TITLE PAGE

Facilitation of chemotaxis activity of mesenchymal stem cells via stromal cell-derived factor-1 and its receptor may promote ectopic ossification of human spinal ligaments

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Abbreviations

MSC, mesenchymal stem cell; SDF-1, stromal cell-derived factor-1; CXCR4, CXC chemokine receptor

4; OLF, ossified ligamentum flavum; FCS, fetal calf serum; SDS, sodium dodecyl sulfate

ABSTRACT

Mesenchymal stem cells (MSCs) have been utilized to elucidate the pathogenesis of numerous diseases. Our recent study showed that MSCs may conduce to the ossification of spinal ligaments. Stromal cell-derived factor-1 (SDF-1) and CXC chemokine receptor 4 (CXCR4) regulate MSC migration. Moreover, their expression is elevated in sites of damage and remodeling in pathological states. We explored the possible role of the SDF-1/CXCR4 axis in chemotactic behavior of MSCs in ossification of spinal ligaments. Specimens of thoracic vertebra ossified ligamentum flavum (OLF) and non-OLF plaques were received from patients whom we had performed spine surgery. Paraffin-embedded tissue sections were prepared for immunohistochemical staining. Cultured MSCs from the ligamentum flavum were prepared for *in vitro* analyses. We observed SDF-1 and CXCR4 localization immunohistochemically in the perivascular area and collagenous matrix of ligaments and in chondrocytes near the ossification front of OLF. And then, immunohistochemical staining showed a close relationship between MSCs and the SDF-1/CXCR4. In the *in vitro* analyses, expression of the SDF-1/CXCR4 and the migratory capacity of MSCs in OLF were remarkably higher compared with non-OLF. Furthermore, the migration of MSCs was up-regulated by SDF-1 and down-regulated by treatment with AMD3100, a specific antagonist for CXCR4. All *in vitro* test data showed a significant difference in MSCs from OLF compared with non-OLF. Our results reveal that the SDF-1/CXCR4 axis may contribute to an MSC-mediated increase in the ossification process, indicating that SDF-1/CXCR4 axis may become a potential target for a novel therapeutic strategy for ossification of spinal ligaments.

INTRODUCTION

Ectopic ossification occurs in human ligaments of spine, resulting in spinal canal narrowing and eventually evoke spinal cord injury and myelopathy with patients suffering from various degrees of neurological symptoms. In severe cases, surgery may be required even if it is still associated with difficult situations such as a higher risk of neurological complications (Choi et al., 2005). Therefore, development of a safe and effective pharmacotherapy to control ectopic ossification is necessary. Up to the present, the cause of ectopic ossification of spinal ligaments has been proved in a widespread way and connected to a variety of factors such as epidemiology, genetics, metabolism, and mechanics (Furukawa, 2006). However, pharmaceuticals having effectiveness for ossification therapy and/or advance suppression is still not clear.

Mesenchymal stem cells (MSCs) have been found in several tissues including, muscle, bone marrow, adipose tissue, synovium, cartilage and ligament. The isolated cells were used for not only tissue repair and regeneration, but also to make clear the pathogenesis of various diseases such as vascular calcification and calcifying tendinopathy (Segawa et al., 2009; Yee et al., 2011). Our previous study showed that ossified ligamentum flavum (OLF) has remarkable accumulation of MSCs compared with non-OLF (control), and chondrocytes around the ossification front in OLF express MSC markers (Chin et al., 2013). Furthermore, an enhancement in the osteogenic differentiation potentiality has been found in MSCs from ossified ligament samples. This leaning toward the osteogenic lineage may be causality in the ossification (Harada et al., 2014; Chiba et al., 2015). Based on these findings, MSCs are thought to be involved in the process of ectopic ossification occurs in spinal ligaments.

Stromal cell-derived factor-1 (SDF-1) and its receptor, CXC chemokine receptor 4 (CXCR4) are expressed in several tissues, and the SDF-1/CXCR4 axis is related to a range of physiological processes such as neovascularization and MSC migration and cytokine secretion (Mirshahi et al., 2000; Petit et al., 2007). Overexpression of SDF-1 and CXCR4 has been found in sites of active damage and remodeling in pathological states (Zgraggen et al., 2014), and a specific CXCR4 antagonist, AMD3100, is used in clinical pathology. At this point, we hypothesized that the SDF-1/CXCR4 axis might have an important influence in ectopic ossification of spinal ligaments. One possibility is that the SDF-1/CXCR4 axis regulates chemotactic behavior of MSCs in ossified ligaments, resulting in MSC migration and ossification. These findings would allow a large insight into the target site for medical treatment.

Therefore, we investigated the both of SDF-1 and CXCR4 expressions in spinal ligaments and the potential effect of the SDF-1/CXCR4 axis on migration by treatment with SDF-1 and AMD3100 in MSCs from OLF and non-OLF. The results we obtained revealed a significant role of the SDF-1/CXCR4 axis in accumulation of MSCs in ossified ligaments, and that the modulation of the SDF-1/CXCR4 axis may be a novel strategy of treatment for ossification of spinal ligaments.

