Upregulated Stromal Cell-Derived Factor 1 (SDF-1) Expression and its Interaction With CXCR4 Contribute to the Pathogenesis of Severe Pterygia

Kyoung Woo Kim, Soo Hyun Park, Seung Hoon Lee, and Jae Chan Kim

Department of Ophthalmology, Chung-Ang University Hospital, Seoul, Republic of Korea

Correspondence: Jae Chan Kim, Department of Ophthalmology, Chung-Ang University Hospital, 224-1, Heukseok-dong, Dongjak-gu, Seoul 156-755, Republic of Korea; jck50ey@kornet.net.

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Purpose. Stromal cell-derived factor 1 (SDF-1) and its interaction with chemokine receptor 4 (CXCR4) have been noted for participating in the wound healing process, and may paradoxically develop hypertrophic scarring. With viewing pterygia as a product of exaggerated wound formation, we evaluated the effects of SDF-1 and CXCR4 on determining the severity of pterygia.

Methods. Human pterygial fibroblasts were cultured from excised tissues. Then, expression levels of SDF-1 and CXCR4 were assessed at both the mRNA and protein levels and analyzed with respect to the severity (grade T1 to T3) of pterygia. Expression patterns of SDF-1 and CXCR4 in pterygium tissues were evaluated by immunohistochemistry. Additionally, to investigate the SDF-1–induced myofibroblast transformation of pterygial fibroblasts, the correlation between SDF-1 and α-smooth muscle actin (α-SMA) expression levels was evaluated. Furthermore, α-SMA levels in pterygial fibroblasts were determined before and after knockdown of SDF-1 and blockade of CXCR4 by AMD3100.

Results. Stromal cell-derived factor 1 and CXCR4 were expressed in identical areas in severe pterygium tissues (grade T3) and CXCR4-immunopositive cells were concentrated at perivascular regions. Stromal cell-derived factor 1 levels in cultured pterygial fibroblasts correlated positively with the severity of pterygia. Stromal cell-derived factor 1 levels had a significant, positive correlation with α-SMA levels in pterygial fibroblasts. Furthermore, each knockdown of SDF-1 expression and blockade of SDF-1/CXCR4 signaling in severe pterygia significantly reduced α-SMA levels.

Conclusions. Stromal cell-derived factor 1 expression is upregulated in severe pterygia, and SDF-1 and CXCR4 interaction may contribute to the myofibroblast transformation, which can be possibly restored through the downregulation of the SDF-1/CXCR4 axis.

Keywords: SDF-1, CXCR4, alpha-SMA, myofibroblast

The development of pterygia may result from reactive wound formation triggered by oxidative stress due to UV light exposure, ocular irritation, and/or inflammation on the ocular surface.[1] Solar radiation, a major pathogenetic factor, induces tissue injury such as elastosis of conjunctival connective tissue[2] and severe damage of Bowman's membrane.[3] However, excessive wound healing beyond the normal repair mechanism may induce dysregulated and inappropriate tissue remodeling, fibroproliferation, enhanced vascularization, and deposition of extracellar matrix (ECM), leading to the formation of hypertrophic scarring.[4] Unlike that of the skin, scarring on the ocular surface is not simply limited to a cosmetic problem, but can result in vision loss or symblepharon and motility restriction-related diplopia, which have a devastating effect on quality of life and psychosocial function.[5]

Stromal cell-derived factor 1 (SDF-1), the ligand of chemokine receptor 4 (CXCR4) was initially identified as supporting the bone marrow niche[6–8] and has attracted attention as an upregulator of organ repair,[9–14] and is expressed in human by pericytes, endothelial cells, fibroblasts, and neurons.[15] Stromal cell-derived factor 1 enhances wound healing by recruiting CXCR4-expressing cells into wound areas.[16] According to previous studies, exaggerated SDF-1/CXCR4 signaling contributes to the development of hypertrophic scarring,[17–19] and conversely, anti–SDF-1 treatment attenuates tissue fibrosis in vivo.[20,21]

Myofibroblasts found at the site of tissue injury are considered to play a pivotal role in the healing process. By secreting ECM proteins and the contractile protein α-smooth muscle actin (α-SMA), myofibroblasts promote tissue repair.[22] Additionally, these cells are also reported to exist in the fibrovascular tissue of pterygia.[23] However, the transformation of fibroblasts into myofibroblasts in the wound area can be an important step of abnormal scar formation.[24,25] Furthermore, the upregulated expression of α-SMA from myofibroblasts may produce the firmer and more solid fibrovascular tissue, and consequently result in the more severe pterygia.

Myofibroblast differentiation is classically known to be stimulated and mediated by TGF-β.[24,26] One study recently reported that SDF-1/CXCR4 signaling cross-talks with TGF-β/Smad signaling to drive myofibroblast differentiation.[27] This report suggests that SDF-1 and its interaction with CXCR4 may function as an integral component to trigger myofibroblast

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Table 1. Grading Criteria of Pterygia Based on Pterygium Body Translucency

Grade T	Characteristics
T1 (atrophic)	Lesion with unobscured and clearly distinguished episcleral vessels underlying its body
T2 (intermediate)	Lesion with indistinct or partially obscured episcleral vessel details underlying its body
T3 (fleshy)	Thick pterygium in which episcleral vessels underlying its body are totally obscured by fibrovascular tissue

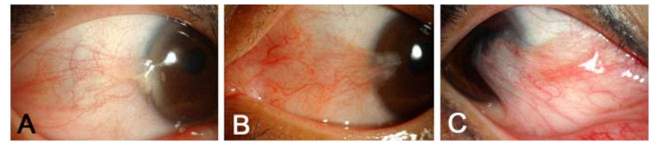

Figure 1. Standard photographs classified by body translucency (grade T). By the grade T classification system, grades T1, T2, and T3 are defined as atrophic pterygium (**A**), intermediate pterygium (**B**), and fleshy pterygium (**C**), respectively.

differentiation, and furthermore in the pathogenesis of aggravation of tissue fibrosis.

