Dual regeneration of muscle and nerve by intravenous administration of human amniotic fluid–derived mesenchymal stem cells regulated by stromal cell–derived factor-1α in a sciatic nerve injury model

Laboratory investigation

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Object. Human amniotic fluid–derived mesenchymal stem cells (AFMSCs) have been shown to promote peripheral nerve regeneration. The expression of stromal cell–derived factor-1α (SDF-1α) in the injured nerve exerts a trophic effect by recruiting progenitor cells that promote nerve regeneration. In this study, the authors investigated the feasibility of intravenous administration of AFMSCs according to SDF-1α expression time profiles to facilitate neural regeneration in a sciatic nerve crush injury model.

Methods. Peripheral nerve injury was induced in 63 Sprague-Dawley rats by crushing the left sciatic nerve using a vessel clamp. The animals were randomized into 1 of 3 groups: Group I, crush injury as the control; Group II, crush injury and intravenous administration of AFMSCs (5 × 10⁶ cells for 3 days) immediately after injury (early administration); and Group III, crush injury and intravenous administration of AFMSCs (5 × 10⁶ cells for 3 days) 7 days after injury (late administration). Evaluation of neurobehavior, electrophysiological study, and assessment of regeneration markers were conducted every week after injury. The expression of SDF-1α and neurotrophic factors and the distribution of AFMSCs in various time profiles were also assessed.

Results. Stromal cell–derived factor-1α increased the migration and wound healing of AFMSCs in vitro, and the migration ability was dose dependent. Crush injury induced the expression of SDF-1α at a peak of 10–14 days either in nerve or muscle, and this increased expression paralleled the expression of its receptor, chemokine receptor type-4 (CXCR-4). Most AFMSCs were distributed to the lung during early or late administration. Significant deposition of AFMSCs in nerve and muscle only occurred in the late administration group. Significant enhancement of neurobehavior, electrophysiological function, nerve myelination, and expression of neurotrophic factors and acetylcholine receptor were demonstrated in the late administration group.

Conclusions. Amniotic fluid–derived mesenchymal stem cells can be recruited by expression of SDF-1α in muscle and nerve after nerve crush injury. The increased deposition of AFMSCs paralleled the expression profiles of SDF-1α and its receptor CXCR-4 in either muscle or nerve. Administration of AFMSCs led to improvements in neurobehavior and expression of regeneration markers. Intravenous administration of AFMSCs may be a promising alternative treatment strategy in peripheral nerve disorder.

Abbreviations used in this paper: AFMSC = amniotic fluid–derived MSC; BDNF = brain-derived neurotrophic factor; CMAP = compound muscle action potential; CNTF = ciliary neurotrophic factor; CXCR-4 = chemokine receptor type-4; DMSO = dimethyl sulfoxide; FBS = fetal bovine serum; GDNF = glia-derived neurotrophic factor; MSC = mesenchymal stem cell; NT-3 = neurotrophin-3; SDF-1α = stromal cell–derived factor-1α; SFI = sciatic function index.

Several approaches have been proposed to facilitate peripheral nerve regeneration, including application of an electrical field, transplantation of stem cells, and administration of neurotrophic factors.13,16,18,21

Key Words • amniotic fluid–derived mesenchymal stem cell • stromal cell–derived factor-1α • nerve regeneration • sciatic nerve crush injury • peripheral nerve

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The implantation of embryonic stem cells, neural stem cells, and MSCs has led to some degree of peripheral nerve regeneration. Cell replacement, trophic factor production, extracellular matrix molecule synthesis, guidance, remyelination, microenvironmental stabilization, and immune modulation have been postulated as possible mechanisms for regeneration.2,13,24

Amniotic fluid has been shown to be a novel source for therapeutic transplantation of stem cells. Amniotic fluid–derived stem cells express characteristics of both mesenchymal and neural stem cells; these cell types have been used to treat various neurological disorders.26 In our previous study, we demonstrated that transplantation of AFMSCs promoted peripheral nerve regeneration through the secretion of neurotrophic factors.16,18 Furthermore, we found that improvements in regeneration were achieved through modulation of the inflammatory process, as well as neurotrophic factor gene transfer.2,15,17 These results demonstrated that AFMSCs are a novel source for peripheral nerve regeneration.

