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Canine bone marrow peri-adipocyte cells could therapeutically benefit acute spinal cord injury through migration and secretion of hepatocyte growth factor to inflammatory milieu.

Running head:

Potential of BM-PACs for SCI therapy

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Abstract

Spinal cord injury (SCI) is a common neurological disorder in dogs. A secondary injury that occurs in the acute phase causes expansion of inflammation, resulting in lesion extension and further loss of function. Mesenchymal stem cells (MSCs) have trophic effects and the ability to migrate toward injured tissues; therefore, MSC-based therapy is considered promising for the treatment of canine SCI. We recently reported that bone marrow peri-adipocyte cells (BM-PACs) can be obtained from canine bone marrow and have stem cell potential superior to that of conventional bone marrow MSCs (BMMSCs). However, their therapeutic potential for SCI have been still unknow. Here, we first evaluated the ability of BM-PACs to secrete hepatocyte growth factor (HGF) and their migration ability toward inflammatory milieu in vitro. BM-PACs can secrete HGF in response to pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, and exhibit migration ability toward these cytokines. Next, BM-PACs were intravenously administered into nude mice with acute SCI to analyze the homing ability and therapeutic effects of HGF secreted by BM-PACs. BM-PACs homed to the injured spinal cord, where the HGF expression level increased 7 days after administration. Intravenous administration of BM-PACs induced functional recovery and pathological improvement, indicated by less demyelinating area, more preserved axons, and less glial scar formation compared with the mice only received vehicle. These findings suggest that the intravenous administration of BM-PACs can be a novel therapeutic intervention for acute canine SCI.

Key words: mesenchymal stem cell (MSC), acute spinal cord injury (SCI), intravenous administration, hepatocyte growth factor (HGF), pro-inflammatory cytokines

Introduction

Spinal cord injury (SCI), mainly due to intervertebral disc herniation, is a common neurological disorder in dogs and causes a series of complications ranging from back pain to total lower-limb paralysis [1]. Currently, SCI management includes surgical decompression, pharmacological treatment, and physical rehabilitation [2, 3]. However, owing to the lack of axonal regeneration in the central nervous system [4], regaining loss of motor function is usually difficult, especially when the spinal cord is severely damaged. Secondary injury following primary mechanical damage in acute SCI is defined as further progressive destruction of the tissue surrounding the necrotic core, and leads to various histological changes including edema, inflammation, and necrotic/apoptotic cell death [5, 6, 7]. Thus, the inhibition of secondary injury may be an important therapeutic strategy to treat SCI.

Mesenchymal stem cells (MSCs) are multipotent stem cells used in various disease models as candidates for regenerative medicine. Another feature of MSCs is their ability to secrete various trophic factors that contribute to tissue protection and repair [8]. Hepatocyte growth factor (HGF) is a growth factor secreted by MSCs [9] and has been proven to be a robust neurotrophic factor that has anti-inflammatory properties, promotes motor neuron survival, stimulates angiogenesis, and induces axon regeneration [10, 11, 12]. Several studies have also shown that the administration of exogenous human HGF into a rodent SCI model in the acute phase reduces cavity and glial scar formation, protects myelinated fibers, and promotes functional recovery [13, 14].

The inflammatory response is a major pathological feature of acute SCI. Pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, are released by neurons and microglia and are significantly upregulated within 6 h post-injury [15, 16]. These cytokines play essential roles in recruiting circulating leukocytes and monocytes, astrocyte activation, and immune regulation [6]. Recent studies have reported that TNF-α and IL-1β also affect the biological function of MSCs. When exposed to TNF-α or IL-1β *in vitro*, MSCs showed an enhanced ability to secrete growth

factors including HGF and vascular endothelial growth factor (VEGF) [17, 18]. Furthermore, it is also known that MSCs have a migratory ability towards these pro-inflammatory cytokines [19, 20]. These findings indicate the possibility that systemically administrated MSCs home to the injured spinal cord depending on the inflammatory environment following acute SCI and deliver HGF, contributing to therapeutic effects.

We previously reported that bone marrow peri-adipocyte cells (BM-PACs) adhering to adipocytes in canine bone marrow can be easily isolated using ceiling culture technique [21]. BM-PACs showed superior stem cell properties, including significant proliferation and multi-differentiation ability, compared to conventional canine bone marrow MSCs (BMMSCs) harvested by adhering to the culture of mononuclear cells in the bone marrow. Although BM-PACs have not been yet identified from other species than dogs, it is expected that BM-PACs share other beneficial properties such as secretion of trophic factors and homing ability with MSCs, and can be a useful cell source for SCI in dogs.

In this study, to investigate therapeutical potential of BM-PACs for SCI, we assessed the *in vitro* HGF secretion ability in response to stimulation with TNF-α and IL-1β of BM-PACs comparing with conventionally cultured BMMSCs. The migration ability of BM-PACs toward these pro-inflammatory cytokines was also evaluated. Subsequently, as a preclinical study for dogs with SCI, BM-PACs were systemically administered to nude mice with acute SCI. Homing ability and HGF secretion ability of BM-PACs was assessed *in vivo*, and therapeutic effects were also evaluated.

Materials and methods

Animals

Bone marrow was collected from four healthy beagles (female, 1–2 years old, 9–11 kg, Kitayama Labes Co. Ltd., Nagano, Japan) under general anesthesia maintained with 2% isoflurane. Robenacoxib (2mg/kg) was given subcutaneously as an analgesic before bone marrow aspiration. Twenty-eight

BALB/c-*nu/nu* mice (female, 8 weeks old, 17–22 g, Japan SLC, Inc., Shizuoka, Japan) were used as the SCI model. All mice were anesthetized during the surgical procedure and received analgesia. All animal experiments were approved by the Animal Care Committee of the Graduate School of Agriculture and Life Sciences, University of Tokyo, Japan (Approval number: P16-196).