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MATERIALS AND METHODS

Spinal ligament samples

Spinal ligament samples of the ligamentum flavum (LF) were collected *en bloc* from 12 patients who received surgery at the thoracic vertebral level. The samples of ossified LF were obtained from 6 patients (4 males and 2 females; mean age at surgery: 69 years; range: 56–77 years) who had posterior decompression surgery for OLF. As a control, non-ossified LF samples were obtained from 6 patients (5 males and 1 female; mean age: 50 years; range: 22–81 years) who had posterior surgery for spinal tumor, syringomyelia or burst fracture. No patients had evidence of congenital osteoarthropathy or rheumatism. Study protocol was approved by the Human Ethics Review Committee of Hirosaki University Hospital and sufficient informed consent was obtained from each patient.

Cell isolation and culture

We isolated MSCs as described previously (Harada et al., 2014). Cells were identified as MSCs by fluorescent-activated flow cytometry. MSCs were stained using an anti-human CD34 mouse antibody coupled with PE and an anti-human CD105 mouse antibody coupled with PerCP-Cy5.5, respectively (BD Biosciences, San Jose, CA, USA). Cells at first passage (1×10^6) were harvested and washed with phosphate-buffered saline (PBS) containing 20 µl/ml fetal calf serum (FCS). Cells suspended in PBS (50 µl) containing specific antibodies were incubated for 45 min on ice. The cells were washed with PBS, filtered through a 35-µm strainer (BD Bioscience, Bedford, MA), and suspended in PBS (1 ml), and analyzed by using a FACSAria™ II (BD Biosciences). For cell sorting, gates were defined as negative for

CD34 and positive for CD105 according to the intensity of fluorescence from the isotype control. Data

were analyzed by using BD FACSDiva™ software v6.1.3 (BD Biosciences).

Preparation of tissue

Non-ossified and ossified LF were fixed with formaldehyde (100 mg/ml) at 4°C for 1 week.

Samples of ossified LF were further decalcified with KC-X solution (Falma, Tokyo, Japan) for about 4

days. Then, serial 4 μm-thick sections were prepared using paraffin-embedded specimen and processed to

hematoxylin and eosin staining and immunohistochemistry.

Immunohistochemical staining

Immunohistochemical analysis was accomplished as described previously (Chin et al., 2013).

Briefly, antigen retrieval and blocking of non-specific protein binding were performed after

deparaffinization of sections. Then, the sections were incubated overnight at 4°C with a single primary

antibody or a mixture of two primary antibodies diluted in 10 mg/ml BSA. Primary antibodies against

MSC markers included a mouse monoclonal anti-CD73 antibody (1:50 dilution; Abcam, Cambridge, MA,

USA), rabbit polyclonal anti-CD90 antibody (1:100 dilution; Abcam), and mouse monoclonal

anti-CD105 antibody (1:50 dilution; Dako, Tokyo, Japan). A mouse monoclonal anti-SDF-1 antibody

(1:50 dilution; Abcam); rabbit polyclonal anti-SDF-1 antibody (1:50 dilution; Abcam); mouse

monoclonal anti-CXCR4 antibody (1:100 dilution; Abcam), and rabbit polyclonal anti-CXCR4 antibody

(1:500 dilution; Abcam) were used to detect the chemokine SDF-1 and its receptor, CXCR4.

For single staining, the sections were treated with secondary and tertiary agents, and then N, N′

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-diaminobenzidine was used to visualize peroxidase deposition at antigenic sites. Finally, the sections were lightly counterstained with hematoxylin. And then, we calculated the prevalence of SDF-1 and CXCR4 positive cells in the collagenous matrix area. The ratio of positive cells to nucleated cells was defined prevalence.

For double staining, sections were incubated with antibodies with green and red-fluorescent dyes, and finally the sections were lightly counterstained with DAPI. After staining with double immunofluorescence, the sections were observed under a confocal laser scanning microscope (Digital Eclipse C1si/C1 plus; Nikon Instruments, Japan). Image data was saved as raw TIFF files without further thresholding or filtering (e.g., without background subtraction).

CXCR4 expression analysis by using real-time polymerase chain reaction (PCR)

Total RNA was extracted from cultured MSCs using an RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. Genomic DNA contamination was eliminated by DNase treatment. First-strand cDNA was synthesized from total RNA (1 μg) using a standard random hexamer priming technique. Real-time PCR was carried out with Power SYBR Green PCR Master Mix on an ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Specific primer pairs for CXCR4 [GenBank accession no.: NM_003467; forward primer (5'-3'): CTGCCCTCCTGCTGACTA; reverse primer (5'-3'): GGGTAGAAGCGGTCACAGA] were designed by using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Conditions for PCR reaction were: 95°C for 1 min, and then 50 cycles of 95°C for 15 s and 60°C for 1 min. All samples were analyzed in parallel for glycerol 3-phosphate dehydrogenase (G3PDH) expression as an internal standard.

The data of real-time PCR experiments were represented as Ct values. The Ct value is defined as the

cycle number at which the amount of PCR product exceeds the threshold level. We used the comparative

Ct method to compare mRNA expression in samples with that in the control in each experiment.

