

Regulation of Apoptosis of Hypertrophic Chondrocytes

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Tumor necrosis factor- α inhibits chondrogenic differentiation of synovial fibroblasts through p38 mitogen activating protein kinase pathways

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Abstract We previously reported that synovial fibroblast-like cells (SFs) can be differentiated into chondrocytes through activin receptor-like kinase (ALK) 3 activation. The aim of this study was to clarify the effect and signaling pathways of tumor necrosis factor (TNF)- α on the chondrogenic differentiation of SFs. Primary SFs from patients with rheumatoid arthritis (RA) were treated with recombinant human bone morphogenetic protein-2 or transduced with a constitutively active mutant of the ALK3 gene (ALK3^{CA}) with or without TNF- α , and then cultured in pellets. Expression of chondrocyte-specific genes was analyzed by real-time polymerase chain reaction or by histological analysis. Inhibitors of mitogen-activating protein kinase (MAPK) pathways or adenovirus vectors carrying a dominant-negative mutant of the I κ B kinase 2 gene (AxIKK2^{DN}) were used to analyze the signaling pathways of TNF- α . Expression of chondrocyte-specific genes was induced in SFs either by rhBMP-2 treatment or by ALK3^{CA} transduction, which was strongly suppressed by TNF- α treatment. TNF- α markedly increased the p38 MAPK pathways in SFs, and inhibition of p38 MAPK activation partially restored the inhibitory effect of TNF- α on the chondrogenic differentiation of SFs. Combination therapy BMP-2 and anti-TNF- α agents especially targeting

p38 MAPK might be a good approach to stimulating neo-chondrogenesis in the damaged joints in RA.

Keywords Rheumatoid arthritis (RA) · Synovial fibroblast-like cell (SFs) · TNF- α · Chondrogenic differentiation · p38 MAPK

Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease of unknown etiology characterized by invasive synovial hyperplasia leading to progressive joint destruction [1]. Rheumatoid synovial cells are not only morphologically characterized by their transformed appearance but are also phenotypically transformed to proliferate abnormally [2–4]. They invade bone and cartilage by producing an elevated amount of proinflammatory cytokines and metalloproteinases, and by inducing the differentiation and activation of osteoclasts, multinucleated cells primarily responsible for bone resorption [5]. We and other groups previously reported that synovial fibroblast-like cells (SFs) obtained from inflammatory joints of RA patients express high levels of receptor activator of nuclear factor kappa B ligand (RANKL) and support osteoclast differentiation from monocyte-macrophage lineage precursors in the presence of osteotropic factors such as 1 α ,25-dihydroxyvitamin D₃ [6–9].

In contrast to such catabolic activity, SFs have anabolic effects leading to bone and cartilage generation. Nishimura et al. [10] demonstrated the chondrogenic differentiation of SFs when they were cultured in the presence of TGF- β , and de Bari et al. [11, 12] showed that multipotent mesenchymal stem cells (MSCs) could be isolated from human synovial tissues which differentiated into chondrocytes as well as

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osteoblasts, adipocytes, and myotubes under the proper culture conditions. More recently, Sekiya and co-workers [13] reported that synovium-derived MSCs display greater in vitro chondrogenic ability than other mesenchymal tissues. We previously reported that SFs undergo chondrogenic differentiation by transducing constitutively active activin receptor-like kinase (ALK)3, also known as the bone morphogenetic protein (BMP) type IA receptor [14]. These observations have led us to speculate that synovial tissues contain multipotent cells with chondrogenic potential through BMP signaling pathways, and they might be involved in the repair process of articular cartilage defects.

It has been widely recognized that proinflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1, IL-6 and IL-17 play a crucial role in joint destruction in RA [11]. In particular, the pathologic role of TNF- α has attracted a great deal of attention because of the remarkable success of biologic agents targeting TNF- α , which are not only effective in ameliorating the clinical symptoms of RA patients, but also suppress bone destruction and maintain joint integrity [15–17]. In this study, we found that TNF- α suppressed chondrogenic differentiation of SFs induced by recombinant BMP-2 treatment or ALK3^{CA} transduction, and demonstrated the possible involvement of p38 mitogen activating protein kinase (MAPK) pathways in the inhibitory effect against TNF- α .