Based on overall SDF-1 function in tissue fibrogenesis, it is possible that SDF-1 may mediate the formation of severe forms of pterygia characterized by fibrovascular scarring through dual processes: (1) the upregulation of SDF-1 expression, and (2) the involvement in myofibroblast transformation via the interaction with CXCR4. We therefore investigated SDF-1–related biochemical signaling as a candidate mechanism determining the severity of pterygia.

Methods

This prospective study was approved by the institutional review board of Chung-Ang University Hospital. All procedures were performed according to the tenets of the Declaration of Helsinki, and informed consent was obtained from all patients. Pterygium samples were collected from 78 eyes of 59 patients between September 2011 and March 2013. In 19 patients who had pterygia in both eyes among the whole 59 patients, pterygium tissues were harvested from each eye and were considered as two separate samples. Specimens were harvested intra-operatively during standard pterygium removal surgery. All surgeries were performed by one surgeon (JCK) to ensure consistent tissue harvest technique. Patients with a history of previous ocular surgery, ocular trauma, ischemic cardiovascular disease, hematologic disorder, and/or malignant disease were excluded.

Preoperatively, each patient underwent complete ocular examination by one ophthalmologist, and the phenotype of pterygium was graded by translucency of the pterygium body (grade T) as per the Tan classification system, which has been previously validated as a marker of pterygial severity.[28,29] The grade of pterygium body translucency was evaluated based on grading criteria (Table 1) and with the aid of standard photographs (Fig. 1). Among the grade T1 to T3 pterygia, pterygia with grade T3 were defined as severe pterygia.

Study Design

Our study design was outlined as follows:

1. Investigate SDF-1 and CXCR4 expressions in excised pterygium tissues and in cultured pterygial fibroblasts;
2. Evaluate the contribution of SDF-1 upon increase of the myofibroblast population in pterygia;
3. Analyze the alteration of myofibroblast transformation after knockdown of SDF-1 in cultured pterygial fibroblasts of severe pterygia; and
4. Evaluate the effect of disruption of SDF-1/CXCR4 signaling by CXCR4 blockade on myofibroblast transformation in cultured pterygial fibroblasts of severe pterygia.

First of all, the expression pattern of SDF-1 and CXCR4 was observed in a few severe pterygium tissues. Then, to investigate the differences of SDF-1 and CXCR4 expressions according to pterygial severity, Western blotting and semiquantitative RT-PCR were performed in cultured pterygial fibroblasts from all of 78 eyes. Thereafter, in randomly selected 40 eyes irrelevant to their pterygial severity among the total 78 eyes, the correlation between SDF-1 and α-SMA expression in pterygial fibroblasts was analyzed to evaluate the contribution of SDF-1 upon increase of the myofibroblast population in pterygia. Moreover, to examine the potential therapeutic role of attenuation of SDF-1 action in severe pterygia, alteration of α-SMA expression in pterygial fibroblasts was analyzed before and after each of knockdown of SDF-1 using SDF-1 siRNA and CXCR4 blockade using AMD3100 in 10 highly SDF-1– and α-SMA–expressing eyes with severe pterygia.

Histologic Evaluation of SDF-1 and CXCR4 Expression in Pterygium Tissues

Three severe pterygium tissue samples (grade T3) were obtained intra-operatively. Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Briefly, all paraffin sections (4 μm) were deparaffinized in xylene and rehydrated, and endogenous peroxidase was quenched. Cryostat sections were placed on gelatinized slides and fixed in cold acetone. Tissue sections were equilibrated in tris-buffered saline (TBS), blocked in nonimmune serum (Zymed Laboratories, South San Francisco, CA), and incubated with monoclonal mouse antibodies against human SDF-1 (1:2000; Abcam, Inc., Cambridge, MA) or CXCR4 (1:2000; Abcam, Inc.) overnight at 4°C. Sections were washed in TBS before adding biotinylated secondary antibody, washed again, incubated for 1 hour with peroxidase-conjugated streptavidin, and then the presence of peroxidase was revealed by adding substrate-chromogen (3-amino-9-ethycarbazole) solution. The sections were then counterstained with hematoxylin, examined under an optical microscope (Axioskop 40; Carl Zeiss, Göttingen, Germany), and photodocumented.

Specimen Collection and Culture of Human Pterygial Stromal Fibroblasts

Pterygium specimens were obtained at the central portion of the pterygium body at the time of excision. These specimens were used for explant cultures to generate human pterygial body fibroblasts. Cells were cultured in alpha-Modified Eagle's Medium (alpha-MEM; Gibco-Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; WelGENE, Daegu, South Korea) and 100 units/mL penicillin/streptomycin (WelGENE). Thereafter, upon attaining 80% to 90% confluence, fibroblasts were subcultured with 0.05% trypsin and 0.85 mM EDTA in calcium-free MEM with a 1:3 to 1:4 split for three passages. Subconfluent pterygial fibroblasts were

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Table 2. Sequences of PCR Primers

Gene	Primer Sequence	Product Size, bp
SDF-1		
Sense	5-ATGAACGCCAAGGTCGTGGTC-3′	282
Antisense	5-TGGCTGTTGTGCTTACTTGTTT-3′	
CXCR4		
Sense	5-GGCCCTCAAGACCACAGTCA-3′	352
Antisense	5-TTAGCTGGAGTGAAAACTTGAAG-3′	
α-SMA		
Sense	5′-CCGTGATCTCCTTCTGCATT-3′	175
Antisense	5′-CTGTTCCAGCCATCCTTCAT-3′	
β-actin		
Sense	5′-ATCCGCAAAGACCTGT-3′	293
Antisense	5′-GGGTGTAACGCAACTAAG-3′	
GAPDH		
Sense	5′-TGTGGTCATGAGTCCTTCCA-3′	296
Antisense	5′-CGAGATCCCTCCAAAATCAA-3′	

washed with PBS and then were cultured in serum free media overnight before drug treatment in each experiment.