Reactions were carried out in triplicate for each cell sample. Results are expressed as the mean ± standard

deviation (SD) (n = 6). Graphs depict relative expression levels compared with the control (non-OLF

MSCs) unless otherwise indicated.

CXCR4 expression analysis by Western Blotting

After MSCs from non-OLF and OLF patients (6 cell samples each) reached confluency, the cells

were lysed in RIPA buffer [250 mM Tris-HCL, pH 7.6, 150 mM NaCl, 10 mg/ml NP-40, 10 mg/ml

Na-deoxycholate, and 10 mg/ml sodium dodecyl sulfate (SDS)]. After a Bradford protein assay, proteins

samples (3 μg each) were resolved by SDS/polyacrylamide gel electrophoresis and then transferred to

polyvinylidene difluoride membranes (Immobilon-FL; Millipore Corporation, Billerica, MA, USA). The

membranes were blocked with 1 mg/ml dry skim milk in PBS with 1 μl/ml Tween 20 and incubated with

primary antibodies against CXCR4 (rabbit polyclonal antibody, 1:500 dilution; Abcam) or β-actin (rabbit

polyclonal antibody, 1:1000 dilution, Abcam, UK) and then an Alexa Fluor 680-labelled goat anti-rabbit

secondary antibody (1:1000; Abcam). Proteins were detected using the Odyssey Imaging System

(LI-COR Biosciences, Lincoln, NE, USA).

Cell migration analysis

Cell migration was assayed by the scratch wound-healing method using an *in vitro* incisional

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wound model as reported previously (Liang et al., 2007). MSCs were cultured in 6-well plates (BD

Biosciences, New Jersey, USA) with α-minimum essential medium (MEM) containing 100 µl/ml FCS. At

80% confluence, FCS in the medium was reduced to 20 µl/ml to avoid cell proliferation during the assay

period. After 1 day of incubation, scratch wounds of 1 mm in width were created in confluent monolayers

by using an aseptic 1000 µl pipette tip. After detached cells were washed out, the wounded monolayers

were cultured for 24 h. Images of the wound area were obtained at 0 and 24 h using a phase contrast

microscope (OLYMPUS, Tokyo, Japan). The area between the wound edges was analyzed using NIH

ImageJ software (Bethesda, Maryland, USA). The wound area was defined as: wound healing (%) = [1-

(wound area (t = 24 h)/wound area (t = 0 h))] × 100. Data was averaged over six independent experiments.

Each experiment was repeated 3 times. MSCs were treated with CXCR4 inhibitor AMD3100 (Abcam,

Cambridge, UK) at a final concentration 300 nM to determine the dependency of migration on CXCR4.

Cell chemotaxis assay

Chemotactic migration was evaluated as described previously (Mori et al., 2012) in chemotaxis

chambers (Chemotaxicell, Kurabo, Osaka, Japan) inserted into a 24-well culture plate. MSCs (2×10^4)

were spread into the upper chamber, and the lower chamber was filled with PBS with or without SDF-1 at

various dosages (10, 30, and 100 ng/ml) in α-MEM containing 20 µl/ml FCS. The cells were incubated

for 48 h at 37°C with 5% CO_2. The chambers were washed two times with PBS and then fixed with 100

mg/ml formaldehyde for 5 min, followed by washing two times with water. The chamber was stained

with methyl green for 20 min, washed three times with water, and then the number of stained cells in the

filter, which had migrated from the upper chamber, was counted under a microscope.

Statistical analysis

All data analyses were performed by KyPlot 5.0 software (Kyenslab, Tokyo, Japan). Differences among groups were analyzed using one-way analysis of variance with the Student-Newman-Keuls post-hoc correction procedure. *P*-values less than 0.05 were regarded as statistically significant.

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RESULTS

Localization of SDF-1 and CXCR4 in spinal ligament tissues

SDF-1 and CXCR4 distributions in spinal ligaments were analyzed immunohistochemically. Figure 1 shows representative images. Single immunostaining showed SDF-1 expression and both SDF-1 and CXCR4-positive cells around blood vessels in both tissues of OLF and non-OLF (control). In non-OLF samples, little expression of SDF-1 and a small number of SDF-1 and CXCR4-positive cells were observed poorly in the perivascular area (Fig. 1A). Conversely, in OLF tissues, there was abundant angiogenesis, and high expression of SDF-1 and great numbers of SDF-1 and CXCR4-positive cells were observed around blood vessels (Fig. 1A). Next, we examined SDF-1 and CXCR4 expressions within the collagenous matrix. In control non-OLF tissues, a small number of fibroblast-like cells were observed in the collagenous fibers with regular arrangement. Moreover, only few fibroblast-like cells were positive for SDF-1 and CXCR4. Conversely, numerous fibroblast-like cells were positive for SDF-1 and CXCR4 in OLF tissues. In addition, the expression of SDF-1 was observed at a very low level in control non-OLF tissues in comparison with its strong expression in OLF tissues among the irregularly arranged and fragmented collagenous fibers (Fig. 1B and 1D). In OLF tissue samples, we observed intense expression of SDF-1 and a large number of chondrocyte-like cells expressing both of SDF-1 and CXCR4 around the ossification front (Fig. 1C).