Local delivery of AFMSCs in peripheral nerve injury made these treatment scenarios possible.2,15–18 Intravenous administration of stem cells in brain and heart ischemia models was shown to be successful.14,22 However, most of the intravenously administered stem cells were deposited in the lung, and this made the stem cell treatment difficult in other organs.7 Increased expression of stem cell homing factors such as CXCR-4/SDF-1α in either a small subpopulation of stem cells or a gene transfer increased the number of cells that migrated to injured organs.28 Thus, the integration of homing factors in stem cells or injured tissue should increase the success of cell transplantation.

Stromal cell–derived factor-1α is expressed in various tissues of adult rats. Upon nerve crush injury, the SDF-1α mRNA level transiently increased after lesioning. It reached a maximum of 175% at 2 days after injury and declined to the control level at 7 days after injury.8 The expression of SDF-1α in the nerve crush injury started to increase at 3 days and reached a plateau at 10–14 days.25 Bone marrow MSCs weakly expressed CXCR-4, and only a small proportion of the subpopulation exhibited strong expression. Amniotic fluid–derived MSCs expressed the characteristics of MSCs but showed only a slight difference in surface markers. In this study, we investigated the effect of different concentrations of SDF-1α on the migration ability of AFMSCs in vitro. Furthermore, we attempted to determine the AFMSC distribution in various tissues and the amount of cell deposition as related to SDF-1α expression in nerve and muscle tissue. In addition, we investigated the amount of AFMSC deposition related to neurobehavior, electrophysiological function, and nerve regeneration outcomes.

Methods

Although many of the methods listed in this section have been published previously,2,25 we choose to detail them again here for clarity.

Crush Models

Sixty-three Sprague-Dawley rats weighing 250–300 g were used in this study. The rats were anesthetized using 4% isoflurane during induction, followed by a maintenance dose (1%–2%). The left sciatic nerve was exposed under a microscope using the gluteal muscle-splitting method. A vessel clamp (B-3, pressure 1.5 g/mm²; S&T Marketing) was applied 10 mm from the internal obturator canal for 20 minutes. The crush site was sutured with 9-0 nylon over the epineurium as a mark.16 The animals were randomized into 1 of 3 groups: Group I, crush injury as the control (crush group, n = 27); Group II, crush injury and intravenous administration of 5 × 10⁶ AFMSCs for 3 days immediately after injury (AFS 0 days, early administration; n = 18); and Group III, crush injury and intravenous administration of 5 × 10⁶ AFMSCs for 3 days, 7 days after injury (AFS 7 days, late administration; n = 18). All animals received rehabilitation therapy on a metal mesh every week. Food and water were provided ad libitum before and after the experiments. The animals were kept in a temperature-controlled environment at 20°C and were exposed to alternating light and dark cycles of 12 hours. All animals were treated and cared for in accordance with the guidelines recommended by the Ethics Committee of Taichung Veterans General Hospital.

Preparation and Culture of Human AFMSCs

Human amniotic fluid samples (20 ml) were obtained by amniocentesis performed between 16 and 20 weeks of gestation for fetal karyotyping. For culturing amniocytes, 4 primary in situ cultures were set up in 35-mm tissue culture–grade dishes using Chang medium (Irvine Scientific). Microscopic analysis of Giemsa-stained chromosome banding was performed, and the rules for metaphase selection and colony definition were based on the basic requirements for prenatal cytogenetic diagnosis in amniocytes.26 For culturing AFMSCs, nonadhering amniotic fluid cells in the supernatant medium were collected on the fifth day after primary amniocyte culture and maintained until completion of fetal chromosome analysis. The cells were then centrifuged and plated in 5 ml of β-minimum essential medium (β-MEM; Gibco-BRL) supplemented with 20% FBS (HyClone) and 4 ng/ml basic fibroblast growth factor (R&D Systems) in a 25-cm² flask and incubated at 37°C with 5% humidified CO₂.18 Amniotic fluid–derived MSCs were labeled with Hoechst 33342 before grafting. In brief, AFMSCs were subcultured in a 10-cm culture dish until cell confluence was reached. We added 1 ml of 1 µg/ml Hoechst 33342 (Sigma-Aldrich) to the culture dish titrated to a concentration of 0.1 µg/ml, which was incubated at 37°C with 5% humidified CO₂ for 16 hours Then, AFMSCs labeled with Hoechst 33342 were obtained for transplantation.