Total RNA Isolation and Semiquantitative/Real-Time Quantitative RT-PCR

RNA isolation was performed using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For semiquantitative RT-PCR, total RNA was reverse transcribed into complementary (c)DNA (cDNA synthesis kit; Takara Bio, Inc., Otsu, Japan). Equal amounts of samples were used for PCR amplification of cDNA with primers specific for human SDF-1 or CXCR4.

Real-time quantitative RT-PCR (qRT-PCR) was performed using SYBR Premix Ex Taq (Takara Bio, Inc.). SybrGreen fluorescence of the amplified cDNA products of SDF-1 and α-SMA was quantified using the CFX96 Real-Time PCR Detection System (BioRad, Hercules, CA) and an appropriate standard curve from autonomous qPCR assay reactions. Relative gene quantities were obtained using the comparative cycle threshold (Ct) method after normalization to a reference gene (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]). The results of the qRT-PCR analysis are presented as the average amount of each gene expressed relative to average GAPDH expression.

The specific primers that were used for SDF-1, CXCR4, α-SMA, β-actin, and GAPDH are shown in Table 2.

Western Blot Analysis

Western blot analysis of cultured pterygial fibroblasts was performed as previously described.[30] Primary mouse monoclonal antibodies against human SDF-1 (1:1000; Abcam, Inc.), CXCR4 (1:1000; Abcam, Inc.), and α-SMA (1:1000; Millipore, Billerica, MA) were diluted in TBS, applied to the membrane, and incubated overnight at 4°C. Secondary antibodies were then diluted in TBS (1:2000), applied to the membrane, and incubated for 1 hour at room temperature. The protein signal after the application of secondary antibody was visualized using an enhanced chemiluminescence Western blotting detection kit (Pierce Biotechnology, Inc., Rockford, IL). β-actin was used as a loading control. Image analysis of the immunobands was performed using ImageJ software ver. 1.46 (National Institutes of Health, Bethesda, MD).

Table 3. Demographics of Pterygium Cases

Grade T	Number of Eyes	Number of Male Patients	Age Mean ± SD
T1	15 (19.2%) from 13 patients	8 (61.5%)	55.3 ± 10.6
T2	18 (23.1%) from 12 patients	9 (75.0%)	50.3 ± 7.1
T3	45 (57.7%) from 34 patients	15 (44.1%)	48.9 ± 13.3

Immunostaining of Cultured Pterygial Fibroblasts

Pterygial fibroblasts were stained with anti–SDF-1 (1:50; Abcam, Inc.), anti-CXCR4 (1:50; Abcam, Inc.), and anti–α-SMA (1:50 Dako, Glostrup, Denmark) antibodies. Immunofluorescence staining was performed as described previously.[30]

Targeted Knockdown of SDF-1 With Specific siRNA

Stromal cell-derived factor 1–specific siRNA duplexes were synthesized, desalted, and purified by Bioneer (Daegu, South Korea). Briefly, cultured pterygial body fibroblasts were split into 60-mm dishes so that 24 hours later they were 70% to 80% confluent. At that time, each dish was transfected using RNAiMax reagent (Invitrogen) according to the manufacturer's instructions. The efficacy of knockdown of SDF-1 was assessed by qRT-PCR and Western blotting.

Treatment of Cultured Pterygial Fibroblasts With Antagonist of CXCR4

Cultured pterygial fibroblasts were stimulated with 2 μg/mL of AMD3100 (Sigma-Aldrich, St. Louis, MO), a specific antagonist of CXCR4, for 1 hour. The levels of α-SMA in cultured pterygial fibroblasts were analyzed by qRT-PCR and Western blotting before and after AMD3100 treatment.

Statistical Analysis

Statistical analysis was performed using SPSS software version 19.0 (SPSS, Inc., Chicago, IL). All average values were expressed as mean ± SE. A P value less than 0.05 was considered to be statistically significant.

Results

In this study, pterygium body tissues were collected from 78 eyes of 59 consecutive patients (32 males and 27 females). In 19 patients among the total 78 eyes, the pterygium was found in both eyes. Subject age averaged 50.4 ± 11.9 years (mean ± SD) and ranged from 18 to 81. Detailed demographic data of patients and pterygia according to the grades are shown in Table 3.

SDF-1 and CXCR4 Expression in Tissues of Severe Pterygia

To observe the expression pattern of SDF-1 and CXCR4 in pterygia, we investigated the localization of SDF-1– and/or CXCR4-positive cells using immunohistochemical stain analysis in three grade T3 pterygial specimens (Figs. 2A–C, i).

Stromal cell-derived factor 1–immunopositive cells were detected in the entire layer of the stroma, including the deep stroma and substatia propria, as well as in the basal layer of the epithelium. Moreover, SDF-1 and CXCR4 expression was prominent in cells at identical locations in the stroma (dotted lines and circles in Figs. 2A–C). In addition, copious cellular CXCR4 expression at the perivascular area that is contiguous

