jastec, Japan; console: Avance-I system, Bruker BioSpin, Germany), with a volume coil for transmission (Bruker BioSpin), and 2-ch phased array coil for reception (Rapid Biomedical, Germany) dedicated to examining spinal cord lesions in live mice. DTI, and T2-weighted (T2WI) MRI were conducted 4 weeks after injury in mice assigned to the PBS, F3.LacZ, and F3.CD.IFN groups. DTI data sets were acquired with a respiratory-gated gradient-echo echo-planar diffusion-weighted pulse sequence, based on the Stejskal-Tanner diffusion preparation(41). Scanning parameters of the DTI were as follows: repetition time (TR), 3000 ms; echo time (TE), 34 ms; flip angle, 90°; field of view (FOV), 25.6 × 12.8 mm; data matrix, 128 × 128; reconstructed image resolution,
200 × 100 μm^2; slice thickness, 1.0 mm; b-value, between 0.68 and 1000 s/mm^2; motion-probing gradient (MPG) orientations, 30 axes and 5 B0 images; number of averaging (NA), 1; number of EPI segment, 4; fat saturation pulse, on. T2WIs were acquired through rapid acquisition with relaxation enhancement (RARE) sequence as follows: TR, 4200 ms; TE, 12 ms; effective TE, 36 ms; FOV, 25.6 × 12.8 mm; data matrix, 256 × 128; reconstructed image resolution, 100 × 100 μm2; slice thickness, 1.0 mm; fare factor, 8; NA, 8; fat saturation pulse, on. Mice were placed under anesthesia with a mixture of 1.5% isoflurane, 40% oxygen, and 58% air delivered via tracheal intubation prior to T2WI and DTI analysis. Vital signs (respiration, heart rate, and body temperature) of the anesthetized animals were monitored using a MRI-compatible monitoring and gating system (Model 1025; SA Instruments Inc., Stony Brook, NY). Respiratory-gated acquisition was used to increase the image quality by minimizing breathing-related image artifacts whenever necessary. To reduce motion artifacts, animals were immobilized on an acrylic bed, and a specially designed head positioner (Rapid Biomedical) for gated imaging was attached to each animal's front thorax. Diffusion tensor analysis was performed using ParaVision with Jive software (Bruker BioSpin). Three

eigenvectors (e1, e2, e3) associated with the largest eigenvalue (λ1) were visualized as a color map (left-right, red; posterior-anterior, green; superior-inferior,blue) (34).

Statistical analysis

The statistical significance of observed differences was determined by ANOVA (StatView; SAS Institute, Cary, NC), and Bonferroni's correction was employed for multiple comparisons. All reported P-values are two-tailed; a value of $P < 0.05$ was considered statistically significant.

Results

Molecular signature of reactive astrocytes following SCI

A laser-captured microdissection technique was used to procure specific areas from each heterogeneously damaged tissue structure. In order to analyze the molecular signaling of reactive astrocytes following SCI, the sites of lesions that were expected to form a glial scar were captured, and a microarray was used to profile the expression of 30,000 genes on day 10 after dorsal hemisection of the spinal cord at the T9–T10 level. We evaluated gene expression as either significantly upregulated or significantly downregulated by using the fold-level criteria (>2-fold change: the significance of upregulation was set at >2 and that of downregulation at <0.5). Data analysis identified 488 significantly upregulated genes and 226 downregulated genes in several different categories. Complete lists of genes demonstrating significant expression changes are listed in Supplementary Table 1. Only 1.6% of the probe set corresponding to individual genes was expressed at a level >2.0-fold after SCI. After the microarray data were imported into MetaCore, functional gene networks were generated to integrate reactions and interactions around the identified genes of interest. Significantly activated pathways are displayed in Fig. 1A ($p < 0.05$). In this study, we focused on TLRs

that may be related to IFN-β as reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative RT-PCR revealed that TLR-4 was markedly upregulated in the lesioned astrocytes (Fig. 1B).

F3 cells migrate to SCI lesion sites in mice

We evaluated the efficient migration of intravenously injected F3 NSCs tagged with DiI or lacZ to SCI sites in mice (Fig. 2A). It was revealed that a large number of DiI-labeled F3 cells migrated to the lesion site (Fig. 2B), and immunohistochemical staining with anti-β-galactosidase (β-gal) antibody demonstrated that β-gal-positive cells were also extensively distributed in the lesioned area (Figs. 2C, D). All NSCs were found to be located in or around the injured parenchyma, indicating that there was specificity of migration. Conversely, in the control injured animals injected with PBS alone, no β-gal-positive cells were observed in the spinal cord (Figs. 2E, F). IFN-β expression was also detected at the SCI site of the animals injected with 2×10^6 F3.CD.IFN by RT-PCR (Fig. 2G).