Expression change of the SDF-1/CXCR4 axis in MSCs

Because high expression of SDF-1 and CXCR4 was observed in OLF tissues, which was similar

to our previously described expression change in MSCs (Chin et al., 2013), we investigated the relationship between SDF-1 or CXCR4-positive cells and MSCs, which are identified by the expression of CD73, CD90 and CD105, in collagenous matrix and the ossification front of OLF samples. Double immunofluorescence staining was performed for SDF-1/CD73, SDF-1/CD90, SDF-1/CD105, CXCR4/CD73, CXCR4/CD90, and CXCR4/CD105. The results showed co-localization of SDF-1 or CXCR4 -positive cells and MSC marker-positive cells (Fig. 2A). To confirm these discoveries, we assayed the CXCR4 mRNA levels by quantitative real-time PCR in non-OLF and OLF groups. CXCR4 mRNA was extremely expressed in MSCs from OLF samples, but it was expressed at a very low level in MSCs from non-OLF samples (Fig. 2B). Statistical analysis indicated a substantial increase in CXCR4 expression of MSCs derived from OLF than non-OLF ($p < 0.01$). Protein expression analysis showed a similar result (Fig. 2C).

Chemotaxis activity assay of the SDF-1/CXCR4 axis in MSCs

We evaluated the migratory capacity of MSCs derived from both non-OLF and OLF groups by a scratch wound assay. After 24 h of culture in the medium containing 20 μl/ml FCS to avoid cell proliferation, MSCs from OLF samples migrated into >45% of the scratched area, whereas MSCs from non-OLF samples migrated into <18% of the scratched area. It showed a significant difference between these ($p < 0.01$) (Fig. 3A). To find out the effect of activating the SDF-1/CXCR4 pathway on the regulation of MSC migration *in vitro*, we first determined the regulatory role of CXCR4 in MSC migration by the scratch wound assay. The migratory activity of MSCs derived from the non-OLF group pre-treated with AMD3100 showed a decrease in comparison with that of MSCs without AMD3100

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pre-treatment. Furthermore, MSCs derived from the OLF group showed a significant degradation of

migratory activity in response to AMD3100 ($p < 0.01$) (Fig. 3B). Next, we examined whether SDF-1

pre-treatment influenced the chemotaxis of MSCs toward the SDF-1 gradient in a transwell migration

analysis. After PBS or SDF-1 treatment for 24 h, the total number of MSCs that migrated through the

porous membranes of transwell chambers was obviously higher in the OLF group than the non-OLF

group in all treatments ($p < 0.01$). The higher migration ability of MSCs from the OLF group was

consistent with the results of the scratch wound assay. Additionally, 10 ng/ml SDF-1 pre-treatment

resulted in an approximately 2-fold increase in the number of migrated MSCs compared with the PBS

control. In addition, 30 and 100 ng/ml SDF-1 pre-treatment resulted in an increase of the MSC migration

ability in response to SDF-1 treatment in a dose-dependent manner in both non-OLF and OLF groups

(Fig. 3C).

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DISCUSSION

Immunodetection has identified the SDF-1/CXCR4 axis in multiple organs *in vivo*, which resides in both collagenous matrix and small blood vessels (Zgraggen et al., 2014; Liu et al., 2016). However, there was no report describing the detailed expression in human spinal ligaments *in situ*. Here, we show the existence of the SDF-1/CXCR4 axis in both non-OLF and OLF *in vivo*. Furthermore, we observed a high expression in ossified spinal ligaments from OLF patients, which is similar to the expression change in MSCs observed previously (Chin et al., 2013). Fibroblast-like cells in the collagenous matrix and chondrocyte-like cells around the ossification front, which may be derived from MSCs and contribute to ectopic ossification through endochondral ossification and aggregation of abnormal fibrocartilage or cartilaginous cells (Yee et al., 2011; Chin et al., 2013), expressed SDF-1 and CXCR4. Furthermore, double immunostaining showed the coexistence of SDF-1/CXCR4 and MSC markers. Real-time PCR and western blot analyses showed that the expression level of CXCR4 in MSCs from OLF was significantly higher than that in MSCs from non-OLF. These results suggest a close relationship between MSCs and the SDF-1/CXCR4 axis, and that they committed the presence/development of ectopic ossification.