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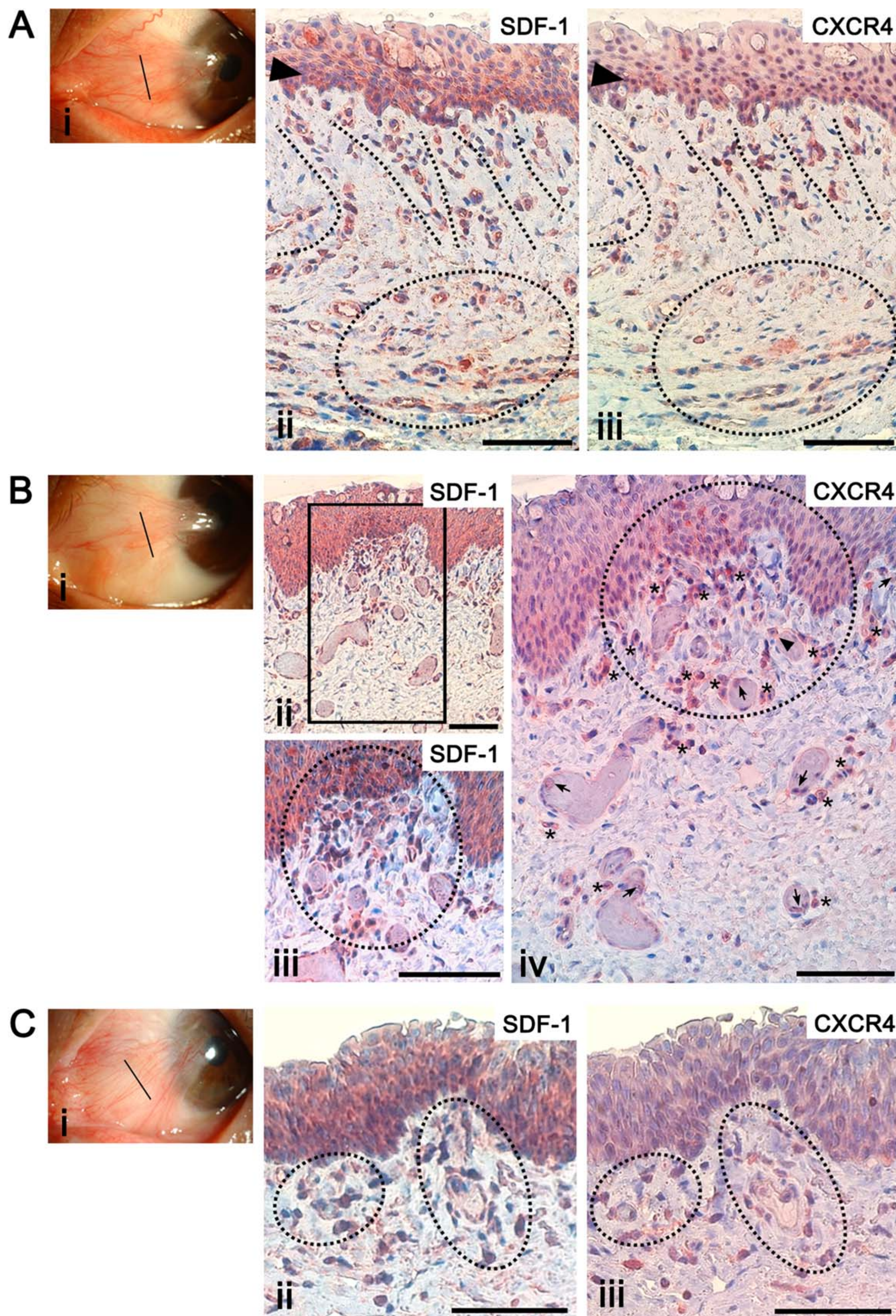

Figure 2. Expression of SDF-1 and CXCR4 in three representative tissues with severe pterygia (grade T3). In immunohistochemical staining for SDF-1 and CXCR4 of specimens from the pterygium body (*line* in [**A–C**, **i**]), both SDF-1 and CXCR4 expression were prominent in cells at identical locations in the stroma ([**A–C**], *dotted lines* and *circles*). (**A**) Full-thickness samples showed the existence of SDF-1– and CXCR4-positive cells in whole layers of the stroma including the deep stroma and substantia propria (**ii**, **iii**), as well as in the basal layer of the epithelium ([**ii**, **iii**],

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arrowheads). (**B**) In addition, copious cellular CXCR4 expression in the perivascular area [(**iv**], *asterisks*) contiguous with intravascular CXCR4-positive cells ([**iv**], *arrows*) was noted. *Scale bars*: 200 μm.

with the intravascular CXCR4-positive cells was noted (Fig. 2B, iv).

Expression of SDF-1 and CXCR4 in Cultured Pterygial Fibroblasts According to Pterygial Severity

To determine the existence of SDF-1– and CXCR4-positive fibroblasts in pterygium, and to analyze differential SDF-1 and CXCR4 expressions according to pterygial severity, the expression pattern of both proteins was examined at the mRNA level using semiquantitative RT-PCR, and at the protein level using Western blot analysis.

Based on pterygial severity (grade T), protein expression of SDF-1 was significantly higher in pterygia with grade T3 compared with pterygia with grade T1 ($P < 0.001$) or with grade T2 ($P < 0.001$) (Fig. 3A). In addition, a trend toward higher SDF-1 expression was found in pterygial fibroblasts with higher grade T at the mRNA level (Fig. 3B).

According to pterygial severity, we noted that there was a significant positive correlation between pterygial severity and expression level of SDF-1 protein (Pearson's correlation coefficient $R = 0.514$, $P < 0.001$, Fig. 4A). However, although a positive tendency was observed, there was no statistically significant correlation between pterygial severity and CXCR4 levels (Pearson's correlation coefficient $R = 0.172$, $P = 0.134$, Fig. 4B).