F3.CD.IFN cells inhibit growth of primary astrocytes, but not TLR-4-deficient astrocytes

To quantify the inhibitory effect of F3.CD.IFN on astrocyte growth, DiI-labeled F3 cells were co-cultured with primary normal astrocytes in different ratios. As shown in Fig. 3A and B, the number of astrocytes significantly decreased with a reduction in the astrocyte:F3 ratio in the F3.CD.IFN group. When they were co-cultured with F3.CD.IFN with added 5-FC (F3.CD.IFN + 5-FC), there was no growth inhibitory effect on cultured astrocytes. To evaluate whether TLR-4 is necessary for IFN-β to exert this inhibitory effect on astrocyte growth, DiI-labeled NSCs were also co-cultured with astrocytes derived from TLR-4-deficient mouse in 40:1 ratio. As shown in Fig. 3C and D, the number of TLR-4-deficient astrocytes did not change. Thus, the inhibitory effect by the F3.CD.IFN cells was not exerted in the absence of TLR-4.

Intravenous administration of F3.CD.IFN-β NSCs spares host neural tissue and enhances axonal regeneration and TLR-4 blockade cancels regeneration

To determine the effects of the intravenous administration of F3.CD.IFN cells on neuronal regeneration and the inhibition of glial scar formation, the expression of glial fibrillary acidic protein (GFAP) and neurofilament (NF) at the injury site was immunohistochemically evaluated. SCI was shown to result in a severe loss of neurons at the injury site, as well as the extensive growth of reactive astrocytes forming a glial scar. Hematoxylin and eosin (HE) staining revealed severe destruction of the dorsal half of the spinal cord above the central canal (Fig. 4A), and the formation of a granular scar was clearly detected at the injury site. Immunohistochemical staining for GFAP showed hypertrophy of GFAP-positive reactive astrocytes even in the area ventral to the central canal (Fig. 4B). The intensity of GFAP staining was significantly decreased in the F3.CD.IFN-treated group as compared to that observed in the other groups. GFAP density was calculated quantitatively using NIH imaging (mean ± SEM): sham group, 17 ± 0.5; PBS, 38 ± 2.9; F3.LacZ, 35 ± 2.3; F3.CD.IFN, 28 ± 1.2; F3.CD.IFN + 5FC, 35 ± 1.1 (Fig. 4C). In the PBS and F3.LacZ groups, NF-stained transverse sections demonstrated significant loss of neural fibers extending to the ventral side (Fig. 4D), while only partial preservation of neural axons was detected in the F3.CD.IFN + 5FC group. Compared to these findings, sections from the F3.CD.IFN group revealed the dramatic preservation of fiber number and alignment, which was confirmed by the quantification of NF density: sham, 31 ± 0.4; PBS, 21 ± 1.5; F3.LacZ, 21 ± 1.3; F3.CD.IFN, 30 ± 1.8; F3.CD.IFN + 5FC, 24 ± 3.5) (Fig. 4E). Next, the expression of GFAP and NF at the injury site was compared in mice treated with F3.CD.IFN in the presence or absence of the TLR-4 inhibitor OxPAPC. While the dorsal half of the spinal cord was destroyed equally in every group, the anterior half of the spinal cord was found to be markedly preserved in the F3.CD.IFN group when compared to the F3.CD.IFN+OxPAPC group (Fig. 5A). The intensity

of GFAP staining was also significantly decreased in the F3.CD.IFN group as compared to that observed in the F3.CD.IFN+OxPAPC and F3.LacZ groups (Fig. 5B). NF-stained transverse sections also demonstrated a significant loss of neural fibers in the F3.CD.IFN+OxPAPC and F3.LacZ groups, while neural fiber was dramatically preserved in sections from the F3.CD.IFN group (Fig. 5C).

Behavioral studies

To determine whether F3.CD.IFN administration improved motor function after SCI, we assessed functional recovery using two independent behavioral tests: the Basso mouse scale (BMS) and the inclined plane test. Animals were evaluated for locomotor recovery at 1, 2, 3, 4, 6, and 8 weeks after injury.