Materials and methods

Chemicals and antibodies

Alpha-modified minimum essential medium (α -MEM) and fetal bovine serum (FBS) were purchased from Gibco BRL, Life Technologies Inc. (Rockville, MD, USA). Recombinant human BMP-2 (rhBMP-2) was obtained from Astellas Pharma Inc. (Tokyo, Japan). Recombinant human interleukin (IL)-1 β and tumor necrosis factor (TNF)- α were purchased from Sigma-Aldrich Inc. (St Louis, MO, USA). Anti-p38 MAPK, anti-phospho-p38 MAPK (Thr180/Tyr182) and anti-MAPKAPK-2, anti-JNK, anti-phospho-c-Jun, anti-ERK, anti-phospho-ERK, anti-I κ B, anti-phospho-I κ B antibodies were obtained from Cell Signaling Inc. (Beverly, MA, USA). Anti-Smad1 antibody was from Upstate Inc. (Lake Placid, New York). Anti-phospho-Smad1/Smad5/Smad8 antibody, which recognizes the phosphorylated form of Smad1, Smad5, and Smad8 was purchased from Cell Signaling Inc. Anti-hemagglutinin (Anti-HA) antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). HRP-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG were from Promega Co. (Madison, WI, USA). Rabbit polyclonal anti-rat type II collagen and type X collagen antibodies

were purchased from LSL (Tokyo, Japan). A p38 inhibitor SB203580 and a JNK inhibitor SP600125 were from Biomol Inc. (Plymouth Meeting, PA, USA). An ERK inhibitor PD98059 was from Cell Signaling Inc.

Isolation of SFs from human synovial tissues

Synovial cells were obtained as previously described [6, 18, 19]. In brief, with enzymatic digestion, human synovial cells were isolated from synovial tissues of the knee joints of 24 female patients (age range 43–78 years; mean 64.9 years) who fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for RA [20] at the time of total knee arthroplasty operations. Written informed consent for the subsequent experiments was obtained from each patient. Cells were suspended in α -MEM containing 10% FBS and were cultured in monolayers. After three to five passages, subcultured cells were composed of morphologically uniform synovial fibroblastic cells (SFs) that were free of macrophages. They were untreated or treated with rhBMP-2, TNF- α , or MAPK inhibitors, or infected with adenovirus vectors, and subjected to pellet cultures.

Constructs and gene transduction

The recombinant adenovirus vectors carrying HA-tagged constitutively active ALK3 with CAG (cytomegalovirus IE enhancer + chicken β -actin promoter + rabbit β -globin poly(A) signal) promoter (AxALK3^{CA}) were kindly provided by Dr. Kohei Miyazono (The University of Tokyo) [21]. The adenovirus vector carrying the dominant negative I κ B kinase 2 gene (Ser177 and Ser181 to Ala mutant, AxIKK2^{DN}) was constructed as previously reported [22]. SFs were infected with adenovirus vectors following a method previously described [6, 23]. In short, subconfluent SFs were incubated with a small amount of medium (α -MEM without serum) that contained recombinant adenoviruses for 2 h at 37°C at the indicated multiplicity of infection (MOI), and then ten times more medium containing 10% FBS was added. Infected cells were subjected to the pellet culture 24 h after the infection.

Pellet cultures of isolated SFs

Subconfluent SFs were trypsinized and cell numbers were ascertained. Aliquots of 5×10^5 cells were spun down at $500 \times g$ in 15 ml polypropylene conical tubes in 5 ml of α -MEM with ascorbate 2-phosphate (0.1 mM) and 10% FBS. Within 24 h of incubation, the cells formed a single, free-floating pellet. The medium was changed every 2–3 days, and duplicate pellets were harvested after 7 days for real-time polymerase chain reaction (PCR), or after 4 weeks for

histological and immunohistochemical analysis. To examine the chondrogenetic differentiation of SFs, cells were pretreated with rhBMP-2 (100 ng/ml) or infected with AxALK3^{CA} before being subjected to the pellet culture. The effects of TNF- α treatment with or without various inhibitors on chondrogenic gene expression in SFs were also examined.