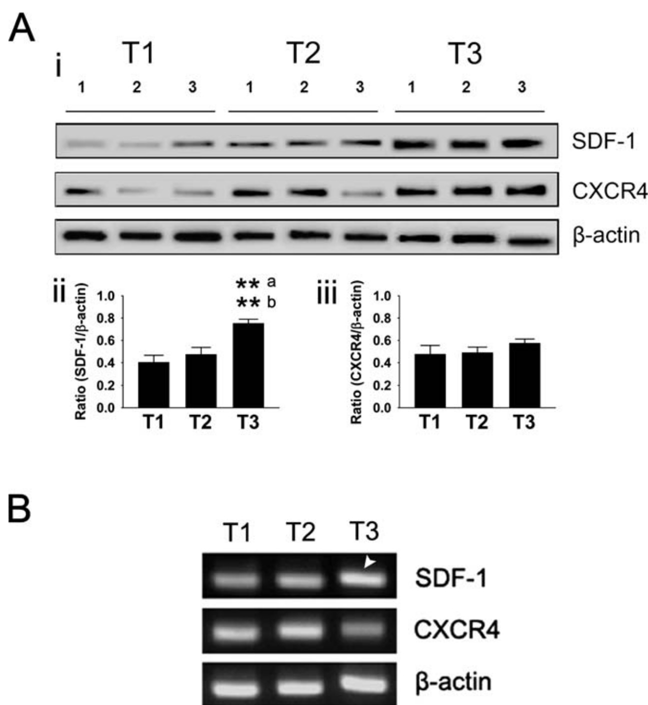

Figure 3. Expression of SDF-1 and CXCR4 in cultured pterygial fibroblasts according to pterygial severity (Grade T). Western blot analysis showed that the density of the SDF-1 immunobands were more prominent in pterygia with grade T3 than in pterygia with grade T1 or T2 (**A**, **i**), and that the differences were statistically significant (**A**, **ii**). Although there was a positive correlation tendency of CXCR4 expression towards the higher grade T as in Western blotting (**A**, **i**), the statistical analysis aiming the whole cases revealed no significance (**A**, **iii**). Representative data of expression of SDF-1 and CXCR4 at the mRNA level in cultured pterygial fibroblasts revealed tendencies toward higher expression of SDF-1 ([**B**], *arrow*) in grade T3 pterygia. However, there was no elevation of CXCR4 mRNA expression correlating with Grade T, similar to what was seen by Western blotting. $^{**}P < 0.001$; a, T3 vs. T1; b, T3 vs. T2.

Correlation Between SDF-1 Expression and Myofibroblast Accumulation in Pterygia

Myofibroblasts play an essential role in producing hypertrophic scarring. As hypothesized, SDF-1 may aggravate myofibroblast transformation under the interaction with CXCR4. As a first step to testify this hypothesis, we analyzed the expression of SDF-1 and α-SMA based on cross-sectional data, using 40 samples of randomly selected cultured pterygial fibroblasts out of the entire 78 samples.

Western blot analysis revealed that higher protein levels of α-SMA correlate with a trend toward higher SDF-1 levels (Fig. 5A), which was found to be statistically significant (Pearson's correlation coefficient $R = 0.753$, $P < 0.001$, Fig. 5B). Immunofluoroscent analysis of fibroblasts to detect SDF-1 and α-SMA revealed high expression of α-SMA in severe pterygium (grade T3), which was also associated with high expression of SDF-1 (Fig. 5C). Because the presence of α-SMA is a phenotypic hallmark of myofibroblasts, these results indicate that SDF-1 may play a role in increasing the myofibroblast population in pterygia.

SDF-1 siRNA-Induced Attenuation of Myofibroblast Transformation in Pterygia

To more specifically elucidate the role of SDF-1 in myofibroblast accumulation, we determined the levels of α-SMA after knockdown of SDF-1 protein using SDF-1 siRNA in cultured pterygial fibroblasts from 10 highly SDF-1– and α-SMA–expressing eyes with severe pterygia (grade T3). After in vitro delivery of SDF-1 siRNA to the pterygial fibroblasts, the expression of α-SMA was significantly reduced to 0.46-fold ($P = 0.005$) of control at the mRNA level as determined by real-time qRT-PCR, and to 0.43-fold ($P = 0.028$) of control at the protein level as determined by Western blotting (Fig. 6B). Moreover, immunofluorescence staining also showed a decrease of cellular expression of α-SMA after knockdown of SDF-1 (Fig. 6C).

Role of SDF-1/CXCR4 Interaction in Myofibroblast Transformation in Pterygia

Because CXCR4 is the cognate receptor of SDF-1, we wondered whether the disruption of SDF-1/CXCR4 interaction as well as knockdown of SDF-1 protein contributes to transformation of pterygial fibroblasts into myofibroblasts. To determine this, the levels of α-SMA were measured in cultured pterygial fibroblasts also from 10 highly SDF-1– and α-SMA–expressing eyes with severe pterygia (grade T3) before and after the treatment with AMD3100, a CXCR4-specific inhibitor.

Pterygial fibroblasts treated with AMD3100 attenuated α-SMA levels to 0.67-fold ($P = 0.027$) of control at the mRNA level as measured by real-time qRT-PCR, and to 0.29-fold ($P = 0.008$) of control at the protein level as measured by Western blotting (Fig. 7B). Moreover, immunofluorescent staining revealed a reduction in cellular expression of α-SMA after AMD3100 treatment (Fig. 7C).