One week after SCI, on the day of PBS or NSC administration, all injured animals exhibited a mean BMS score of <3, indicating extensive ankle movement without stepping ability. Animals with SCI receiving only PBS (PBS group) or F3.LacZ.NSCs (F3.LacZ group) spontaneously recovered to a BMS score of 3–4 (plantar placing of the paw or occasional plantar stepping) by 4 weeks after injury, and further recovery was limited. Animals treated with F3.CD.IFN followed by 5-FC administration (F3.CD.IFN + 5-FC group) had slightly better outcomes. In contrast, mice injected with F3.CD.IFN in the absence of 5-FC (F3.CD.IFN group) showed significant improvement in BMS scores compared to the other 3 groups, whereby the F3.CD.IFN group exhibited a mean BMS of 4.9 ± 0.5 at 4 weeks, and the PBS, F3.LacZ, and F3.CD.IFN + 5-FC groups attained mean scores of 3.7 ± 0.4, 3.6 ± 0.5, and 4.0 ± 0.5, respectively. Differences between the mean BMS scores of the F3.CD.IFN group and the other groups were statistically significant 4 weeks after injury and thereafter ($p < 0.05$) (Fig. 6A).

The inclined plane test was performed to examine additional post-SCI locomotor function. There was significant recovery of performance 2 weeks following the injury in the F3.CD.IFN

group, whereas the performance of the other 3 groups remained severely impaired ($p < 0.05$). None of the groups recovered to pre-injury levels of performance (Fig. 6B). Finally, the improvements in the BMS scores and inclined plane test results that were observed in the F3.CD.IFN group were shown to be inhibited by the OxPAPC TLR-4 inhibitor (Fig. 6C, D).

Electrophysiology

The mean motor nerve evoked-action potentials (MEP) amplitude observed in the sham operation group was approximately 600 μV at both 4 and 8 weeks after injury. This amplitude was reduced to a range of approximately 150–200 μV in the PBS, F3.LacZ, and F3.CD.LacZ+5-FC groups at 4 and 8 weeks, while animals in the F3.CD.IFN group showed significantly better recovery, to approximately 300 μV at both 4 and 8 weeks after injury ($p < 0.05$) (Fig. 7A).

However, OxPAPC administration in the F3.CD.IFN group significantly worsened the mean MEP amplitude to 178 μV and 228 μV at 4 and 8 weeks, respectively ($p < 0.05$)(Fig. 7B).

Assessment of regenerative sprouting of CST axons by 7-T MRI

Four weeks after dorsal hemisection, in vivo proton diffusion tensor imaging (DTI) was performed for noninvasive evaluation of regenerative axonal sprouting (Fig. 8). We visualized the diffusion tensor of the thoracic spinal cord. The eigenvectors associated with the tract were depicted as colors according to their orientation: blue illustrates a longitudinal (superior-inferior) orientation. The continuity of this longitudinal tract demonstrated functional regeneration of the axons. DTI was performed 4 weeks after the injury in mice that received PBS, F3.LacZ, or F3.CD.IFN. In the PBS and F3.LacZ groups, the injury site and its surrounding area displayed high signal intensity in T2-weighted sagittal sections, indicating

spinal cord edema or necrotic tissue alteration, whereas this high intensity was not detected in the F3.CD.IFN group. The DTI of the injured spinal cord in the PBS and F3.LacZ groups revealed discontinuity of longitudinal fibers, which were mostly visible in the F3.CD.IFN group.

Discussion

In the present study, we transplanted intravenously human NSCs transduced with IFN-β gene in SCI mice and the animals exhibited extensive suppression of glial scar formation and preservation and/or regeneration of nerve fibers in the lesioned spinal cord. Significant neurobehavioral and electrophysiological recovery was attained, as measured by the Basso mouse scale for locomotion, inclined plane test and transcranial MEPs. Evaluation with 7.0-T MRI also confirmed axonal regeneration. Systematic evaluation of spinal cord 10 days after SCI with gliosis-specific microdissection, genome-wide microarray and MetaCore pathway analysis revealed the upregulation of TLR-4 in the spinal cord lesion site. We therefore focused on the functional role of TLR-4 signaling in reactive astrocytes, as there is a strong relationship between TLR-4 and IFN-β in glia (8). TLR-4 in the presence of IFN-β was shown to suppress the SCI-induced proliferation of reactive astrocytes within lesion site. Furthermore, as mice that were administered with IFN-β-expressing NSCs and TLR-4 inhibitor simultaneously gained less functional recovery than mice in which only IFN-β-expressing NSCs were injected, we have verified that TLR-4 plays an extremely important role in the effects of IFN-β during astrocytic gliosis. These results suggest that IFN-β delivery by intravenous injection of genetically engineered NSCs is capable of inhibiting glial scar formation after SCI, and promotes functional recovery in the presence of TLR-4.