Cell culture

Under general anesthesia, canine bone marrow was harvested from the humeral bones, and BM-PACs and BMMSCs were isolated as described previously [21]. Briefly, the bone marrow was carefully placed on Ficoll-Paque (GE Healthcare, Buckinghamshire, UK) and centrifuged at 430*g* for 30 min. For the culture of BM-PACs, the top layer, including adipocytes, was collected, and washed two times with Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Carlsbad, CA, USA). The collected cells were placed in 25-cm^2 flasks filled with DMEM supplemented with 20% FBS and antibiotics/antimycotic (100 U/ml of penicillin G, 100 g/ml of streptomycin sulfate, and 0.25 g/ml of Amphotericin B) for ceiling culture. The flasks were incubated at 37 °C, 5% CO_2. For BMMSCs, after density gradient centrifugation, the middle layer, including mononuclear cells, was harvested, and washed twice with DMEM supplemented with 10% FBS. The erythrocytes were eliminated using hemolysis buffer, and the remaining cells were filtered through a 70-μm nylon mesh. The isolated mononuclear cells were plated in 90-mm petri dishes containing 10 ml of DMEM supplemented with 10% FBS and incubated at 37 °C in a humidified 5% CO_2 incubator. After reaching 80% confluence, BM-PACs and BMMSCs were harvested and cryopreserved with STEM-CELLBANKER (TakaraBio, Shiga, Japan) in liquid nitrogen. After thawing, cryopreserved cells (P0) were plated in 90-mm petri dishes. The proliferated cells (P1) were detached and used for further experiments.

115 RT-PCR

116 To determine the HGF secretion ability in response to TNF-α and IL-1β, BM-PACs and BMMSCs (P1)
117 independently isolated from 4 dogs were plated at a density of 1×10^4 cells/cm^2 in 90-mm petri dishes.
118 After cells reached at 80% confluence, medium was replaced to a low-serum medium (DMEM
119 supplemented with 1% FBS) containing 1, 10, 50, 100 ng/ml of recombinant canine TNF-α (R&D
120 Systems, Inc., Minneapolis, USA) or recombinant canine IL-1β (Kingfisher Biotech, Inc., Saint Paul,
121 USA). After 24 h, cells were harvested, and total RNA was extracted using TRI reagent (Molecular
122 Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. cDNA was
123 synthesized using ReverTra Ace qPCR RT Master Mix with gDNA remover (Toyobo, Osaka, Japan).
124 RT-PCR was performed using the THUNDERBIRD SYBR qPCR mix kit (Toyobo, Osaka, Japan).
125 Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a reference gene. The PCR cycling
126 conditions were as follows: initial denaturation at 50 °C for 2 min and 95 °C for 10 min, followed by
127 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The forward and reverse primers used for specific
128 amplification of *GAPDH* and *HGF* were as follows: TGACACCCACTCTTCCACCTTC,
129 CGGTTGCTGTAGCCAAATTCA, and AAAGGAGATGAGAAACGCAAACAG,
130 GGCCTAGGCAAGCTTCAGTAATACC, respectively. *HGF* mRNA expression was compared
131 between BM-PACs and BMMSCs after normalization relative to *GAPDH* expression.
132

133 Enzyme-linked immunosorbent assay (ELISA)

134 BM-PACs were cultured in 12-well plates treated with low serum medium (1% FBS) overnight. BM-
135 PACs were stimulated with TNF-α (100 ng/ml) or IL-1β (10 ng/ml) for 24 h. The culture medium was
136 collected, and HGF concentrations were measured using an ELISA kit (Wuhan USCN Business Co.,
137 Ltd., Wuhan, China) according to the manufacturer's instructions. To estimate the HGF production

from each cell more accurately, the DNA content in each well was measured and HGF production was expressed by values of HGF content divided by the DNA content.

Chemotaxis assay

A chemotaxis assay was performed to analyze the migration ability of cells towards TNF-α and IL-1β. In the upper chamber (Falcon, Oxnard, CA, USA), 1×10^5 cells were placed in 200 μl of low-serum (1% FBS) medium, and an equal aliquot of low-serum medium with or without TNF-α or IL-1β was added to the lower chamber. The concentrations of both cytokines were adjusted to 12.5, 25, 50, 100, 150, and 200 ng/ml. After 24 h of incubation in a 37 °C, 5% CO_2 incubator, the cells transferred to the lower side were fixed with 95% ethanol for 5 min and then stained with crystal violet. The total number of migrated cells was counted at 200x magnification in 5 random fields.

Spinal cord injury and cell transplantation

BM-PACs were seeded in 90-mm Petri dishes 1 week before transplantation. At 90% confluence, BM-PACs were collected and stained with Vivotack680 (PerkinElmer, Spokane, WA, USA) according to the instructions to track the distribution of transplanted cells *in vivo*. BALB/c-nu/nu mice were anesthetized with 2% isoflurane. The vertebral column was exposed between T9 and T11, and the lamina at T10 was completely removed. Severe spinal cord injury was induced by an 80 kdyn force using an Infinite Horizon Impactor (Precision Systems and Instrumentation, Lexington, KY, USA). The animals were then randomly assigned to the BM-PAC and control groups. For the BM-PAC group, 1×10^6 BM-PACs (suspended in 100 μl of the vehicle (DMEM)) labeled with Vivotrack680 were administered from the femoral vein within 6 h post-SCI. For the control group, 100 μl of the vehicle was injected. For pain management and infection prophylaxis, each animal received a subcutaneous injection of lactated Ringer's solution (50 ml/kg) containing buprenorphine (0.5 mg/kg) and

enrofloxacin (5mg/kg), and their bladders were manually expressed twice daily until voluntary urination was established.

In vivo fluorescence imaging

All mice administrated BM-PACs labelled with Vivotrack680 were subjected to *in vivo* fluorescence imaging under general anesthesia. Intravenously administered cells were tracked using an *in vivo* imaging system (IVIS; Spectrum, Caliper Life Science, Hopkinton, MA, USA) within 6 h, at 3 and 7 days after cell administration. Then, 7 mice in each group were sacrificed at 7 days after cell administration for immunohistochemistry (n = 3) and western blot (n = 4) analysis. The remaining 7 mice in each group were continuously used for *in vivo* fluorescence imaging and BMS scoring once a week up to 6 weeks after cell administration.

Western blot analysis

At 7 days after cell administration, 4 mice in each group were deeply anesthetized with overdose isoflurane and intracardially perfused with ice-cold saline. The injured spinal cord tissue (1-cm long) was then removed and transferred to 1 ml of lysis buffer containing 10 mM NaF, 2 mM Na_3VO_4, and protease inhibitor cocktail (Roche, Mannheim, Germany). The spinal cord was homogenized and centrifuged to collect the supernatants. A Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to adjust the protein concentration to 1 mg/ml, and 10 μl of the protein samples was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad, Hercules, CA, USA) containing 8% gel acrylamide for electrophoresis. The isolated proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% skim milk for 1 h at room temperature and then incubated with a rabbit monoclonal anti-HGF antibody (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or

mouse monoclonal anti-actin antibody (1:1000, EMD Millipore, Billerica, MA, USA) at 4 ℃ overnight. Thereafter, the membrane was washed and incubated with goat anti-rabbit or goat anti-mouse IgG antibody (1:10000, EMD Millipore, Billerica, MA, USA) for 1 h at room temperature. Immobilon Forte Western HRP substrate (EMD Millipore, Billerica, MA, USA) was used for chemiluminescent detection. The fluorescence signal was analyzed using the ChemiDoc XRS+ system (Bio-Rad, Hercules, CA, USA) with Image LabTM software.