Immunoblotting

All the extraction procedures were performed at 4°C or on ice. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed with M-PER (Pierce Chemical Inc, Rockford, IL, USA) containing 2 mM Na₂VO₄, 10 mM NaF and 10 μ g/ml of aprotinin, according to the manufacturer's instructions. Lysates were prepared by centrifugation at 14,000 \times g for 10 min. An equal amount (15 μ g) of proteins was separated by electrophoresis on SDS-PAGE with 7.5–15% Tris–Glycine gel (DRC, Tokyo, Japan). After electrophoresis, proteins were electrotransferred onto nitrocellulose membranes (Bio-Rad Inc, Hercules, CA, USA). After being blocked in 3% BSA/TBST, immunoblotting with specific antibodies was performed with ECL Western blotting reagents (Amersham Life Sciences Inc, Arlington Heights, Illinois, USA), following the manufacturer's protocols.

Histology and immunostaining

Cultured pellets were fixed with 3.7% formaldehyde, embedded in paraffin, and cut into sections of 4- μ m thickness. Representative sections were subjected to Alcian blue staining, and immunohistochemistry. Alcian blue staining was performed according to the protocol described previously [24]. Briefly, after deparaffinization, sections were stained with 0.5% Alcian blue 8GX (Wako, Osaka, Japan) in 0.1 N HCl for 1 h. For immunostaining with anti-type II collagen or anti-type X collagen antibody, the sections were treated with 2.5% hyaluronidase (Sigma-Aldrich) for 30 min at room temperature, and then with 3% hydrogen peroxidase in methanol for 30 min at room temperature to inactivate the endogenous peroxidase. After non-specific staining was blocked by incubating in PBS with 1% bovine serum albumin (BSA; Seikagaku, Tokyo, Japan) for 1 h at room temperature, the sections were stained with anti-type II or type X collagen antibody at a dilution of 1:100 for 24 h at 4°C. The sections were then rinsed with PBS and incubated with a horseradish peroxidase (HRP)-conjugated goat antibody against rabbit IgG (Dako, Glstrup, Denmark) for 20 min at room temperature. After being washed with PBS, the sections were immersed in a diaminobenzidine solution for 5 min at room temperature to detect the immunoreactivity of type II

or type X collagen. The sections were counterstained with methyl green. For morphological comparison, colonies containing more than four cells were selected and photographed.

Total RNA extraction and real-time PCR

Total RNA was isolated from SFs pellet cultures using an RNA extraction kit (ISOGEN[®], Wako) following the supplier's protocol. Complementary DNA (cDNA) was synthesized from 500 ng of total RNA with ExScript[™] RT reagent Kit (Takara Bio Inc, Shiga, Japan). For real-time PCR and data analysis, the ABI Priss Sequence Detection system 7000 was used. Primers were designed based on sequences obtained from GenBank and amplicons of 50–250 base pairs with a melting temperature between 55 and 60°C were selected. Aliquots of first-strand cDNA (1 μ g) were amplified with qPCR Mastermix Plus for SYBR[®] Green I (Nippon Gene Co, Tokyo, Japan) under the following conditions: initial denaturation for 10 min at 94°C, followed by 40 cycles consisting of 15 s at 94°C and 1 min at 60°C. The expression ratio was calculated from the difference in threshold cycles at which an increase in reporter fluorescence above a baseline signal could first be detected among three samples, and was averaged for duplicate experiments. The primers we used in real-time PCR were as follows:

Type II collagen, 5'-GGTGGCTCCATTTCAGCTA-3' & 5'-TACCGGTATGTTTCGTGCAG-3',
 Aggrecan, 5'-TATGTGAGGAGGGCTGGAAC-3' and 5'-TCACCACACAGTCTCTCCA-3',
 Type X collagen, 5'-AGGAATGCCTGTGTCTGCTT-3' and 5'-ACAGGCTACCCAAACATGA-3',
 GAPDH, 5'-GAAGGTGAAGGTCGGAGTCA-3' and 5'-GAAGATGGTGATGGGATTTC-3'.

Statistical analysis

Statistical analyses were performed using the two-tailed unpaired *t*-test for the real-time PCR. The data were expressed as a mean \pm SD. Statistical differences were presented at probability levels of *P* < 0.01 and 0.005.