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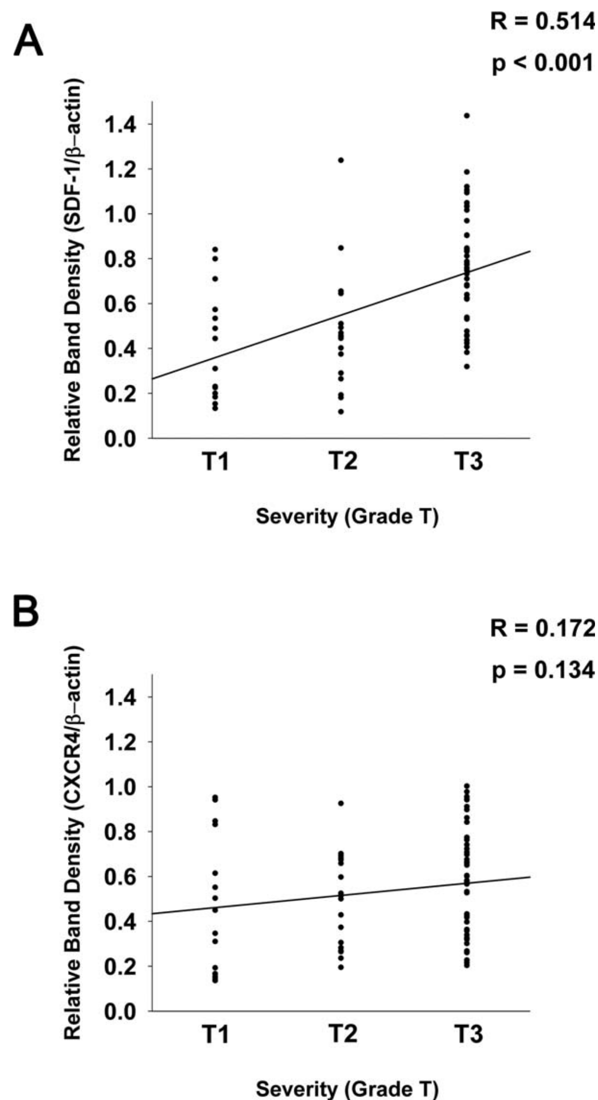

FIGURE 4. Individual expression levels of SDF-1 and CXCR4 in each pterygium severity (grade T1 to T3). (**A**) Linear regression showed a significant positive correlation between SDF-1 levels and pterygial severity. (**B**) However, there was no significant correlation, only a positive tendency, between CXCR4 levels and pterygial severity.

Strong correlation of SDF-1 expression levels with α-SMA levels as well as in vitro downregulation of α-SMA expression in fibroblasts induced by each of knockdown of SDF-1 and disruption of SDF-1/CXCR4 interaction demonstrates that SDF-1 derived from pterygial fibroblasts and its signaling with CXCR4 contributes to the transformation of fibroblasts to myofibroblasts producing severe pterygium, and furthermore to the maintenance of myofibroblast trait.

DISCUSSION

The clinical phenotype of pterygium displays inconsistency and varying severity according to patients. Our intention in this study was to reveal the principle factor determining and differentiating the clinical phenotypes and severities of pterygia. While it is known that SDF-1 recruits CXCR4-positive cells from bone marrow (BM) to injured areas for wound healing and/or tissue fibrosis, the specific relationship of both SDF-1 and CXCR4 with pterygium has thus far been unexplored. This study provides, to our knowledge, the first evidence showing that SDF-1 contributes to the pathogenesis of pterygium, especially in producing severe pterygia.

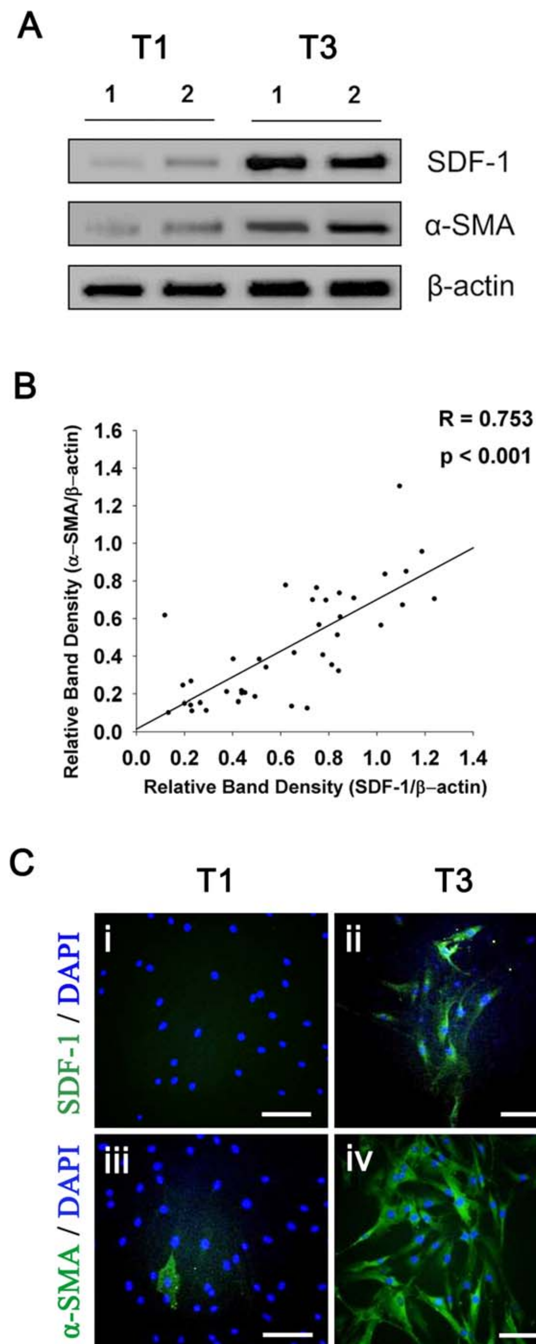

FIGURE 5. Contribution of SDF-1 to increase of myofibroblast population in pterygium. (**A**) Western blotting showed that SDF-1 and α-SMA were highly expressed in pterygial fibroblasts from grade T3 pterygia relative to grade T1 pterygia. (**B**) Linear regression showed a significant positive correlation between the cellular protein levels of SDF-1 and α-SMA. (**C**) Immunofluorescence of pterygial fibroblasts revealed that SDF-1 expression was scanty in a grade T1 pterygium (**i**) compared to the distinct expression in a T3 pterygium (**ii**). In addition, α-SMA was also less expressed in a grade T1 pterygium (**iii**) compared to in a T3 pterygium (**iv**). *Scale bars*: 200 μm.