This protocol was approved by the Institutional Review Board of Taichung Veterans General Hospital, and written informed consent was obtained from all the patients who provided amniotic fluid samples.

Analysis of Functional Recovery

A technician who was blinded to treatment allocation evaluated sciatic nerve function every week after the surgery using the SFI.7 Several measurements were...
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taken from the footprint, and these included the following: 1) distance from the heel to the third toe, the print length (PL); 2) distance from the first to the fifth toe, the toe spread (TS); and 3) distance from the second to the fourth toe, the intermediate toe spread (ITS). All 3 measurements were taken from the experimental (E) and normal (N) sides. The SFI was calculated according to the equation: SFI = 38.3(EPL - NPL/NPL) + 109.5(ETS - NTS/NTS) + 13.3(EITS - NITS/NITS) - 8.8. The SFI is close to 0 in animals with normal nerve function, whereas an SFI of -100 represents total dysfunction.

**Automated Quantitative Gait Analysis**

Computerized gait analysis was performed, as previously described. Briefly, animals had to cross a 100-cm-long glass floor plate, which was placed between Plexiglas walls spaced 8 cm apart in a darkened room. Light from an otherwise completely encased white fluorescent tube entered one of the long edges of the glass floor. The light tube was placed 2 cm from the glass plate so that light entering the 5-mm-thick glass plate was completely internally reflected. Light exited at only those points where a paw touched the glass plate, and this device thereby illuminated the points of contact of the paws. During crossings, the walkway was monitored from below by a Pulnix TM-62EX camera equipped with a wide-angle objective (8.5 mm, Cosmicar). The camera registered the paw-floor contact in pixels of 1.23 mm², whereas the intensity (gray value) of the signal depended on the applied pressure. Higher paw pressure resulted in a larger total area of skin-floor contact and a more intense scatter (brighter pixels). The signal was digitized by a pcImage-SRGB frame grabber board (Matrix Vision GmbH) and subsequently acquired, compressed, and stored by CatWalk software (Noldus Information Technology) for further analysis. All areas containing pixels brighter than a preset analysis threshold were stored. Using the analysis component of the CatWalk program, these areas were assigned to 1 of the paws, and data were exported to a spreadsheet. Quantitative analysis of this data included the following parameters.

1. **Step sequence distribution.** Six different walking patterns or normal step sequence patterns that fall into 3 different categories can be discerned in rats, depending on the sequential placement of the 4 paws.

2. **Regularity index.** This parameter is a measure of interlimb coordination. Interlimb coordination is considered normal when, during uninterrupted locomotion, only normal step sequences are used. The regularity index rates the degree of interlimb coordination as a percentage of complete coordination by the following equation: regularity index = (NSSP × 4/PP) × 100%, where NSSP represents the number of normal step sequence patterns and PP indicates the total number of paw placements. Consequently, extra paw placements and irregular walking on 3 paws would result in a decrease of the regularity index.

3. **Print area.** This parameter was defined as the total floor area in pixels contacted by the paw during stance phase. Possible reasons for the hind limb print area to increase are paralysis of the lower limb leading to a deficiency in plantar stepping or paw/toe dragging during part of the step cycle. A decrease in this parameter can be indicative of mechanical allodynia.

4. **Base of support.** The distance in millimeters between the 2 hind paws is defined as the base of support. This distance is measured perpendicular to the direction of walking.

5. **Duration of the swing and stance phases.** Since the duration of the stance or swing phase depends on the animal’s walking speed and degree of dysfunction, these parameters are transformed to a fraction of the total step duration according to the following formula: fraction stance or swing phase = [(time in stance or swing phase)/(time in single step)] × 100%. Durations of the swing or stance phase and total step are expressed in seconds.