Results

The inhibition of rhBMP-2- or ALK3^{CA}-induced chondrocyte-specific gene expression by pro-inflammatory cytokines

We first investigated the effect of pro-inflammatory cytokines on chondrocyte-specific gene expression in SFs. SFs

were untreated or pretreated with TNF- α together with rhBMP-2 (100 ng/ml) for 3 days and then subjected to pellet cultures. As shown in Fig. 1a, the expression of type II collagen and aggrecan genes was increased in rhBMP-2-treated cells after 7 days of culture, which was dose-dependently suppressed by TNF- α treatment, by real-time PCR. Expression of type X collagen was slightly reduced by rhBMP-2 treatment, and greatly upregulated by co-treatment with TNF- α , indicating that hypertrophic differentiation of SFs was promoted by TNF- α (Fig. 1a). IL-1 β also displayed a similar effect on SFs, but with much less efficiency (data not shown).

We next examined the effect of TNF- α on chondrocyte-specific gene expression in SFs induced by AxALK3^{CA}

infection. As previously reported, type II collagen and aggrecan gene expression was induced in AxALK3^{CA}-infected pellet cultures [14], which effect was suppressed by co-treatment with TNF- α in a dose-dependent manner (Fig. 1b). In addition, the expression of the type X collagen gene was strongly upregulated by the treatment with TNF- α (Fig. 1b).

Inhibition of BMP2-induced cartilagenous matrix production by pro-inflammatory cytokines

Next, the effect of TNF- α on cartilagenous matrix production of SFs was histologically examined. Cells were subjected to pellet cultures and treated with TNF- α

Fig. 1 Effects of TNF- α on chondrocyte-specific gene expression or on intracellular signaling pathways in SFs induced by rhBMP-2 treatment or ALK3^{CA} transduction.

a, b Gene expression in SFs after 7 days of pellet culture was analyzed by real-time PCR analysis. TNF- α treatment dose-dependently suppressed rhBMP-2 or AxALK3^{CA}-induced type II collagen (COL II) and aggrecan gene expression and increased type X collagen (COL X) gene expression. **c** Histological analysis. SFs pellets after 4 weeks of culture were fixed and subjected to Hematoxylin-Eosin staining, Alcian Blue staining and immunostaining with anti-type II collagen (COL II) or anti-type X collagen (COL X) antibody. Clear Alcian blue staining and type II collagen immunostaining were observed in rhBMP-2-treated or AxALK3^{CA}-infected pellets, which was suppressed by co-treatment with TNF- α . Clear type X collagen immunostaining was observed in TNF- α -treated cultures. **d, e** Activation of Smad1/5/8, MAP Kinases and NF- κ B pathways was determined by immunoblotting. NC represents the negative control. The adenovirus vector carrying the β -galactosidase gene (AxLacZ) was used as a control virus. Bar 50 μ m