Chemokine signaling of SDF-1 with CXCR4 is necessary during wound healing. However, when exaggerated, the signaling can misdirect the wound healing process, resulting

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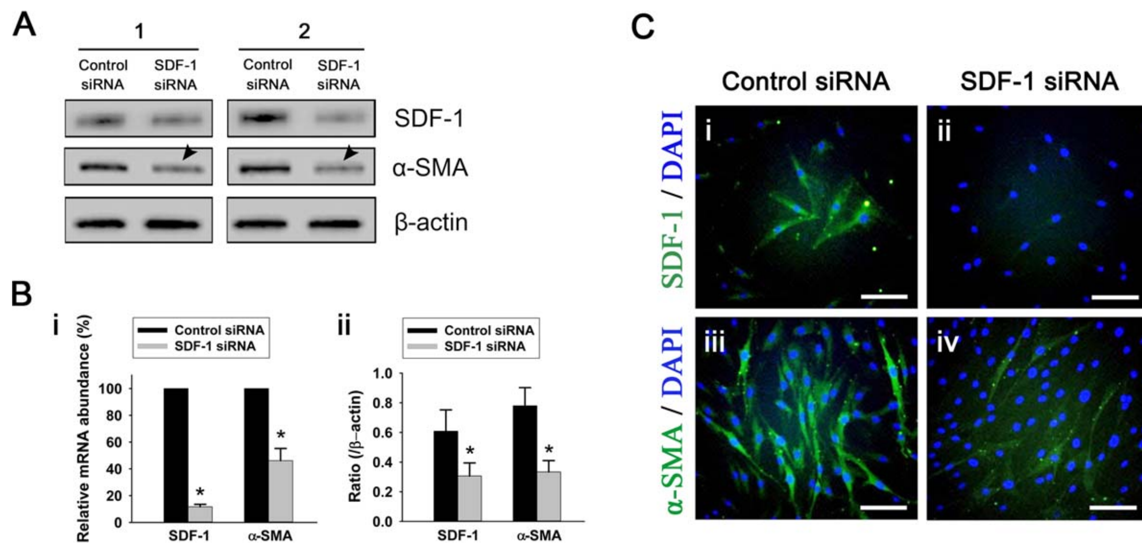

FIGURE 6. Stromal cell-derived factor 1 siRNA–induced attenuation of myofibroblast transformation in severe pterygium. (**A**) Western blot analysis of SDF-1 and α-SMA revealed that knockdown of SDF-1 protein in fibroblasts from severe pterygia (grade T3) attenuated expression levels of α-SMA (*arrowheads*). (**B**) α-Smooth muscle actin expression after in vitro delivery of SDF-1 siRNA in pterygial fibroblasts decreased significantly compared with controls at both the mRNA (**i**) and protein levels (**ii**). (**C**) In immunofluorescence staining, SDF-1 expression in pterygial fibroblasts (**i**) was attenuated following SDF-1 siRNA delivery (**ii**). Accordingly, prominent expression of α-SMA in pterygial fibroblasts (**iii**) showed the decrease of α-SMA expression after the knockdown of SDF-1 (**iv**). *Scale bars*: 200 μm.

in a fibroproliferative disorder.[31] In our study, expression of SDF-1 was correlated positively with pterygial severity. That is to say, SDF-1 could be considered to perform a significant role in producing severe pterygia (grade T3), characterized by a phenotype of fibrovascular scarring. However, although there was a positive correlation tendency, there was no statistically significant correlation between pterygial severity and CXCR4 levels. Because CXCR4 is a cognate receptor of SDF-1, we thought that this was probably due to the negative feedback loop between the two that was suggested by Fruehauf et al.[32] In addition, Ding et al.[31] reported that the lack of positive finding of CXCR4 signaling in postburn hypertrophic scar is probably due to differentiation of CXCR4-expressing cells into other repair cells in the later stages of wound healing. This may also explain why there was low correlation between pterygial severity and CXCR4 levels (Figs. 3A, iii, 4).

In immunohistochemical staining of three severe pteygium specimens, SDF-1 and CXCR4 expression was detected in the same lesions in the stroma. In addition, CXCR4-positive cells were found predominantly at the perivascular areas, and some