Basso Mouse Scale (BMS)

Behavioral tests were performed using the Basso Mouse Scale (BMS) to evaluate the functional recovery of mice once weekly for 6 weeks after cell administration.

Histopathology

Under general anesthesia with overdose isoflurane, mice were transcardially fixed with 4% paraformaldehyde solution. The spinal cord was removed and fixed in 4% paraformaldehyde at 4 ℃ overnight. Then, the spinal cord tissue was dehydrated with 20% and 30% sucrose in sequence at 4 ℃ overnight. After dehydration, the spinal cords were embedded in an OCT compound (Sakura Finetek, Torrance, CA, USA) and cut longitudinally into 20-μm-thick sections. To identify HGF expression in BM-PACs at the injury site, immunostaining for HGF was performed using 1-week samples. Subsequently, slides were washed thrice with PBS and then blocked with 10% normal goat serum (NGS) at room temperature for 1 h. The slides were then incubated with anti-HGF antibody (1:100) at 4 °C overnight. Goat anti-rabbit IgG Alexa 488 (1:400, Abcam, Cambridge, UK) and DAPI (1:1000, PromoCell, Heidelberg, Germany) were added and incubated in the dark at room temperature for 1 h. Slides were mounted using ProLong Gold Antifade Mounting Medium (Invitrogen, Carlsbad, CA, USA).

Using 6-week samples, Luxol fast blue (LFB) staining was performed to measure the spared myelinated area. A picture of the 3-mm-long median sagittal plane at the lesion epicenter was captured using a 2× objective lens. LFB-positive areas were measured using ImageJ software (NIH, Bethesda, MD, USA). Spared axons and glial scar formation were evaluated by immunohistochemistry using the following antibodies: rabbit monoclonal anti-NF200 (1:1000; Sigma-Aldrich, St. Louis, MO, USA) and rat monoclonal anti-glial fibrillary acidic protein (GFAP; 1:200; Invitrogen, Carlsbad, CA, USA), followed by goat anti-rabbit IgG Alexa 488 and goat anti-rat IgG Alexa 586 (1:400, Abcam, Cambridge, UK). Slides with no primary antibody were used as negative control.

Statistical analysis

All data are presented as the mean ± standard deviation. One-way ANOVA followed by Tukey's multiple-comparisons test was used to analyze the results of the RT-PCR and chemotaxis assays. Dunnett's test was used for the ELISA experiments. Differences in BMS scores were assessed using the Mann-Whitney U test to identify differences between the BM-PAC and control groups. The remaining data were analyzed using the unpaired two-tailed Student's t-test. Differences were considered statistically significant at $p < 0.05$. SPSS statistical software version 22.0 (SPSS, Chicago, Illinois, USA) was used for analysis.

Results

TNF-α and IL-1β stimulation increased HGF secretion by BM-PACs and BMMSCs

BM-PACs and BMMSCs showed similar HGF expression at basal levels, and no significant difference was observed. After stimulation with TNF-α, HGF mRNA expression showed a tendency to upregulate in both cell types, however, a significant upregulation was only detected in BM-PACs

stimulated at 100 ng/ml TNF-α. After IL-1β stimulation, the expressions of *HGF* mRNA increased by over 4 times in BM-PACs and a significant upregulation was observed at 10 ng/ml IL-1β, whereas those of BMMSCs were relatively unchanged. (Fig. 1A)

HGF protein content in the supernatant was low before stimulation and significantly increased after stimulation with 100 ng/ml TNF-α and 10 ng/ml IL-1β. When the cells were stimulated with 10 ng/ml IL-1β, significantly larger amounts of HGF protein were detected in the culture supernatant of BM-PACs than in that of BMMSCs (Fig.1B).

Migration of BM-PACs toward TNF-α and IL-1β

The migration assay revealed that BM-PACs tended to migrate to higher concentrations of TNF-α (Fig. 2A). The lowest TNF-α concentration with a significant difference between the groups was 100 ng/ml (Fig. 2C). In contrast, IL-1β had no significant effect on the migration of BM-PACs (Fig. 2B, D).

Distribution of fluorescence signals of Vivotrack680-labbeled BM-PACs

As shown in Fig. 3A, within 6 h after transplantation, fluorescence signals were detected in the lungs. However, the signal intensity at the injured spinal cord became stronger after three days. The peak signal was observed 1 week after administration and became weaker after 2–3 weeks. No fluorescence signal was apparent after 4 weeks. To determine the specific location of the fluorescence signal in the organs, some mice were sacrificed one week after administration. As shown in Fig. 3B, the fluorescence signal was detected in the injured spinal cord, lungs, and liver.

HGF expression in the injured spinal cord

Immunostaining was performed using the spinal cords of mice sacrificed 1 week after administration. As shown in Fig. 4, Vivotrack680-labeled cells were observed at the lesion epicenter,

and HGF expression was observed. Western blot analysis further revealed that HGF concentration significantly increased in the injured spinal cord of the BM-PACs group when compared to that of the control group.

Functional outcome

After inducing SCI, all mice were in a paralyzed state of the hind limbs (score = 0). The BM-PAC group showed better recovery of motor function than the control group, in which spontaneous recovery was also observed. At the 2nd, 5th, and 6th week after transplantation, the scores of the BM-PACs group were significantly higher than those of the control group. At the 6th week after transplantation, the average score of the BM-PACs group reached nearly 4 points (mean score = 3.93), indicating that mice occasionally walked with weight bearing (Fig. 5).

Intravenous administration of BM-PACs improved tissue sparing in the injured spinal cord

Histological analysis of the injured spinal cord was performed 6 weeks after transplantation. The LFB-positive area in the BM-PAC group was significantly larger than that in the control group (Fig. 6A, B). Immunohistochemistry for NF200 and GFAP also revealed that the BM-PAC group tended to have more axons and less glial scar formation at the lesion epicenter than the control group.