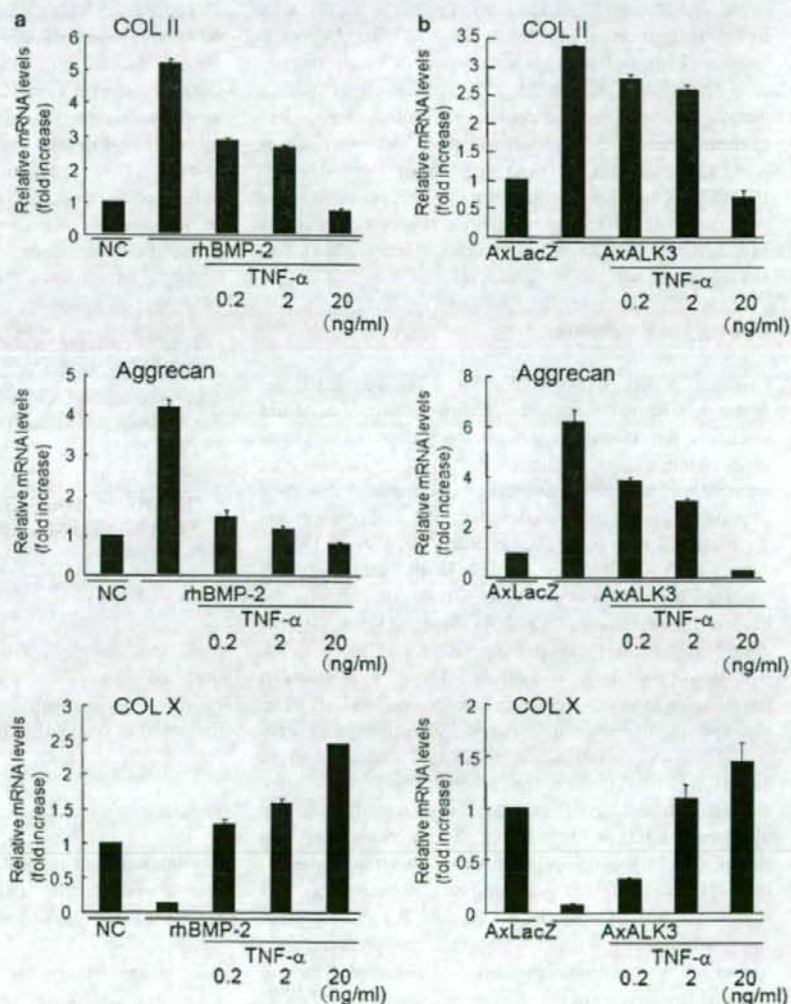
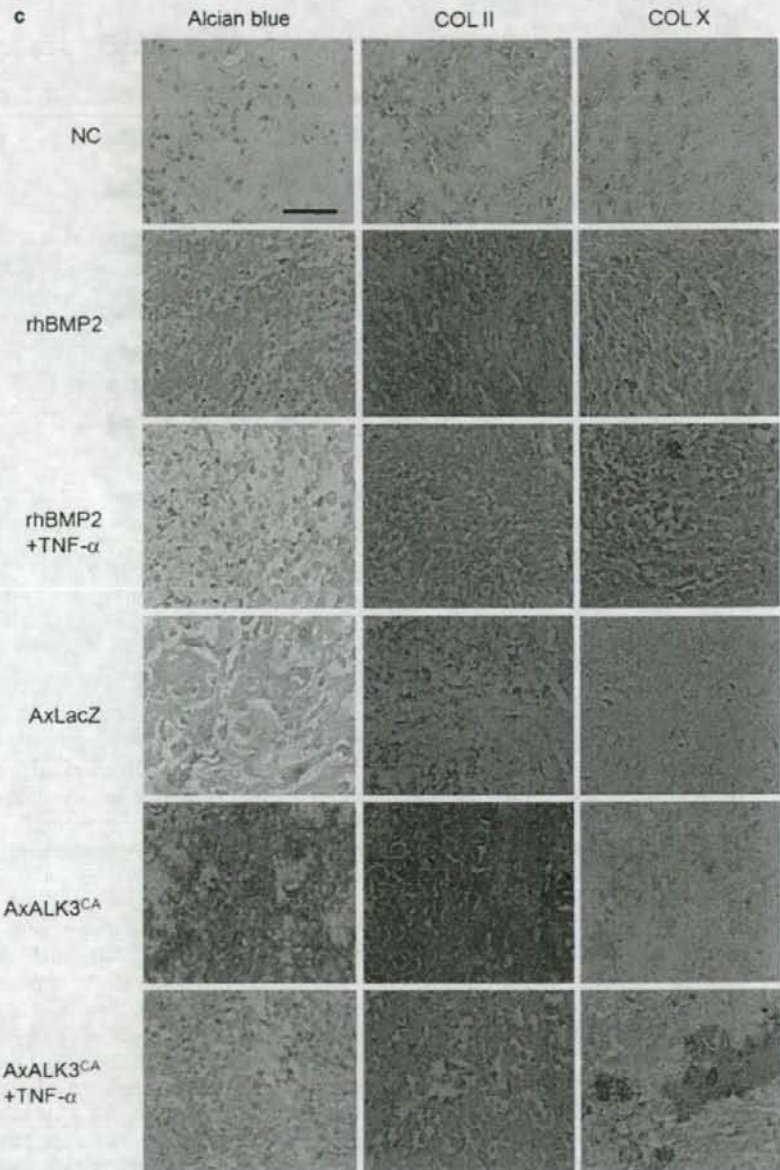