The present results showed a large amount of vascularization and high expression of the chemokine SDF-1 and its receptor CXCR4 in OLF tissues. In addition, MSCs derived from OLF showed an increased migration ability in response to SDF-1. Vascular activation is also observed in chronic inflammatory diseases (Baluk et al., 2005), and various proinflammatory chemokines have been reported to exhibit angiogenic properties (Zgraggen et al., 2014; Kiefer et al., 2011). Particularly, the chemokine SDF-1 and its receptor, CXCR-4, have been found to have an angiogenic activity (Mirshahi et al., 2000; Pablos et al., 1999). Elevated expression of the SDF-1/CXCR4 axis and vascularization have been found

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in lesion sites. Blood vessels are thought to be an origin of MSCs, providing conclusive proof that neovascularization is related to heterotopy calcification of tissues (Bautch et al., 2011; Lounev et al., 2009). In our previous study, MSCs were not only in the perivascular area, but also diffused over ligament tissue in OLF, suggesting that MSC migration plays an important part in the ligament ossification process (Chin et al., 2013). Recently, it has been reported that the SDF-1/CXCR4 axis is an important regulator of stem cell migration and homing (Liu et al., 2016; Kokovay et al., 2010), and MSCs synthesize and secrete multiple paracrine factors that influence their own migration (Boomsma et al., 2012; Liang et al., 2012). Therefore, we suspect that SDF-1 is secreted from MSCs as a division of the repairing process caused by mechanical stress that leads to collagenous fiber rupture in spinal ligaments. In addition, we consider that the SDF-1/CXCR4 axis plays an important part in vascularization and accumulation of MSCs in neoplastic blood vessels and collagenous matrix through their migration.

MSCs are suspected to play an important part in the ectopic ossification process of OLF (Chin et al., 2013), and the SDF-1/CXCR4 axis is involved in a range of physiological processes such as endochondral bone development (Toupadakis et al., 2012; Murata et al., 2012). Moreover, an interesting study has proved that circulating bone marrow-derived osteoblast progenitor cells are recruited to the bone-forming site in a bone-forming model via the SDF-1/CXCR4 axis (Otsuru et al., 2008). Although the relationship between MSCs and the SDF-1/CXCR4 axis in ectopic ossification remains controversial, these reports and our results suggest that MSCs may be related to the ectopic ossification of OLF through up-regulation of the SDF-1/CXCR4 axis. The regulatory mechanism of MSCs and the SDF-1/CXCR4 axis in ectopic ossification should be pursued further in the future.

Increased expression of the SDF-1/CXCR4 axis has been reported in various diseases such as

colitis and arthritis, and these studies indicate the SDF-1/CXCR4 axis involved disease maintenance and progression (De Klerck et al., 2005; Mikami et al., 2008). Indeed, inhibition of CXCR4 exerts anti-inflammatory effectiveness in various inflammatory diseases (Lukacs et al., 2002). Furthermore, the experiment on animals showed the therapeutic effect of psoriasis (a chronic inflammatory skin disease) after treatment with the CXCR4 antagonist AMD3100, which is associated with decreased inflammatory vascularization and cell accumulation (Zgraggen et al., 2014). In the current study, we found that inhibition of SDF-1/CXCR4 axis signaling by AMD3100 significantly abolished the migration ability of MSCs in the OLF group, whereas no remarkable decrease was detected in the non-OLF group. These observations indicate that one of the noteworthy findings is that blocking CXCR4 is directly related to the reduced migration ability of MSCs derived from OLF samples. According to the above results, we suggest that the SDF-1/CXCR4 axis may serve as an up-to-date therapeutic target for ossification of spinal ligaments in reducing angiogenesis and MSCs accumulation. Future studies such as using the ossification of spinal ligaments model animal to investigate the *in vivo* ossification suppressant effect of the CXCR4 antagonist AMD3100 may elucidate the role of the SDF-1/CXCR4 axis and its relationship with MSCs in the ectopic ossification process of OLF.

Figure 4 depicts a possible role of SDF-1/CXCR4 in ectopic ossification of spinal ligaments. Authentic role of MSCs is a repair of tissue which has damaged by mechanical stress, inflammation, *etc.* (Maxson et al. 2012). The MSCs are promoted by SDF-1/CXCR4 axis and migrate into damaged area of the ligament, resulting in the recovery of the ligament. This process occurs in normal tissues. However, in OLF patient tissues, MSCs have become transformed one with osteogenic nature through genetic or epigenetic change (Chiba et al. 2015). Promoted MSCs into the damaged area evoked ossification of the

tissue instead of repairment. Furthermore, the transformed MSCs have lost an anti-angiogenic ability (unpublished our observation), then MSCs permit vascular invasion and facilitate ossification of ligament through endochondral ossification process (Yamamoto et al. 2002, Shi et al. 2016). It is supported by a clinical observation in which blood loss after surgery of patients with ossified spinal ligament was significantly higher than that of patients without ossification (Kishiya et al. 2009). This observation suggests an increase in angiogenesis in ossified spinal ligaments.

The first limitation of this study is the absence of sufficient data characterizing MSCs in the tissue sections. Confirmation of the CD73, CD90, and CD105 surface markers is required because MSCs have no unique antibody. In the future, four color staining procedure with CD73, CD90, CD105, and SDF-1 or CXCR4 will supply a more accurate representation of the relationship between MSCs and the SDF-1/CXCR4 axis. Second, AMD3100 only induced about a 50% decrease in wound healing of both OLF and non-OLF samples. Because there are other chemokine-chemokine receptor axes in addition to SDF-1-CXCR4 in the recruitment of MSCs, the potential roles of other chemokine receptors expressed by MSCs, such as transforming growth factor β receptors (Labour et al., 2016), should be considered in future studies.