Discussion

In this study, we first assessed HGF production in BM-PACs and BMMSCs following treatment with various concentrations of TNF-α and IL-1β. Although significant upregulations of *HGF* gene were detected only in BM-PACs treated with 100 ng/ml TNF-α and 10 ng/ml IL-1β, BM-PACs as well as BMMSCs secreted a considerable amount of HGF in response to those pro-inflammatory cytokines.

The discrepancy between gene and protein expression levels was probably because that *HGF* gene expression levels markedly increased at an earlier period after stimulation and gradually attenuated after 24 h, while HGF protein continuously accumulated in the medium. Although the expressions of receptors for TNF-α and IL-1β were not detected in this study, the significant HGF secretion in response to these cytokines suggested that TNF-α and IL-1β receptors may be present in both BM-PACs and BMMSCs. It has been also known that human MSCs secret HGF in response to comparable concentrations of TNF-α and IL-1β, and Zhang et al. reported that pre-incubation of human BMMSCs with neutralizing antibody for TNF receptor type II resulted in a significant reduction of *HGF* gene expression [17, 18]. The result of ELISA also suggested that IL-1β induces HGF production more strongly than TNF-α. Due to the difference of responsiveness to TNF-α and IL-1β may be caused by their expression levels, the expressions of the receptors for TNF-α and IL-1β in canine BM-PACs and BMMSCs should be further investigated.

The chemotaxis assay further proved that BM-PACs have a tendency to migrate towards higher concentrations of TNF-α, while these cells did not show a remarkable migratory ability toward IL1-β. Thus, systemically administered BM-PACs have the potential to migrate to an inflammatory site depending not on IL-1β but rather TNF-α concentration.

In vivo imaging analysis revealed that fluorescence signal was detected in the lung within 6 h after administration and distributed toward the lesion site and other tissues, including the lung and liver, within a week. This finding is consistent with previous studies demonstrating that intravenously administered rodent MSCs accumulate in the lungs and gradually move to the liver, spleen, kidney, and bone marrow within 48 h [22]. Further, immunohistochemistry of the lesion revealed Vivotrack680-labbeled cells showed apparent HGF expression. Recent studies have pointed out that the distribution of fluorescent signal indicates redistribution of host immune cells phagocytosed labeled cells [23], however, it was unlikely that host immune cells phagocytosed BM-PACs during systemic circulation

simultaneously expressed HGF in the lesion site. The fluorescent signal was detected at the lesion site as early as 3 days, peaked 7 days after administration, and gradually declined after 2 weeks. These findings suggested that BM-PACs were phagocytosed by host immune cells such as microglia and macrophages and eventually excreted from the host body. However, the fluorescent signals detected in the lesion strongly suggested that BM-PACs successfully migrated into the injured spinal cord.

It has been reported that TNF-α released by neurons and microglia significantly upregulated within 6 h post-injury [15, 16] and the protein concentration reached about 62 pg/mg after 6 h [24]. Bethea et al demonstrated that increase of TNF-α protein in the injured spinal cord persisted for up to 7 days after injury [25]. We demonstrated that the effective concentration of TNF-α to induce migration of BM-PACs was over 100 ng/ml. BM-PACs showed a tendency to migrate to lower concentrations of TNF-α, however, there are likely to be other factors that induce the migration of BM-PACs up to 7 days after SCI. CXCL-12, also known as stromal-derived factor 1α (SDF-1α), is a well-known chemoattractant of MSCs [26, 27] and is upregulated in lesions 7 days post-SCI [28]. IL-1β has also been shown to increase at the lesion site after 1 day and peak between 3 and 7 days after SCI [29]. Based on the results of the chemotaxis assay, IL-1β was unlikely to contribute to the migration of BM-PACs. However, Vivotrack680-labeled BM-PACs expressed HGF in the lesion, and western blot analysis also showed that HGF concentration in the lesion significantly increased when BM-PACs were administrated. These results suggest that the administered BM-PACs secreted HGF at the lesion site. The IL-1β protein concentration in the injured spinal cord persistently increased 3 to 7 days after SCI with a concentration of about 20 ng/g [30], at which concentration HGF secretion from BM-PACs could be expected.

The therapeutic assessment showed that systemic administration of BM-PACs enhanced functional recovery from 2 weeks after SCI, and mice showed a significant increase in the BMS score at 5–6 weeks after SCI. These results indicate that BM-PACs exert their beneficial effects between 1–2

weeks after injury, consistent with the increased HGF concentration at the lesion site. Within 2 weeks post-SCI, oligodendrocyte death is induced by oxidative stress, excitotoxicity, and inflammatory response, leading to demyelination [29, 31], and myelin sparing is closely related to motor function recovery [32]. HGF is a potent antioxidant and immunomodulator that can prevent the apoptosis of neurons and oligodendrocytes [14]. Our histological examination also showed that the myelinated areas were significantly increased in the BM-PAC group. Therefore, it can be speculated that after migration to the injury site, BM-PACs effectively reduced the demyelination process within 2 weeks of SCI through their HGF secretion ability. In addition, BM-PAC-treated mice showed higher NF-200 expression than that of the control group. This may be due to the neuroprotective effect of HGF, which reduces axonal loss [14]. Flavio et al. also reported that HGF could enhance the axonal growth rate [33]. Therefore, the observed NF-200-positive axons may indicate HGF-induced axonal regrowth. Moreover, we observed a reduction in glial scarring. This is consistent with previous reports that the administration of exogenous HGF during the acute phase of injury can reduce glial scar formation by inhibiting the secretion of TGF-β1 and -β2 from astrocytes [14].

Although the mechanisms underlying the therapeutic benefits were not fully elucidate, homing to the lesion site and secretion of tissue-protective factors including HGF probably play important roles in the process of functional and histological recovery. In this study, BM-PACs were systemically administrated within 6 h after SCI, it was likely that BM-PACs could migrate toward injured spinal cord during 7 days after SCI and secrete HGF in response to inflammatory milieu. Thus, optimal time window for administration of BM-PACs can be extend up to 7 days after SCI.

Taking into account the clinical application, the optimal administration methods should be also discussed. We used intravenous administration in this study, but other administration routs such as direct injection [34] and intraperitoneal injection [35] have been applied in the studies of cell-based therapy for various diseases. However, direct injection requires surgical technique and may cause a risk

of iatrogenic injury. According to the previous report of intraperitoneal injection of MSCs to mice experimentally induced inflammatory bowel disease, the therapeutic effect was lower when comparing with intravenous injection [35]. Thus, intravenous administration of BM-PACs is considered to be the most reasonable and appliable for canine patients with SCI.