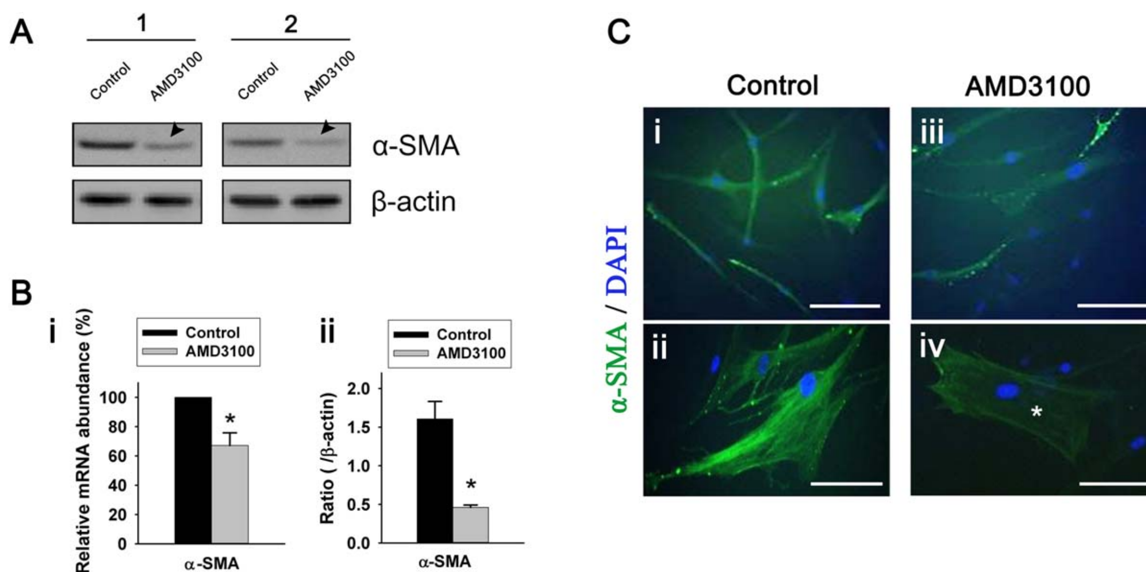

FIGURE 7. Attenuation of myofibroblast transformation by blocking SDF-1/CXCR4 signaling with AMD3100 in severe pterygium. (**A**) Western blotting showed that expression of α-SMA was attenuated in pterygial fibroblasts from severe pterygia (grade T3) after treatment with AMD3100 (*arrowheads*), compared with pretreatment levels (control). (**B**) The difference of α-SMA expression between the AMD3100-treated group and control was statistically significant at both the mRNA (**i**) and protein levels (**ii**). (**C**) In cultured pterygial fibroblasts that highly express α-SMA (**i**), α-SMA expression was decreased after blockade of CXCR4 (**iii**). Immunofluorescent images at higher magnification show attenuation of cytosolic expression of α-SMA after treatment with AMD3100 (*asterisk* in [**C**, **iv**]), in contrast to the prominent expression observed before the treatment (**C**, **ii**). *Scale bars*: 200 μm (**C**, **i**, **iii**); 100 μm (**C**, **ii**, **iv**).

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were also found at the intravascular areas. Chemokine receptor 4 has been known to be expressed also in the BM-derived circulating cells including fibrocytes, and SDF-1 is a well-known recruiter of CXCR4-expressing cells.[24] These cells probably represent the intermediate stage of differentiation of one of the precursors of the monocytes lineage into mature fibroblasts and myofibroblasts at tissue sites.[33] Hence, the authors thought that this phenomenon could suggest the possible transendothelial migration of CXCR4-positive cells into the pterygium to induce fibrosis and to produce severe pterygium. However, although the tissue expression of SDF-1 and CXCR4 in pterygia was first reported in this study, only three specimens were histologically evaluated and, thus, this phenomenon would need to be specified with the BM-derived cell-specific markers and through the large scale study.

Myofibroblast transformation is stimulated by TGF-β; moreover, SDF-1 can cross-talk with TGF-β signaling to promote trans-phenodiffereniation in tumor fibroblasts.[27] Pterygial fibroblasts are known to have acquired many of the features of neoplastic cells.[34] We therefore speculated that SDF-1 itself may also have the potential to boost the transformation of CXCR4-positive pterygial fibroblasts to myofibroblasts in the pterygium. Because Western blot analysis of cultured pterygial fibroblasts revealed the existence of CXCR4-positive fibroblasts and α-SMA-expressing myofibroblasts, first of all, authors intended to validate the correlation between the SDF-1/CXCR4 axis and α-SMA-expression levels. Indeed, in our assay, the expression level of α-SMA, which is a hallmark of myofibroblasts, revealed a significant positive trend following the level of SDF-1 in pterygial fibroblasts. In addition, each of in vitro downregulation of SDF-1 and disruption of SDF-1/CXCR4 signaling using a specific blocker of CXCR4 reduced α-SMA expression. We considered that this phenomenon could propose the possible role of SDF-1 and its signaling with CXCR4 to contribute the myofibroblast transformation and the maintenance of myofibroblast phenotype in resident pterygial fibroblasts. Furthermore, the downregulation of such an axis might potentially suggest a reversible restoration of the activity of pterygial fibroblasts. Although the related mechanistic explanations could not be presented in this study, we found that SDF-1 expression levels based on Western blot analysis in cultured pterygial fibroblasts revealed significant positive correlation with TGF-β expression levels (data not shown). Thus, we imagine that intracellular downstream pathway of SDF-1 signaling may cooperate with TGF-β action, which is well-known to involve in myofibroblast transformation,[24,26] similar to the suggestion by Kojima et al.[27]

However, myofibroblastic features were not completely abolished in vitro by suppression of the SDF-1/CXCR4 axis. It is not yet decided whether the main source of pterygial myofibroblasts is a BM-derived progenitor cells or local fibroblasts. In addition, CXCR4-positive cells may convert to, and settle as, resident fibroblasts, losing their phenotype over time. Thus, if some portion of pterygial myofibroblasts originate from CXCR4-negative lesional tissue fibroblasts, or, alternatively, if CXCR4-positive cells differentiate to other cells, downregulating CXCR4 in the later stage of wound healing, as suggested by Ding et al.,[31] it could be possible that myofibroblast transformation would not be completely impaired under conditions in which the SDF-1/CXCR4 biological axis is inhibited. This hypothesis speculates that other mechanisms could be involved in myofibroblast transformation in pterygium, and additionally proposes a requirement for early suppression of the SDF-1/CXCR4 axis in vivo during pterygium genesis.

Our study has a limitation, which several in vitro assays covering downregulation of SDF-1 through SDF-1–siRNA and suppression of the SDF-1/CXCR4 axis using AMD3100 were performed using relatively small number of samples and, thus, showed the pilot data. However, this study is the first report to propose the possible therapeutic potential of attenuation of SDF-1 action in severe pterygia. Future studies with the large number of cases and in vivo animal experiments are thought to be required to verify our hypothesis more precisely.

In summary, SDF-1 is thought to be strongly involved in producing severe pterygia throughout the two major processes. First, SDF-1 expression from the pterygial fibroblasts is upregulated in the severe pterygia with fleshy pteygium body translucency. Second, SDF-1 and CXCR4 interaction may contribute to the myofibroblast transformation, which can be possibly restored through the downregulation of the SDF-1/CXCR4 axis. Further investigation in this area could help identify SDF-1–related signaling pathways that may serve as new therapeutic targets.

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