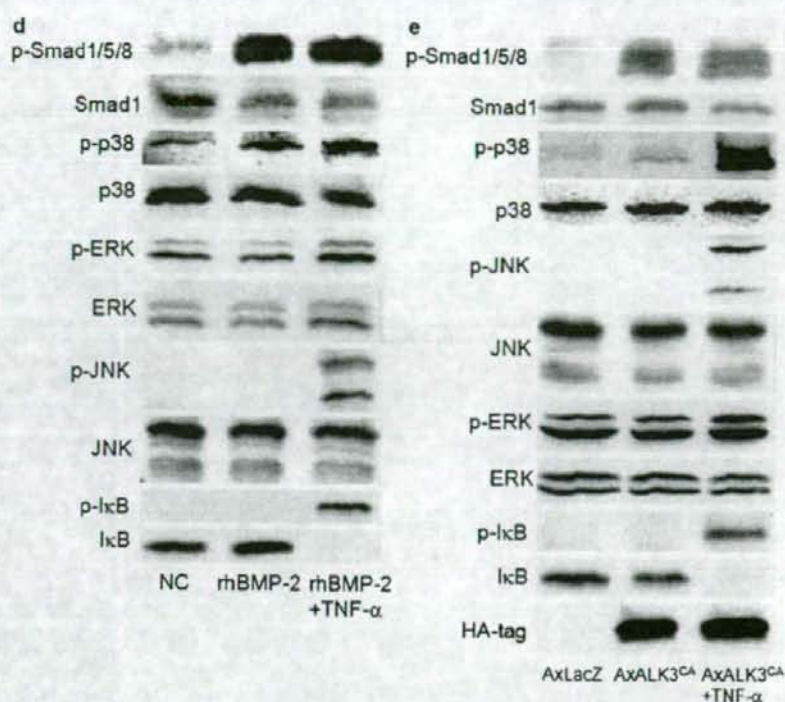
Fig. 1 continued



(20 ng/ml) together with rhBMP-2 (100 ng/ml). After 4 weeks of pellet culture, cells were fixed and sections were prepared for Alcian blue staining and type II and type X collagen immunostaining (Fig. 1c). rhBMP-2-induced cartilaginous matrix deposition, as shown by positive Alcian blue staining, was suppressed by co-treatment with

TNF- α , while positive staining was barely detectable in untreated cultures. Positive immunostaining with anti-type II collagen antibody was observed in rhBMP-2-treated pellets, which was also attenuated by TNF- α . Immunostaining with anti-type X collagen antibody displayed negative staining in rhBMP-2-treated cultures, while TNF- α

Fig. 1 continued



induced a strong positive staining. No positive type II or type X collagen staining was detected in untreated cultures. TNF- α displayed a similar effect on AxALK3^{CA}-infected SFs. TNF- α also suppressed Alcian Blue staining and type II collagen immunostaining, and promoted type X collagen immunostaining in ALK3^{CA}-transduced SFs.

Signaling pathways that regulate the inhibitory effect of TNF- α on the chondrogenic differentiation of SFs

We then proceeded to analyze the signaling pathways that TNF- α suppressed the chondrogenic differentiation of SFs. rhBMP-2 treatment or ALK3^{CA} introduction activated the Smad and p38 MAPK pathways in SFs, as determined by immunoblotting with anti-phospho-Smad1/5/8 and anti-phospho-p38 antibodies (Fig. 1d, e). Pre-treatment of SFs with TNF- α markedly increased the phosphorylation of p38, while no significant difference was observed in the phosphorylation of Smad 1/5/8. TNF- α treatment also stimulated ERK, JNK and NF- κ B pathways in SFs, as determined by immunoblotting with anti-phospho ERK, anti-phospho-c-Jun or anti-phospho-I κ B α (Fig. 1d, e).