Although the therapeutic benefit of intravenous administration of BM-PACs was functionally and histologically demonstrated, there remained some limitations for future clinical use. It remains challenging to obtain a sufficient number of BM-PACs from patients in the acute phase. Therefore, instead of autologous BM-PACs, the effects of allogeneic BM-PACs should be investigated further. Because we used an immunodeficiency nude mouse model to exclude host immune response toward canine BM-PACs, the possibility that therapeutic effects are attenuated when allogeneic BM-PACs are administrated to dogs with normal immune response is concerned. However, it is generally considered that MSCs have low immunogenicity due to a lack of major histocompatibility complex (MHC) II expression and low levels of MHC I expression [36]. We believe that BM-PACs also share these immunological characteristics of MSCs and a comparable therapeutic benefit can be obtained even by allogeneic BM-PACs.

In addition, although the motor function was significantly improved probably owing to HGF secretion from BM-PACs homed to the injury site, the treated mice barely regained to walk with weight bearing. This limitation in functional recovery was likely due to an irreversible loss of neuronal cells after severe SCI and it may require other combination therapies for further improvement. To date, there have been developed a variety of regenerative therapies for SCI, however, replacement of damaged neurons with exogenous neuronal cells derived from various stem cell such as induced-pluripotent stem cells and MSCs [37,38] can be a potential combination therapy with intravenous administration of BM-PACs.

In conclusion, this study demonstrated that canine BM-PACs have therapeutic potential in acute SCI. The abilities of BM-PACs, including homing to the injured spinal cord and HGF secretion in response to pro-inflammatory cytokines, including TNF-α and IL-1β, may impact therapeutic mechanisms. Although there is no direct relationship between the therapeutic effects and HGF secreted from BM-PACs, the therapeutic effects on histological and functional repair were consistent with previous reports, demonstrating the treatment mechanisms of HGF. Thus, systemic administration of BM-PACs is a possible regenerative therapy for acute SCI in dogs.

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Legends

Fig. 1. Changes in *HGF* mRNA and protein secretion of BM-PACs and BMMSCs after TNF-α or IL-1β stimulation. (A) The *HGF* mRNA expression of BM-PACs and BMMSCs was stimulated with TNF-α or IL-1β at different concentrations for 24 h. A significantly higher expression of *HGF* mRNA was induced in BM-PACs treated by 100 ng/ml of TNF-α or 10 ng/ml of IL-1β. (B) Under stimulation with 100 ng/ml of TNF-α or 10 ng/ml of IL-1β, BM-PACs and BMMSCs secreted significantly greater amounts of HGF protein. When the cells were stimulated with IL-1β (10 ng/ml), a significantly larger amount of HGF protein was detected in the culture supernatant of BMMSCs compared with that of BMMSCs (*, # $p < 0.05$, *: vs. control, #: vs. BMMSCs, $n = 4$, each).

Fig. 2. Chemotaxis assay of BM-PACs toward TNF-α and IL-1β. (A-B) Cells that migrated toward the different concentrations of TNF-α or IL-1β were stained. (C) The number of migrated cells was counted. BM-PACs had a tendency to migrate towards a higher concentration of TNF-α and significant differences were observed at concentrations over 100 ng/ml of TNF-α. (D) No significant change of cell migration was observed when BM-PACs were stimulated by the different concentrations of IL-β (**$p < 0.01$, $n = 4$).

Fig. 3. Distribution of BM-PACs after intravenous administration into the nude mice with severe SCI. (A) Representative *in vivo* imaging system (IVIS) images of the mice (lateral and dorsal view) are shown. The fluorescence signal of transplanted BM-PACs was in the lung immediately after administration; however, the signal was intensified in the injured spinal cord area after 3 days and reached the maximum after 1 week. The signal became weaker after 2–3 weeks and almost invisible after 4 weeks. (B) Representative IVIS images of the spinal cord lesion, lung and liver 1 week after administration. The fluorescence signal was observed in the injured spinal cord as well as the lung and liver.

Fig. 4. Immunohistochemistry and western blot analysis to determine the HGF expression at the lesion epicenter. (A) A representative immunohistochemistry image of the injured spinal cord was obtained from the BM-PACs group (a median sagittal section). (B) Magnified images of the lesion epicenter. The red fluorescent cells representing BM-PACs labeled with Vivotrack680 are observed in the injured spinal cord. As indicated by arrowheads, Vivotrack680-positive cells express HGF. (C) Western blot analysis to determine the HGF expression in the lesion site of the BM-PAC and control groups. A representative image of the immunoblot for HGF is shown. The HGF concentration in the injured

spinal cord tissue was increased about four times in the BM-PACs group compared with that in the control group (Bars = 50 μm, *p < 0.05, n = 4).

Fig. 5. Functional recovery evaluated by Basso Mouse Scale (BMS) score. The BM-PAC group showed significantly better functional recovery than the control group, and the score reached nearly 4 points (mean score = 3.93) at 6 weeks after administration. Significant differences were observed at 2, 5, and 6 weeks after transplantation (*p < 0.05, n = 7).

Fig. 6. Histological evaluation of the injured area at 6 weeks after administration. (A) The myelinated areas were stained by LFB. (B) The LFB-positive area was significantly larger in the BM-PAC group than in the control group (*p < 0.05, n = 7). (C) A representative image of immunostaining for NF-200 and GFAP in the BM-PAC and control groups. The BM-PAC group tended to have more axons (NF-200) and less glial scar formation (GFAP).