Three different delivery methods can be considered for SCI cell therapy: intralesional, intrathecal, and intravenous. A cardinal feature of NSCs is their exceptional migratory ability, which has led to their emergence as a therapeutic paradigm in various animal models of neurodegeneration, stroke, and brain tumors (23,24,28,47). Previously, we and others have demonstrated that the transplantation of genetically engineered F3 human NSCs into sites of SCI lesions induces functional improvements in rat SCI models (10,17,18,26). We have also demonstrated that there is

glial and neuronal differentiation of NSCs, indicating that intravenous NSC delivery activates the microenvironment of the injured spinal cord to stimulate self-regeneration (44). Thus, intravenous injection of human NSCs is a promising tool for replacing depleted cells or for delivering therapeutic genes in the treatment of SCI, with the potential to be used in clinical applications. We have also demonstrated that intravenously injected F3 human NSCs migrate preferentially to the SCI site (44). As a novel strategy for SCI treatment, we combined these two studies and examined whether functional recovery and axonal regeneration could be achieved through the intravenous administration of IFN-β-expressing NSCs. F3 NSC is a clonally isolated, multipotent human neural stem cell line that has the ability to self-renew, stably express genes, and

differentiate into neurons, astrocytes, and oligodendrocytes (21). F3 NSCs have previously been used in a clinical trial for gene therapy in glioma patients (A pilot feasibility study of oral 5-Fluorocytosine and genetically-modified neural stem cells expressing E Coli cytosine deaminase for trearment of recurrent high grade glioma-City of Hope National Medical Center, Duarte, CA, USA, June 2010). Inhibition of glial scar formation is a promising approach for the treatment of SCI. Recent studies have focused on the multifaceted roles of astrocytes in response to SCI.

It has previously been demonstrated that selective depletion or ablation of a subpopulation of reactive astrocytes causes widespread tissue disruption and pronounced cellular degeneration (6). Another study has reported that the emergence and migration of reactive

astrocytes are beneficial in limiting inflammatory cell infiltration in the subacute phase of injury, before glial scar completion (33), while others have indicated that the elimination of reactive astrocytes is beneficial for SCI recovery at the subacute stage (37). Considering the above-described contradictory roles of reactive astrocytes after SCI, an ideal treatment strategy may involve inhibiting the aberrant development of hypertrophic reactive astrocytes without eliminating normal quiescent astrocytes, thus preventing a cascading wave of uncontrolled tissue damage. Based on our findings, the timing of NSC transplantation is extremely important. On the basis of our previous studies regarding glial scar formation and effective NSC migration (44), we have determined that the optimal timing of intravenous NSC injection is 7 days after injury.

Critical role of TLR-4 in the effect of NSCs expressing IFN-β

We have previously been successful at restoring functional recovery in a mouse model of SCI by using a liposome-mediated IFN-β gene delivery method (12). IFN-β is a type I IFN that exerts pleiotropic biological effects (3), and acts as a cell-cycle regulator to control the re-entrance of cells undergoing aberrant cell-cycle progression into a senescence-like state (15). Recently, it has been proven that IFNs block the constitutive activation of the MEK-ERK signaling pathway (31,38,39,48), which is activated at the site of SCI (12). IFN-β also blocks the infiltration of neutrophils and proinflammatory cytokines into sites of injury and stimulates the expression of anti-inflammatory cytokines (7). In order to clarify the novel function of IFN-β in the suppression of glial scar formation in this study, we attempted to identify the profile changes in gene expression that occur during astrocytic gliosis. This systematic processing revealed the statistically significant activation of many intriguing pathways ($p < 0.05$) (Fig. 1). Of these, the TLR-4 pathway, which plays an important role in immune regulation in astrocytes (8) and autoregulatory apoptosis in cells bearing the TLR-4 receptor (14,16), was of particular note. TLR-4 can be detected in astrocytes in sites of SCI (16). The

autoregulatory apoptosis of activated astrocytes is known to be initiated by TLR-4 and proceeds by at least 2 pathways, one of which is IFN-β activation (14). Indeed, TLR-4 ligation is essential for the autoregulatory apoptosis of cells bearing this receptor, which ultimately acts to regulate glial scar formation (16). This function requires the presence of IFN-β. Furthermore, TLR-4 deletion significantly impairs the normal progression of SCI repair and functional recovery (16). Our results demonstrate that TLR-4 inhibition results in impaired functional recovery despite the administration of F3.CD.IFN NSCs. Therefore, we can conclude from our findings that the combination of TLR-4 and IFN-β plays a crucial role in regulating post-SCI inflammation and gliosis.