We then investigated which signaling pathways are involved in the inhibitory effect of TNF- α on the chondrogenic differentiation of SFs induced by AxALK3^{CA}. PD98059 is a specific ERK inhibitor, and competitively and reversibly inhibits MEK1 and 2 (IC₅₀ = 4 and 50 μ M), with less inhibitory potency on other MAP kinases [25]. Treatment with PD98059 dose-dependently suppressed TNF- α -induced ERK activation in ALK3^{CA}-transduced SFs without affecting other MAPK activation (Fig. 2a, upper panel), but had no effect on type II collagen, aggrecan or type X collagen expression in the cells (Fig. 2a, middle and lower panels). SP600125 is a specific JNK inhibitor, and competitively and reversibly inhibits JNK1, 2, and 3 (IC₅₀ = 40–90 nM), with less inhibitory potency on other MAP Kinases [26]. Treatment with SP600125 dose-dependently suppressed TNF- α -induced JNK activation in ALK3^{CA}-transduced SFs without affecting other MAPK activation (Fig. 2b, upper panel), but had no effect on type II collagen, aggrecan or type X collagen expression in the cells (Fig. 2b, middle and lower panels). Adenovirus vector-mediated IKK2^{DN} expression suppressed TNF- α -induced NF- κ B activation [27] in SFs, as determined by the phosphorylation of I κ B α (Fig. 2c,

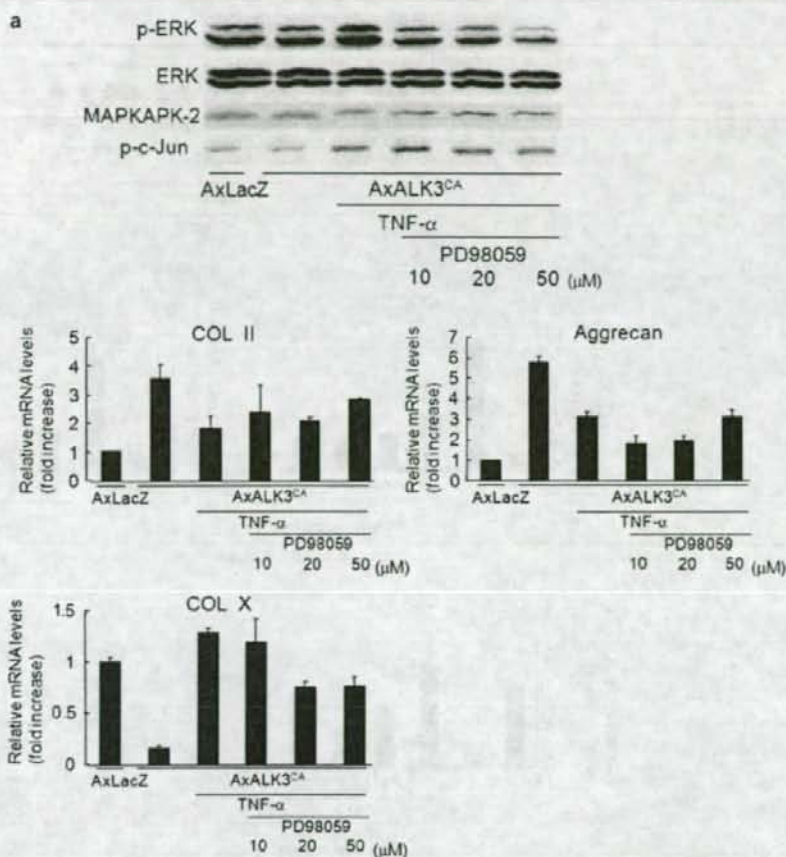


Fig. 2 To analyze the signaling pathways of TNF- α treated with inhibitors of MAPK or adenovirus vector-mediated AxIKK2^{DN} transduction. **a** Effect of an ERK inhibitor PD98059. (Upper) TNF- α treatment induced phosphorylation of ERK, which was dose-dependently suppressed by treatment with PD98059. (Middle and lower) PD98059 treatment did not affect the effects of TNF- α on type II collagen, aggrecan, or type X collagen expression in SFs. **b** Effect of a JNK inhibitor SP600125. (Upper) TNF- α treatment induced phosphorylation of c-Jun, a downstream target of JNK, which was dose-dependently suppressed by treatment with SP600125. (Middle and lower) SP600125 treatment did not affect the effects of TNF- α on type II collagen, aggrecan, or type X collagen expression in SFs. **c** Effect of AxIKK2^{DN} infection. The adenovirus vector carrying the β -galactosidase gene (AxLacZ) was used as a control virus. (Upper) TNF- α treatment induced phosphorylation of I κ B and caused its