In conclusion, our study showed that expression of the SDF-1/CXCR4 axis in human spinal ligaments was higher in OLF, and chondrocytes which exist in the ossification front were positive for SDF-1 and CXCR4. The migratory capacity of MSCs derived from OLF was remarkably higher than that of MSCs derived from non-OLF, which was up-regulated by SDF-1 treatment in a dose-dependent manner. Additionally, the migration activity of MSCs was down-regulated by pre-treatment with a CXCR4 inhibitor, particularly MSCs from OLF. These observations may suggest a role of the

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SDF-1/CXCR4 axis in MSC migration during the pathogenesis of OLF. We suspect that the

SDF-1/CXCR4 axis plays an important role in the ectopic ossification process of OLF.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Chin, Furukawa, Wada, Kumagai, Motomura, Ishibashi.

Conducted experiments: Chin, Furukawa, Kurotaki, Nagasaki.

Performed data analysis: Chin, Furukawa, Kurotaki, Nagasaki.

Wrote or contributed to the manuscript writing and editing: Chin, Furukawa, Wada, Kumagai, Motomura, Ishibashi.

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FOOTNOTES

The authors have no conflicts of interest directly relevant to the content of this article

LEGENDS FOR FIGURES

Figure 1. SDF-1 and CXCR4-positive cells in spinal ligaments. Non-OLF and OLF in the (A) blood vessel region (arrow: blood vessel) and (B) collagenous matrix. (C) The ossification front in OLF. (D) The prevalence in collagenous matrix from three samples per group. $*p < 0.01$ compared with the control (non-OLF group). H&E: hematoxylin and eosin staining; OLF: ossification of the ligamentum flavum. Scale bar = 50 μm.

Figure 2. Interrelationship between the SDF-1/CXCR4 axis and MSCs. (A) Merged images of double immunofluorescence staining for MSC markers and SDF-1 or CXCR4 in OLF. Fibroblast-like and chondrocyte-like cells around the ossification front are yellow and orange, respectively. (B) CXCR4 mRNA levels were analyzed by RT-PCR, and (C) protein expression of CXCR4 was evaluated by western blotting in MSCs isolated from non-OLF and OLF patients. Quantitative analysis of CXCR4 expression after normalization against β-actin. Results are presented as the mean ± SD (n = 6 in each group). Data are represented as the fold change of relative expression levels compared with the control (non-OLF MSCs). $*p < 0.01$ compared with the control (non-OLF group). OLF: ossification of the ligamentum flavum. Scale bar = 50 μm.

Figure 3. Chemotaxis activity assay of the SDF-1/CXCR4 axis in MSCs. (A) Migration activity of MSCs was analyzed by a wound healing assay. Results are presented as the mean ± SD of percentages of the wound healing size from 6 independent experiments in both non-OLF and OLF groups. $*p < 0.01$, compared with the control (non-OLF group). (B) MSCs were pre-treated with AMD3100 (300 nM) and

then analyzed by the wound healing assay to determine the dependency of migration on CXCR4. Results

are presented as the mean ± SD of percentages of the wound healing size from 6 independent experiments

in non-OLF AMD3100-untreated, non-OLF AMD3100-treated, OLF AMD3100-untreated, and OLF

AMD3100-treated groups. The percentages of the wound healing size in the AMD3100-treated samples

were compared with those in the untreated control. (C) MSC migration toward SDF-1 was measured in a

chemotaxis chamber containing various concentrations of SDF-1 (0, 10, 30, and 100 ng/ml). After

incubation, transmigrated MSCs were collected and counted. Results are presented as the mean ± SD

from 6 independent experiments in both non-OLF and OLF groups. Number of cells per 1 mm^2 was

calculated. OLF: ossification of the ligamentum flavum.