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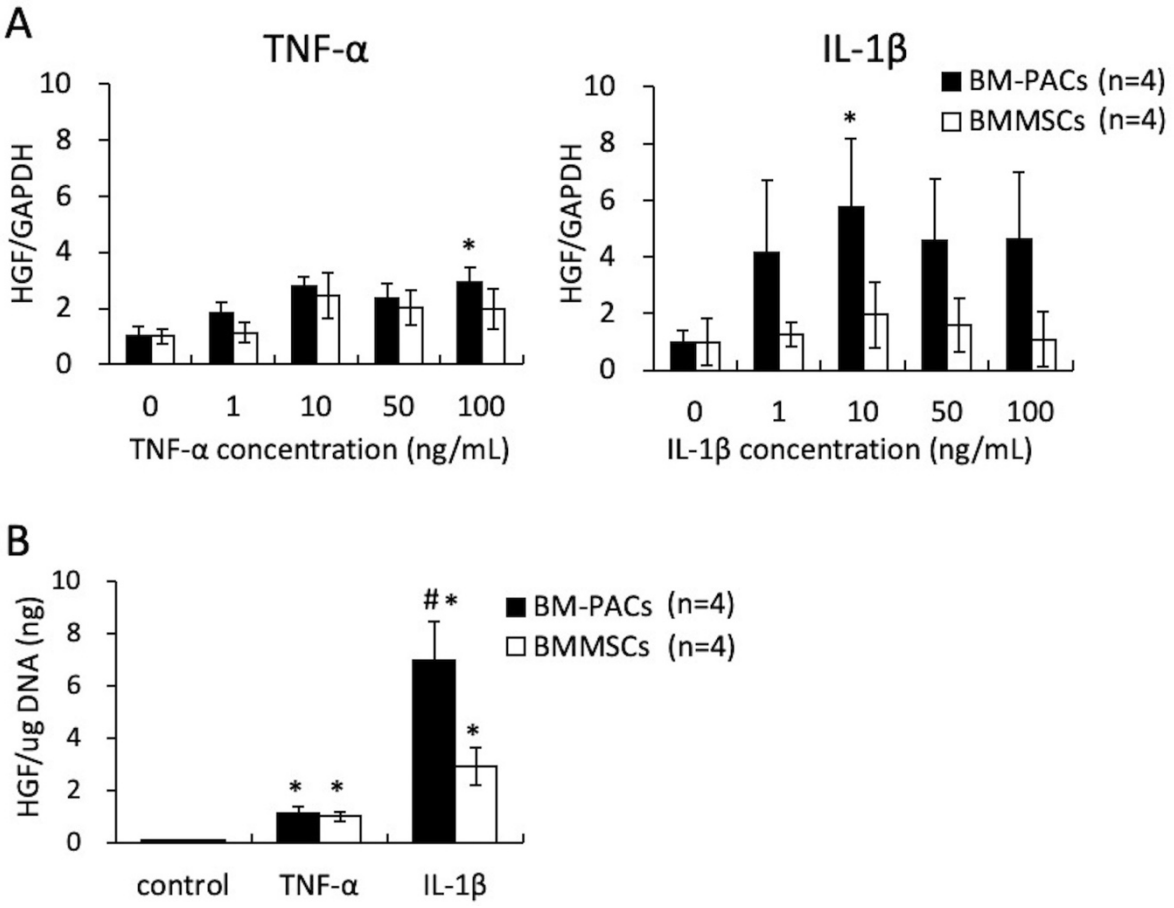
551
552

Fig.1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IL-1β
HGF/GAPDH
BM-PACs (n=4)
BMMSCs (n=4)
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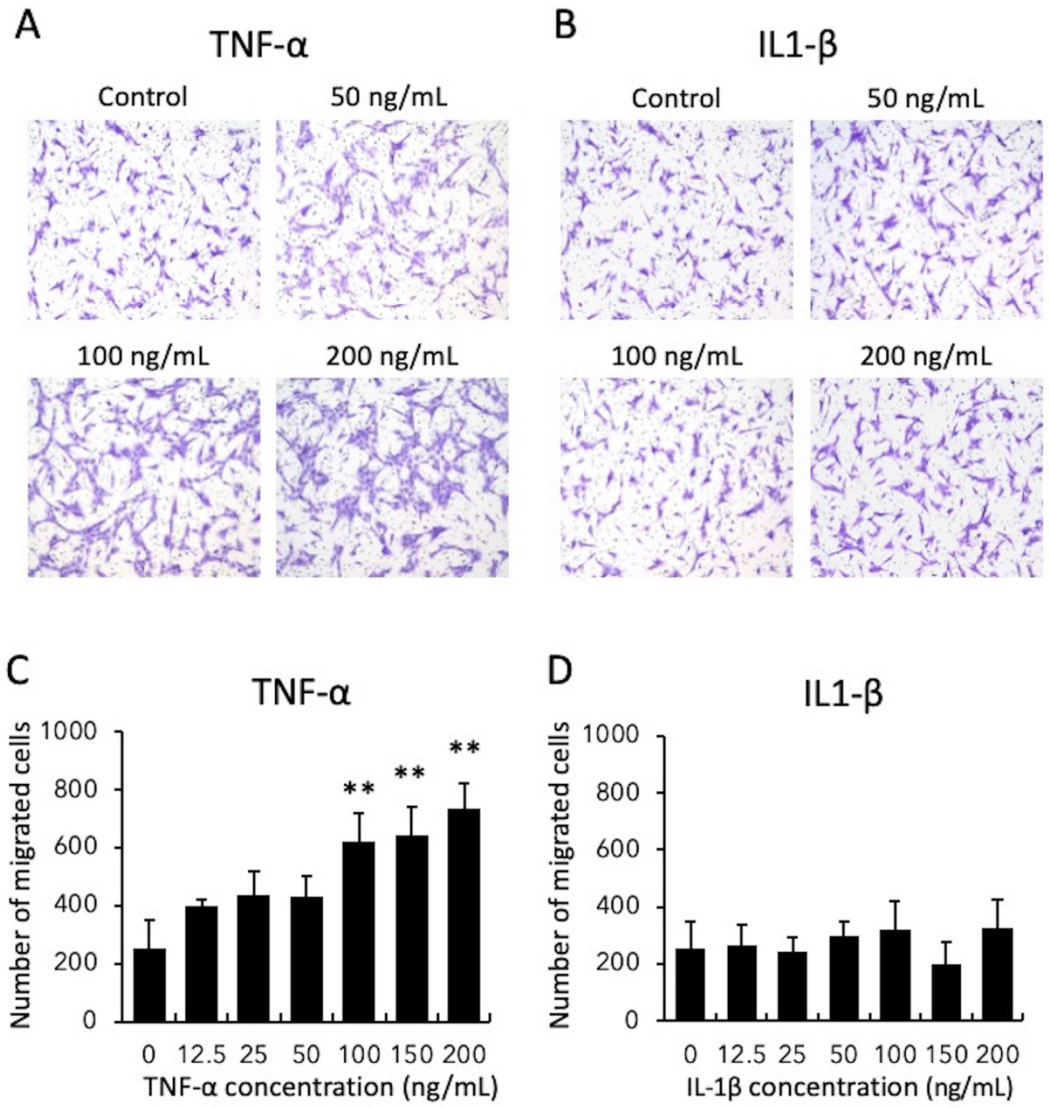
IL-1β concentration (ng/mL)
B
HGF/ug DNA (ng)
control
TNF-α
IL-1β