6. **Hind paw pressures.** This is the mean intensity of the contact area of the hind paw at the moment of maximal paw-floor contact. This parameter is expressed in arbitrary units (intensity arbitrary units).

**Electrophysiological Study**

Sciatic nerves from individual groups were exposed 4 weeks after the crush injury, and electrical stimulation was applied to the proximal side of the injured site. The evoked CMAP amplitudes and conduction latencies were recorded in the gastrocnemius muscle with an active monopolar needle electrode 10 mm below the tibial tuberosity and with a reference needle 20 mm from the active electrode. The stimulation intensity and filtration ranges were 5 mA and 20–2000 Hz, respectively. A similar assessment was performed on the noninjured side. The CMAP data and conduction latency were converted to the ratio of the injured side divided by the normal side to adjust for the effect of anesthesia.

**Western Blot Analysis**

Nerve tissue specimens (10 mm long, with a 9-0 nylon marker in the middle of the nerve) were harvested after treatment at various time points, and proteins were extracted. The gastrocnemius muscle, approximately 10 mm in length over the belly at the corresponding time of nerve sampling, was obtained for analysis. Proteins (50 μg) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a blotting membrane. After blocking with nonfat milk, the membranes were incubated with antibodies against SDF-1α, CXCR-4, and β-actin (Santa Cruz Biotechnology, Inc.) overnight at 4°C. The membranes were incubated with horseradish peroxidase–conjugated secondary antibody and developed using enhanced chemiluminescence Western blotting reagents. The intensity of protein bands was determined by a computer image analysis system (IS1000; Alpha Innotech Corporation).

**Wound Healing Assays**

Amniotic fluid–derivedMSCs were cultured in 12-well plates and grown in medium containing 10% FBS to nearly confluent cell monolayers. A plastic pipette tip was then carefully used to draw a linear “wound” in the cell monolayer of each well. The monolayer was washed twice with phosphate-buffered saline to remove debris.
and the detached cells, and then recombinant SDF-1α (Peprotech) was added in different concentrations (5, 10, and 20 ng/ml); 0.1% of DMSO was added to the control well as the solvent control. The cultures were incubated at 37°C, photographed immediately, and monitored by time-lapse photography (photographed every 20 minutes for 12 hours) using a Carl Zeiss Axiovert with Biostation system (Carl Zeiss). Under the microscope, the number of cells that migrated into the cell-free zone, based on the zero line of the linear “wound,” was evaluated. The experiments were performed in triplicate and were counted in a double-blind manner by at least 2 investigators.

**Transwell Migration Assay**

Transwell membranes were used (8-μm pore size, 6.5-mm diameter; Corning Costar Corporation). The cells were trypsinized, washed, and kept suspended in medium without FBS. Migration-inducing medium (with 10% FBS) was added to the lower wells of the chambers. The upper wells were filled with serum-free medium (20,000 cells per well) that, in some cases, also contained different concentrations of recombinant SDF-1α (5, 10, and 20 ng/ml; Peprotech) or 0.1% of DMSO as the solvent control. The chamber was then placed into a humidified incubator. After 8 hours, assays were stopped by removal of the medium from the upper wells and careful removal of the filters. Filters were fixed with methanol by brief submersion, and cells were subsequently wiped off the upper side using a cotton swab. Filters were stained with 20% Giemsa solution (Sigma Chemical) for light microscopy or 4'-6-diamidino-2-phenylindole for the fluorescent microscopy–based high content screening system (Cellomics). Evaluation of completed transmigration was performed under the microscope, and random fields were scanned (4 fields per filter) for the presence of cells at the lower membrane side only.