Cytosine deaminase gene as a fail safe modulator of uncontrolled proliferation of NSCs

The CD gene examined in this study encodes bacterial enzyme that catalyzes the deamination of nontoxic 5-FC into the highly toxic 5-fluorouracil (FU). We have previously reported improved outcomes in animal models of brain tumors after therapy with this "suicide gene" (11,13,20,40). We therefore applied this strategy in SCI models to investigate the additive efficacy of NSC-delivered CD and IFN-β. NSCs have the ability to migrate to sites of SCI, at which they induce the apoptosis of the surrounding reactive astrocytes via the bystander effect. Thus, we anticipated that the combination of the actions of IFN-β with the 5-FC/CD-mediated bystander effect would exert greater suppression of reactive astrocytes than either treatment alone. However, this combination therapy was not found to be superior to IFN-β alone in vivo or in vitro in this study. Rather, our findings show that the addition of 5-FC to astrocytes treated with F3.CD.IFN did not cause any greater decrease in astrocyte number than treatment with F3.IFN alone. We speculate that sustained expression of IFN-β from F3.CD.IFN is required to produce an inhibitory effect in reactive astrocytes. Further, as NSCs have neuroprotective functions, including the secretion of neurotrophic factors, the presence of NSCs themselves may provide a partial explanation for functional recovery and neuroregeneration after SCI. The F3 NSC line can produce an unlimited number of

differentiated cells in vitro and in vivo, and could therefore be used in transplantation. However, serious problems could arise when transplanted NSCs proliferate uncontrollably. In order to avoid such problems, we generated an F3 human NSC line expressing both the therapeutic IFN-β gene and the CD suicide gene. The expression of CD gene provides a fail safe guard to allow removal of cells in cases of uncontrolled proliferation of grafted cells.

Noninvasive monitoring of axonal regeneration by diffusion tensor imaging

The evaluation of axonal fibers is important to assess the severity of SCI and the efficacy of treatment; however, conventional methods such as tracer injection (e.g., biotinylated dextraamine) in brain parenchyma are technically demanding and highly Invasive (27,35). Because histological examinations are required to evaluate tracer studies, it has been impossible to evaluate axonal fibers in living animals and follow the sequential growth of these axonal fibers in the same animal. In order to evaluate axonal fibers in vivo, we sought a novel method capable of noninvasive evaluation in clinical applications. With this in mind, we determined that MRI is suited for use in the assessment of the state of SCIs. Conventional T1- and T2-weighted MRI of the spinal cord only shows ambiguous images of the various spinal structures, making it difficult to identify the complicated array of directionally oriented nerve fibers. However, DTI is one of the most versatile MRI modalities for longitudinal evaluations of CNS disorders, and is capable of following the orientation of nerve fibers and tracing specific neural pathways such as the CST (19,29,32). In this study, we chose a dorsal transection model because the disruption and regeneration of axons after a transection injury can be more easily evaluated than that following a contusion injury (30,46). Because of the ability to visualize longitudinal axonal tracts in variously oriented neural fibers, DTI has tremendous potential for use in the diagnosis and evaluation of spinal cord diseases. Several researchers have reported successful DTI in the human spinal cord (4,5). To our knowledge, this is the first report of DTI-based visualization of axonal

regeneration in the spinal cord of small rodents. Significant longitudinal axonal regeneration was confirmed by DTI in F3.CD.IFN-β NSC-treated mice 4 weeks after injury.

Conclusions

In conclusion, mice that received intravenous administration of genetically engineered NSCs transduced with IFN-β gene after SCI exhibited extensive suppression of glial scar formation in the lesioned spinal cord. This effect was shown to require the expression of both TLR-4 and IFN-β. Significant neurobehavioral and electrophysiological recovery was attained, as measured by the Basso mouse scale for locomotion, inclined plane test and transcranial MEPs. Axonal regeneration was also visualized noninvasively using DTI. Our results suggest that IFN-β delivery by intravenous injection of genetically engineered NSCs inhibits glial scar formation after SCI and promotes functional recovery through the functions of TLR-4. This novel NSC-based therapy of delivering IFN-β shows promise for the treatment of patients suffering frim SCI.

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