Figure 4. Possible role of SDF-1/CXCR4 in ectopic ossification of spinal ligaments
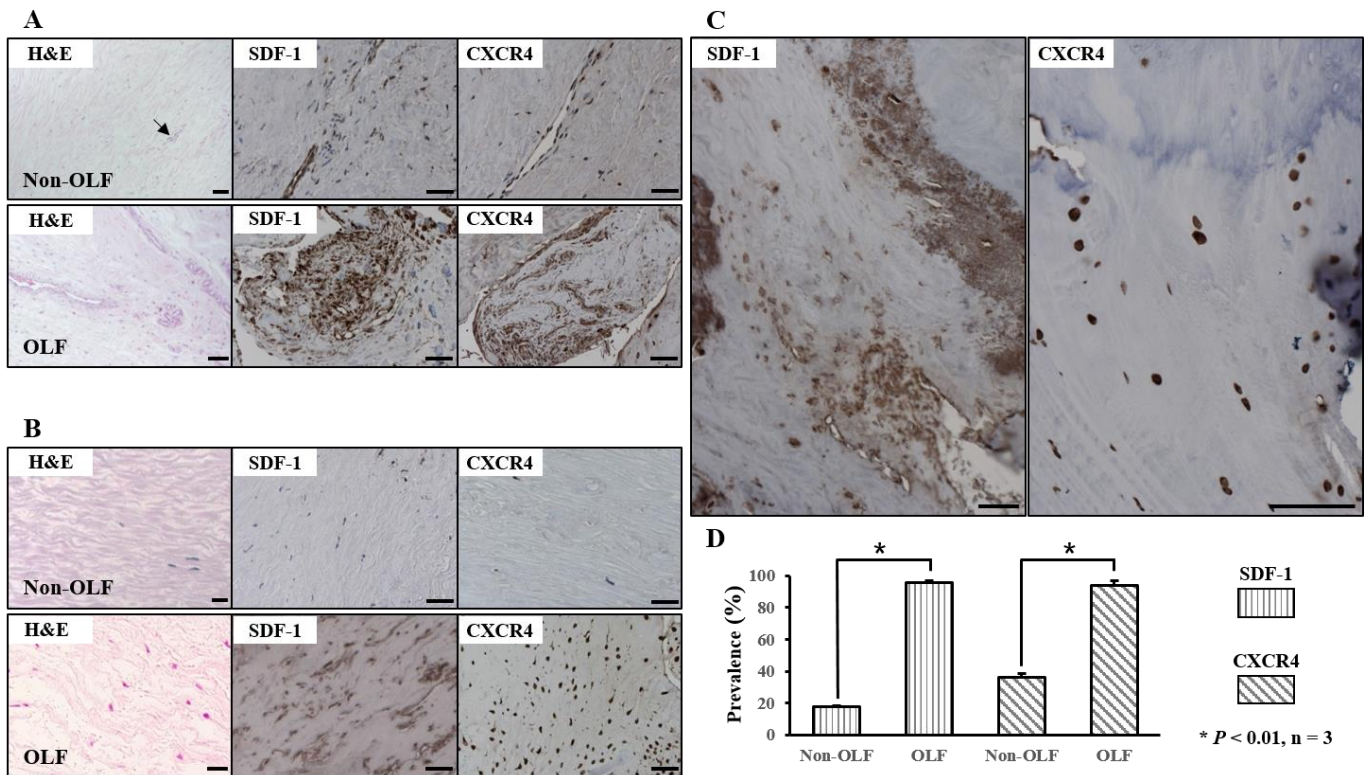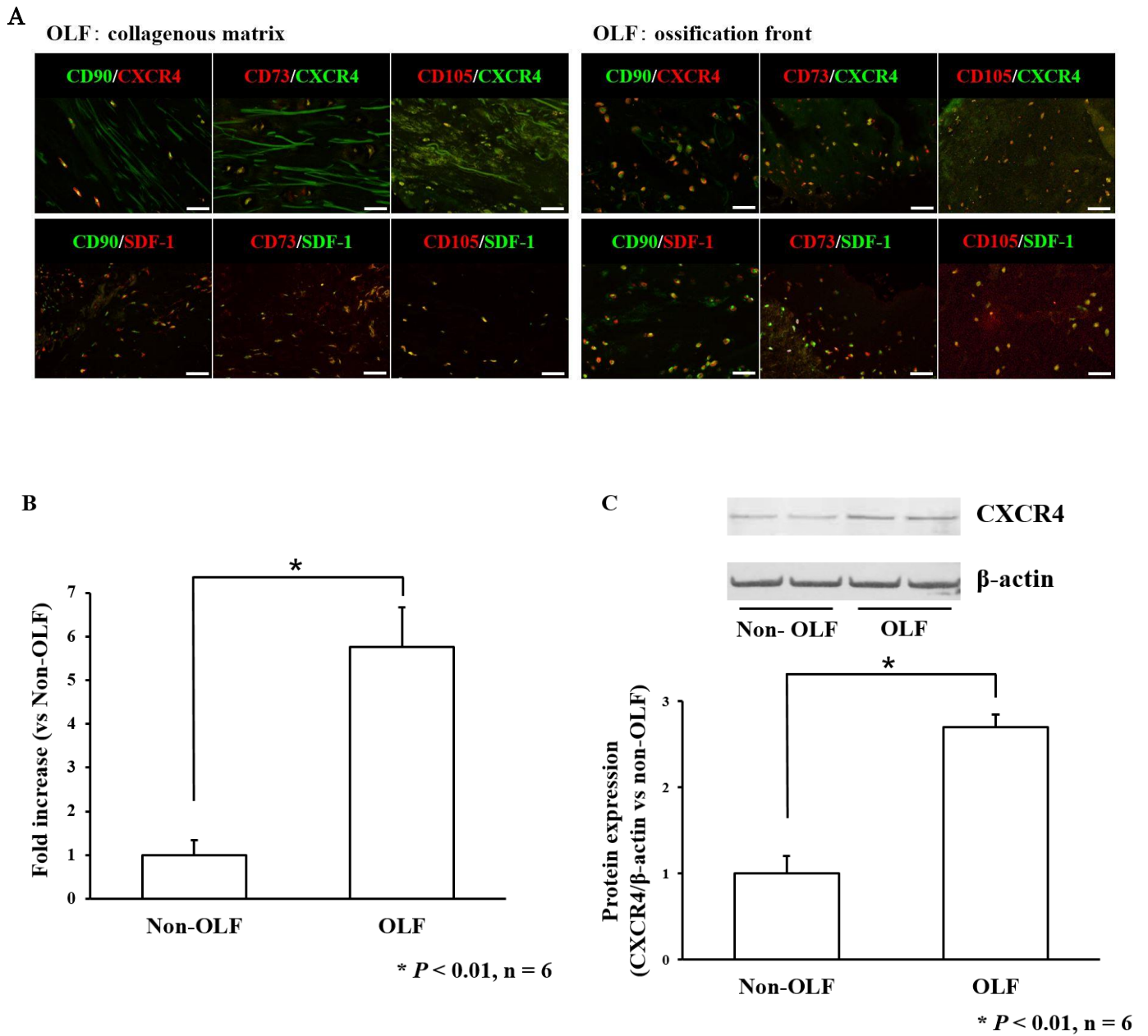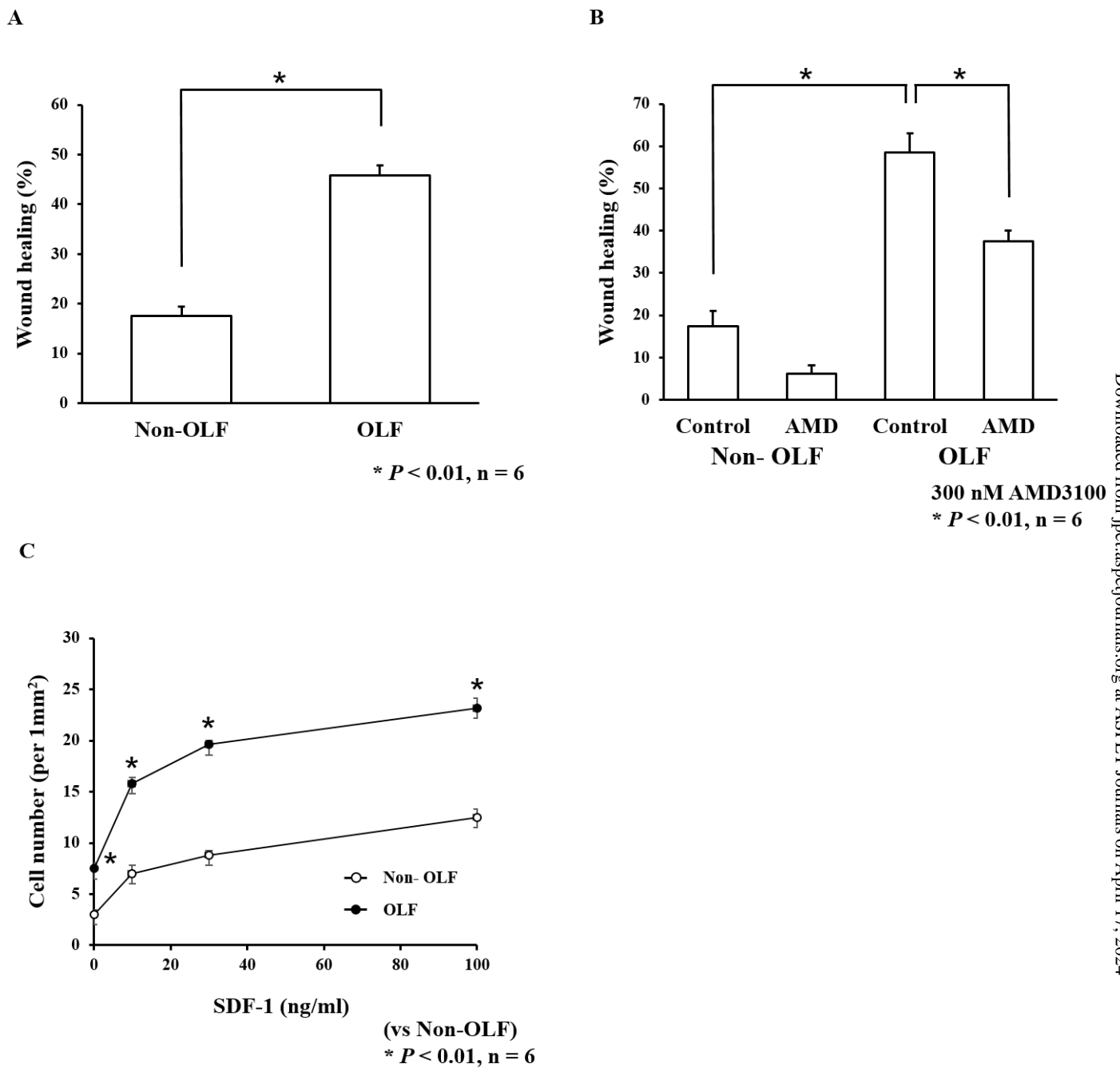
For detail, see Discussion.

Figures

Figure 1

Figure 2

A

B

C

Figure 3

A

B

* P < 0.01, n = 6

300 nM AMD3100
* P < 0.01, n = 6

C

(vs Non-OLF)
* P < 0.01, n = 6

Figure 4