**Immunohistochemical Analysis**

Serial 8-μm-thick sections of sciatic nerve and gastrocnemius muscle were cut using a cryostat and mounted on SuperFrost Plus slides (Menzel-Gläser) and subjected to immunohistochemical analysis using antibodies against GDNF, BDNF, CNTF, NT-3, S100 protein, desmin, and acetylcholine receptor for detection of nerve and muscle regeneration. The immunoreactive signals were observed using goat anti–mouse IgG (FITC, Jackson; 1:200 dilution), anti–mouse IgG (Rhodamine, Jackson; 1:200 dilution), or 3,3′-diaminobenzidine. Among longitudinal consecutive resections, 5 consecutive resections contiguous to a maximum diameter were chosen to be measured. Of 100 squares with a surface area of 0.01 mm² each, 20 squares were randomly selected in an ocular grid and used to count the number of cells. For the determination of the objects of interest (GDNF, BDNF, CNTF, NT-3, S100, desmin, and acetylcholine receptor), 6 nerves in each group were cut longitudinally into 8-μm-thick sections and stained with each antibody. The region of maximum diameter of the resected nerve tissue with crush mark was chosen to be examined. Areas of immunoreactivity (0.2 mm²) appeared dense against the background and were measured by a computer image analysis system (IS 1000; Alpha Innotech).

**Histological Examination**

After neurobehavioral and electrophysiological testing, 6 rats in each group underwent transcardial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) after being reanesthetized. The left sciatic nerve was harvested from the animals after electrophysiological testing, and the nerve tissue was fixed on a plastic plate using stay suturing to keep the nerve straight. The bilateral gastrocnemius muscles were dissected from the bones and sent for measurement of muscle weight.

The obtained nerve tissues (5 mm long) distal to the crush injury (5 mm long) were fixed in 3% glutaraldehyde solution, postfixed in 0.5% osmium tetroxide, embedded, cut transversely into 4-μm-thick sections, and stained with toluidine blue 4 weeks after the crush injury. The number of axons was counted in 10 randomly selected fields (0.1 mm²) at a magnification of 200.

**Statistical Analysis**

Data were expressed as means ± SEMs. The statistical significance of differences between groups was determined by 1-way ANOVA followed by the Dunnett test. For SFI and CatWalk analysis, the results were analyzed by repeated-measures ANOVA followed by the Bonferroni multiple comparison method. A p value < 0.05 was considered statistically significant.

**Results**

**Effect of SDF-1α on Migration of AFMSCs**

Stromal cell–derived factor-1α was observed to mobilize hematopoietic stem cells into the injured tissue, and it also mediated the migration of MSCs to impaired sites in the brain and nerve avulsion injuries. To mimic the AFMSC migration regulated by SDF-1α, the wound healing assay and transwell migration assay were used. In the wound healing test, the migration ability of AFMSCs was measured under conditions of various SDF-1α concentrations. At a concentration of 20 ng/ml, the cells reached full confluence at 12 hours (Fig. 1A). In the transwell migration assay, SDF-1α increased AFMSC migration in a dose-dependent manner (from 0 to 20 ng/ml). Five ng/ml of SDF-1α exerted a significant increase in migration ability compared with 0 ng/ml of SDF-1α (p < 0.05); 10 ng/ml of SDF-1α further increased the migration ability (p < 0.001 compared with 0 ng/ml); and 20 ng/ml reached a maximum migrational effect (p < 0.001 compared with 0 ng/ml). There was also a significant difference between 10 and 20 ng/ml of SDF-1α (p < 0.05; Fig. 1B and C). Based on these data, the migration ability of AFMSCs was regulated by SDF-1α in a dose-dependent manner.

**Expression of CXCR-4/SDF-1α in Nerve and Muscle**

The expression of SDF-1α is increased not only in the bone marrow microenvironment, but also in several other organs such as the nerves, heart, liver, and brain. Its increase likely helps to facilitate the regeneration process. To determine the appropriate time profile for intravenous
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AFMSC administration, the consecutive nerve and muscle samplings at different time points were retrieved for detection of expression of CXCR-4/SDF-1α after nerve injury. In immunohistochemical staining, AFMSCs not only expressed SDF-1α but also its corresponding receptor CXCR-4 (Fig. 2A). After crush injury, SDF-1α started to increase at 3 days (10.5 ± 3.8 intensity arbitrary units) and reached a maximum at 14 days (41.5 ± 4.5 intensity arbitrary units; *p < 0.01; Fig. 2B and C). The increase in SDF-1α expression correlated with an increase in the expression of CXCR-4 (Fig. 2B and C). In muscle sampling, SDF-1α started to increase at 3 days (7.1 ± 3.9 intensity arbitrary units) and reached a maximum at 14 days (44.1 ± 4.6 intensity arbitrary units; *p < 0.01; Fig. 2B and D). This phenomenon was accompanied by altered CXCR-4 expression (*p < 0.01; Fig. 2B and D). Based on the above data, the coupled expression of SDF-1α and CXCR-4 in either muscle or nerve was compatible with similar expression time profiles.