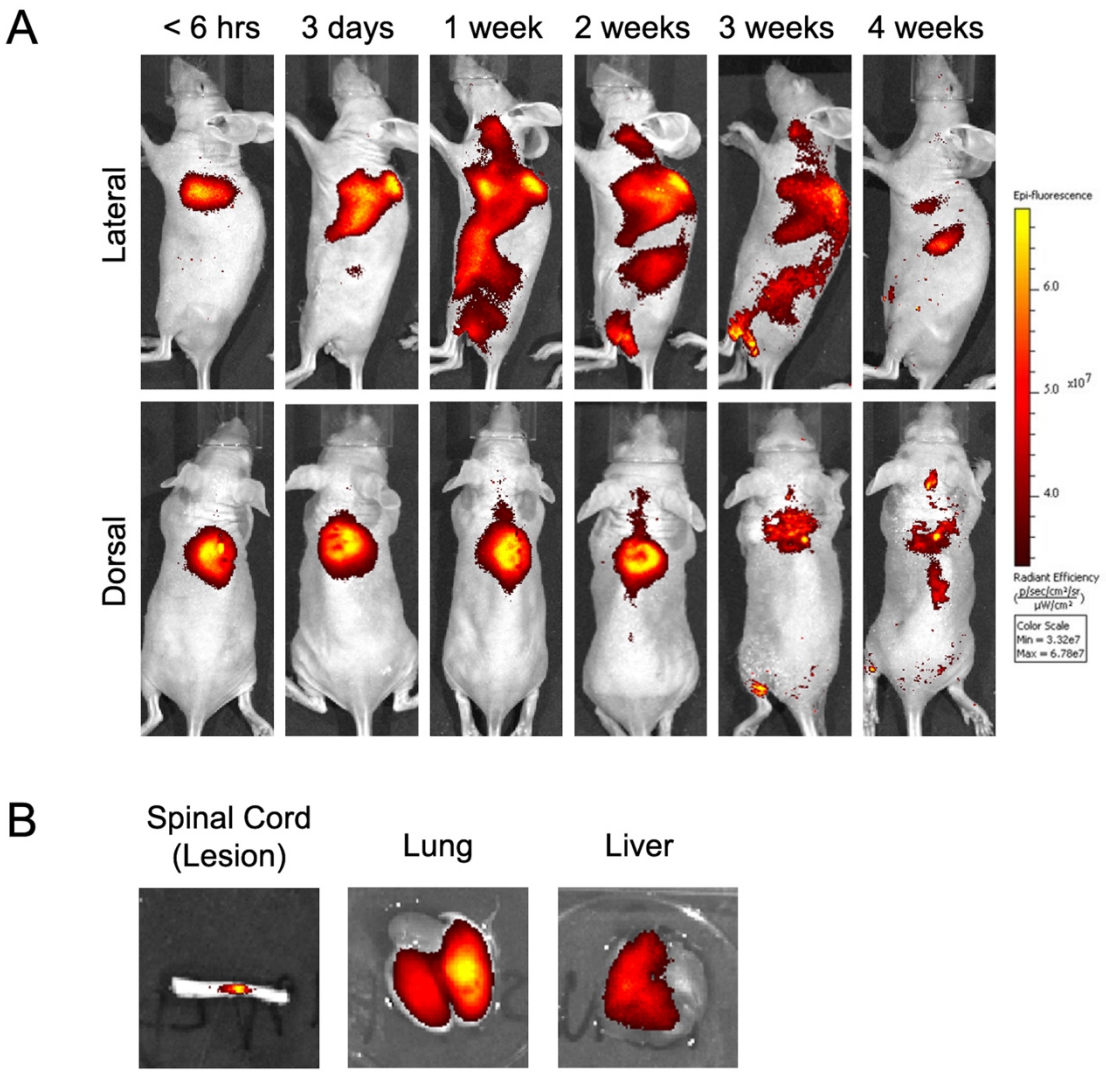
553

Fig.2


A
TNF-α
Control
50 ng/mL
100 ng/mL
200 ng/mL
B
IL1-β
Control
50 ng/mL
100 ng/mL
200 ng/mL
C
TNF-α
Number of migrated cells
1000
800
600
400
200
0
**
**
**
0 12.5 25 50 100 150 200
TNF-α concentration (ng/mL)
D
IL1-β
Number of migrated cells
1000
800
600
400
200
0
0 12.5 25 50 100 150 200
IL-1β concentration (ng/mL)

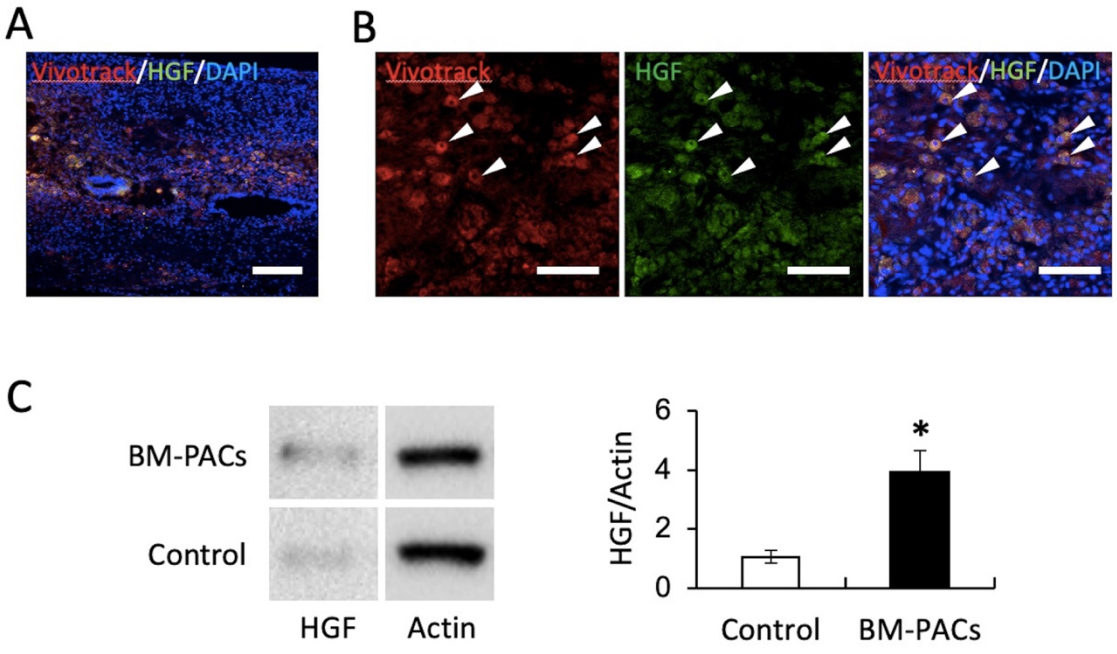
554

Fig.3


A
< 6 hrs
3 days
1 week
2 weeks
3 weeks
4 weeks
Lateral
Dorsal
Epi-fluorescence
6.0
5.0
x10^7
4.0
Radiant Efficiency
Color Scale
Min = 3.32e7
Max = 6.78e7
B
Spinal Cord
(Lesion)
Lung
Liver