Distribution of AFMSCs in Various Organs After Early or Late Intravenous Injection

Amniotic fluid–derived mesenchymal stem cells were administered according to the expression profile of SDF-1α immediately (early) or 7 days (late) after nerve injury. Most of the AFMSCs were distributed to the lung either in early or late administration (Fig. 3A–D). Small amounts of AFMSCs were detectable in the brain or spinal cord either in the early or late injection groups (data not shown). Significant amounts of AFMSCs distributed to the spleen, muscle, and nerve were observed only after late administration, but a few AFMSCs were detected after early administration (Fig. 3E–P).

The distributions of AFMSCs in crushed nerve were deposited around the epineuria and retained for 3 days (Fig. 3O and P) in the late administration group, but there were very few AFMSC deposits in the early administration group (Fig. 3M and N). The allocation of AFMSCs to muscle tissues was mainly retained at the neuromuscular junction (Fig. 3K and L) and was also detectable for 3 days in the late administration group, but there were very few AFMSC deposits in the early administration group (Fig. 3I and J). These data confirmed the hypothesis that the administration of AFMSCs at the peak expression of SDF-1α promoted the significant recruitment of AFMSCs in regenerative organs such as nerve and muscle.

Neurobehavior After Late Administration of AFMSCs

Early or late AFMSC treatment caused a significant
Improvement in SFI compared with the findings obtained in the control group. Early AFMSC administration demonstrated a significant improvement compared with the crush group (p < 0.05). The improvement in the late administration group was statistically more significant than that in the early administration group (p < 0.01; Fig. 4A). The ratio of CMAP was 0.21 ± 0.03 in the crush group, which increased to 0.22 ± 0.02 in the early treatment group and further increased to 0.49 ± 0.03 in the late treatment group (p < 0.01). The ratio of latency was 3.3 ± 0.15 in the crush group, 3.05 ± 0.2 in the early treatment group, and 1.71 ± 0.1 in the late treatment group (p < 0.01). The ratio of muscle weight (left/right) was 0.46 ± 0.01 in the crush group, 0.49 ± 0.02 in the early treatment group, and 0.66 ± 0.01 in the late treatment group (p < 0.05; Fig. 4B). In computerized gait analysis, the mean intensity and printed area showed remarkable improvement in the late AFMSC administration group compared with that in the early AFMSC administration group (p < 0.01; Fig. 5A and B). The early AFMSC administration group showed only slightly more improvement than the crush group (p < 0.05; Fig. 5A and B). The stance duration ratio dropped to half of the original value in crush injury; the late administration group demonstrated significantly greater improvement than the early administration group (p < 0.01). In addition, the early AFMSC administration group showed greater benefits than the crush group (p < 0.05; Fig. 5C). The swing duration ratio showed a substantial decrease in the late administration group compared with that in the early administration group (p < 0.01), whereas the early AFMSC administration group showed only slightly more improvement than the crush group (p < 0.05; Fig. 5D). Based on the SFI, electrophysiology test, and computerized gait analysis, late administration of AFMSCs produced a more favorable outcome than early treatment.

Nerve and Muscle Regeneration After Late Administration of AFMSCs

An increase in axon size correlated with a significant improvement of neurobehavior. In this study, the late treatment group showed a significant increase in the number of axons with 3- to 4-μm diameters (p < 0.01 compared with the crush injury and early groups) as well as...
in the number of axons with 5- to 6-μm diameters (p < 0.01 compared with the crush injury and early groups), but there was no significant increase in the number of axons with smaller diameters (< 2 μm; Fig. 6A and C). The density of the acetylcholine receptor in the gastrocnemius muscle was significantly greater in the early (p < 0.001) and the late treatment groups (p < 0.001) compared with the control group. Furthermore, the acetylcholine receptor density in the late treatment group was greater than that in the early treatment group (p < 0.05; Fig. 6B and D). Based on these findings, late treatment with AFMSCs promoted significantly more regeneration of nerve and muscle tissue function than nontreatment or early AFMSC treatment.

Amniotic Fluid–Derived MSCs Distributed in Nerve and Muscle Tissue

Most of the AFMSCs were distributed to the epineurium and neuromuscular junction of the nerve in the late administration group. Immunohistochemistry of the nerve demonstrated that AFMSCs strongly expressed the neurotrophic factor BDNF and moderately expressed CNTF and NT-3. A portion of AFMSCs expressed the Schwann cell marker S100 (Fig. 7A). Transplanted AFMSCs in innervated muscle showed significant BDNF expression and moderate elevation of CNTF, but weak staining in NT-3. In addition, AFMSCs in muscle tissue expressed the differentiated marker desmin (Fig. 7B). Based on the immunohistochemical study, late intravenously administered AFMSCs were distributed to nerve and muscle, which not only showed the inherited characteristic neurotrophic factors but also the potential for differentiation into nerve and muscle.

**Discussion**

Increased expression of SDF-1α in injured nerve and denervated muscle occurred upon injury, and this result paralleled the elevations in CXCR-4 expression. In late administration of AFMSCs, cells were significantly distributed to nerve and muscle in accordance with the SDF-1α expression profile. However, early administration of AFMSCs did not demonstrate this effect. The increased deposition of AFMSCs produced significant improvement in neurobehavior, electrophysiological function, and myelination of axons, in parallel with the increased density of acetylcholine receptors. These results imply that the timing of AFMSC administration significantly affects stem cell involvement in injured tissue restoration and any subsequent functional recovery.

Local delivery of stem cells in peripheral nerve injury made AFMSC transplantation possible as a beneficial treatment.\(^{2,15–18}\) However, due to a detrimental inflammatory response, secondary injury occurred when the wound was reopened during transplantation, and this inflammatory response had an overall adverse effect on the treatment approach. Intravenous administration of stem cells in brain and heart ischemia models was also
shown to be successful. However, most of the intravenously administered stem cells were deposited in the lung, and this made the stem cell treatment difficult when targeting other organs. In this study, we found that with either early or late treatment, most of the AFMSCs were distributed to the lung, consistent with the results of previous reports. However, a significant amount of AFMSCs were deposited in injured nerve and muscle in the late treatment group. These results confirmed that AFMSCs administered as a late treatment along with increased expression of SDF-1α resulted in significant delivery of AFMSCs to injured nerve and muscle. Thus, the coupling of homing factors in transplanted stem cells with corresponding injury tissue should increase the success of cell transplantation.

Stromal cell–derived factor-1α is expressed in various tissues of adult rats. Upon nerve crush injury, the SDF-1α mRNA level transiently increases after lesioning. This level reaches a maximum of 175% at 2 days after injury and decreases to the control level at 7 days after injury. In this study, we found that the expression of SDF-1α in nerve crush injury appreciably increased at 3 days and reached a plateau at 10–14 days. These trends in SDF-1α expression paralleled changes in expression of CXCR-4. In addition, AFMSCs also expressed the characteristics of the MSC marker SDF-1α/CXCR-4. Thus, the optimal time for delivery of AFMSCs was when SDF-1α was highly expressed. This approach resulted in marked AFMSC deposition in injured tissue and promotion of the nerve regeneration process.

Amniotic fluid–derived stem cells express characteristics of both MSCs and neural stem cells, which have been considered as cell sources for neurological...
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disorders. In our previous study, we demonstrated that transplantation of AFMSCs promoted peripheral nerve regeneration, possibly through the secretion of neurotrophic factors such as CNTF and NT-3. Furthermore, we found that improved outcome of cell transplantation was augmented by the modulation of the inflammatory process, as well as neurotrophic factor gene transfer of GDNF. In this study, the significant deposition of AFMSCs in nerve and muscle correlated with a significant improvement of neurological function, as well as increased nerve myelination and acetylcholine receptor density in muscle. These improvements paralleled enhanced expression of neurotrophic factors BDNF, CNTF, and NT-3, as well as AFMSC deposition. Such findings were consistent with our previous results indicating that transplanted AFMSCs secrete neurotrophic factors that contributed to nerve regeneration.