555

Fig.4


A
Vivotrack/HGF/DAPI
B
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HGF
Vivotrack/HGF/DAPI
C
BM-PACs
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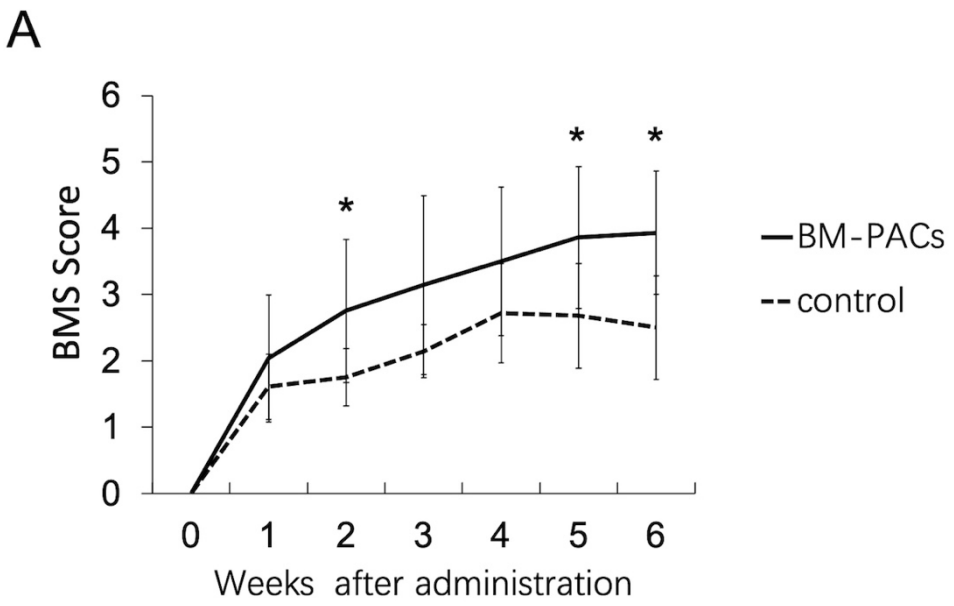
6
4
2
0
*
Control
BM-PACs

556

Fig.5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BMS Score
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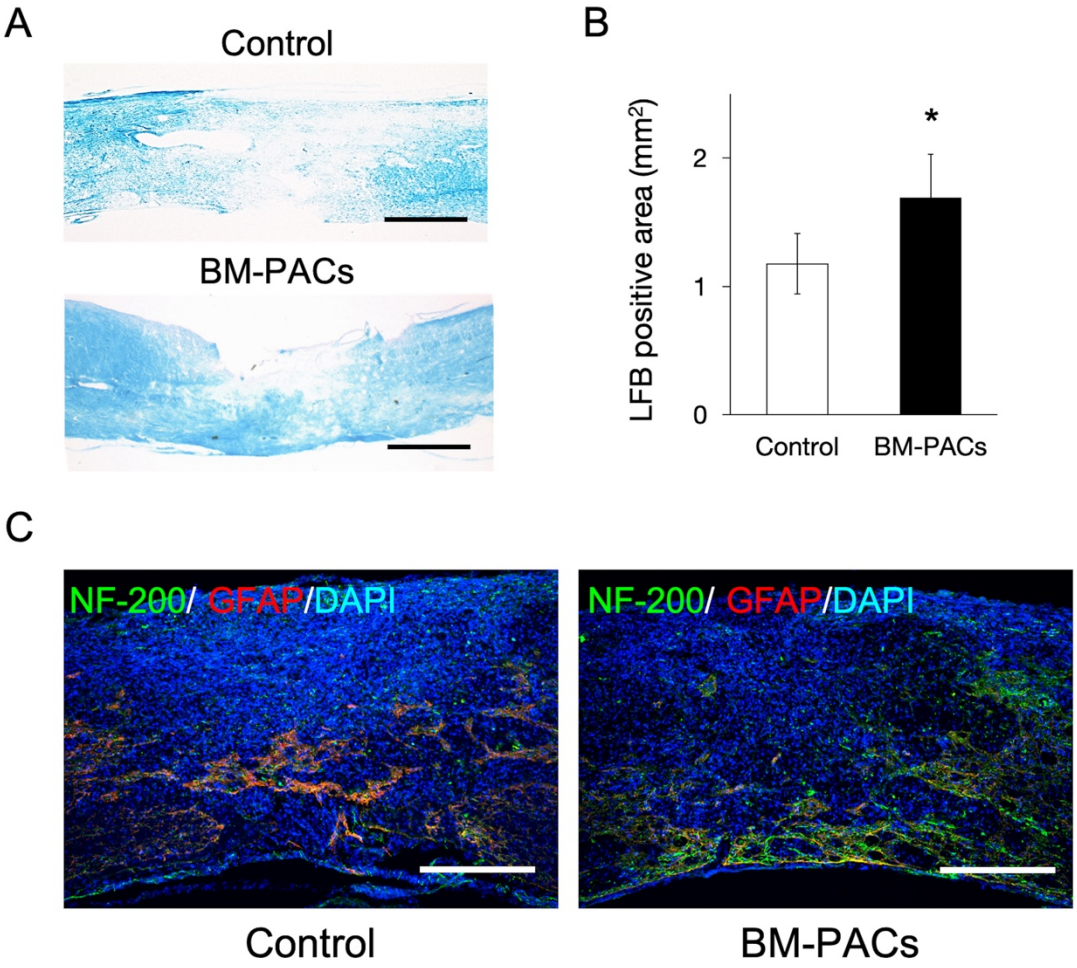
2
3
4
5
6
Weeks after administration
*
*
*
BM-PACs
control

557

Fig.6


A
Control
BM-PACs
B
LFB positive area (mm²)
2
1
0
*
Control
BM-PACs
C
NF-200/ GFAP/DAPI
NF-200/ GFAP/DAPI
Control
BM-PACs