Skeletal muscle fibers are able to successfully regenerate after an injury. Skeletal muscles express several neurotrophic receptors essential for muscle regeneration. The expression of BDNF is found in myogenic progenitor cells responsible for muscle regeneration. The neurotrophic factor NT-3 is essential for muscle spine formation in dystrophic muscle pathology. Receptors of CNTF are expressed in the skeletal muscle fiber, and administration of CNTF reduces denervation atrophy and stimulates myotube formation. In this study, increased expression of BDNF and CNTF as well as scattered expression of NT-3 over the transplanted AFMSCs resulted in neurological improvement with regard to neurobehavioral and myelination. The results were compatible with trophic support from the AFMSCs to augment muscle regeneration.

Animal behavior and electrophysiology are adequate parameters to evaluate neurobehavioral changes. Behavioral and electrophysiological deficits have correlated with the severity of nerve injury. The measurement of SFI is related to the intrinsic muscle function of the feet. In addition, CatWalk is an automated analysis system for evaluating both static and dynamic function of sciatic nerve injury. Furthermore, the CMAP reflects the number of axons innervating the muscles, particularly relating to the amount of acetylcholine receptor density, and decreased conduction latency parallels increased motor function. Furthermore, morphometric analysis of axons with a shift from small axons to large axons indicates nerve regeneration. In this study, we found that late administration of AFMSCs caused a significant improvement in SFI, CatWalk dynamic analysis, electrophysiological function, and density of acetylcholine receptors, and a shift from small- to large-diameter axons. All of these changes led to a significant improvement in neurological outcome after crush injury.

Intravenous administration of stem cells provided the
most feasible route to deliver the cells into injured tissue during the regeneration process. In this study, we found that a significant number of cells and an appropriate time for delivery were required for effective stem cell delivery. The coupling of intravenous AFMSC administration with the time of increased expression of SDF-1α resulted in the greatest benefits in nerve regeneration. However, there was a disparity between the SDF-1α expression profiles in nerve/muscle tissue and serum, and this made it difficult to determine the most appropriate time profile for delivering stem cells to muscle and nerve. Further investigation of the relationship between SDF-1α expression in various injured tissues and in serum is needed to facilitate the identification of an optimal time to deliver the stem cells to injured peripheral nerves.

Several trophic factors might be induced after the nerve injury, which contributed to recruit AFMSCs involved in nerve regeneration. In this study, we administered AFMSCs intravenously according to the SDF-1α expression profile to assess the possibility of AFMSCs being deposited in muscle and nerve. It appeared that a high expression profile of SDF-1α was correlated with a significant amount of AFMSC deposition. We formulated the hypothesis that SDF-1α could recruit AFMSCs to get involved in nerve and muscle regeneration only based on the in vitro study of SDF-1α and CXCR-4 expression in AFMSCs, as well as the AFMSC migration regulated by SDF-1α. To further confirm this hypothesis, knockout of SDF-1α in animals was mandatory. Based on this study, the administration of AFMSCs according to the SDF-1α expression profile could be an appropriate time window for intravenous AFMSC administration to increase AFMSC distribution in nerve and muscle.

Fig. 7. Distribution of AFMSCs in nerve and muscle in late administration. Photomicrographs show double immunohistochemical staining in epineuria (A) and muscle (B) using Hoechst 33342 for BDNF, CNTF, NT-3, and S100. Bar = 100 μm. N = 3 samples tested.

Conclusions

This study showed that AFMSCs can be recruited by expression of SDF-1α in peripheral nerve injury. The increased deposition of AFMSCs paralleled the expression profiles of SDF-1α and its receptor CXCR-4. The extent of AFMSC deposition correlated with the degree of neurobehavioral improvement. Intravenous administration of AFMSCs according to the expression profile of SDF-1α may be a promising treatment strategy following peripheral nerve injury.

Disclosure

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