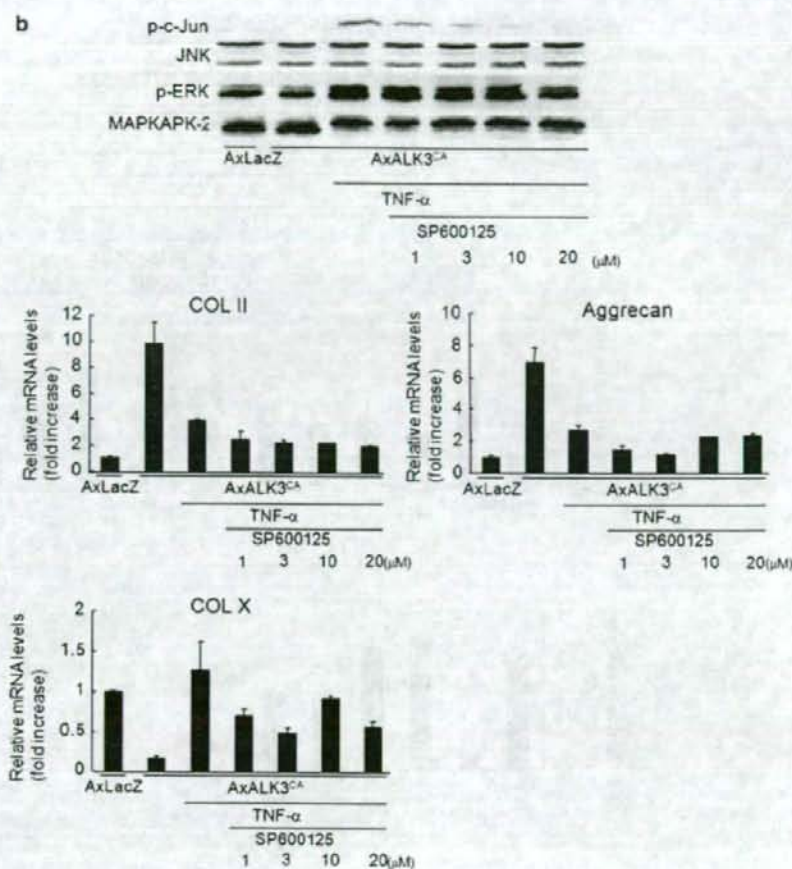
degradation, which was suppressed by AxIKK2^{DN} infection. (Middle and lower) AxIKK2^{DN} infection further suppressed type II collagen and aggrecan expression in SFs compared to AxLacZ-infected cells, and did not affect type X collagen expression. **d** Effect of p38 inhibitor SB203580. (Upper) TNF- α caused a molecular weight shift of MAPKAPK-2 (arrow) as a result of p38 MAPK activation, which was dose-dependently suppressed by SB203580. SB203580 treatment also induced activation of the JNK pathways as determined by immunoblotting with anti-phospho-c-Jun antibody. (Middle) SB203580 at 0.3 μ M partially restored the expression of the type II collagen and aggrecan in SFs, while higher concentrations of the compound rather suppressed the expression of these genes. (Lower) SB203580 dose-dependently suppressed type X collagen expression in SFs. Single asterisk and double asterisks are significantly different. * $P < 0.01$, ** $P < 0.005$

upper panel), but was unable to restore type II collagen or aggrecan expression (Fig. 2c, middle panels). IKK2^{DN} overexpression did not affect type X collagen expression in SFs (Fig. 2c, lower panel).

MAPK-activated protein kinase-2 (MAPKAPK-2) is a downstream target of p38 MAPK, and exhibits a molecular

weight shift from 47 to 50 kDa upon phosphorylation by p38 MAPK activation [28]. TNF- α induced a molecular weight shift of MAPKAPK-2 in ALK3^{CA}-transduced SFs, which was dose-dependently suppressed by the p38 MAPK inhibitor SB203580 (Fig. 2d, upper panel) [29, 30]. SB203580 restored the inhibitory effects of TNF- α on type

Fig. 2 continued



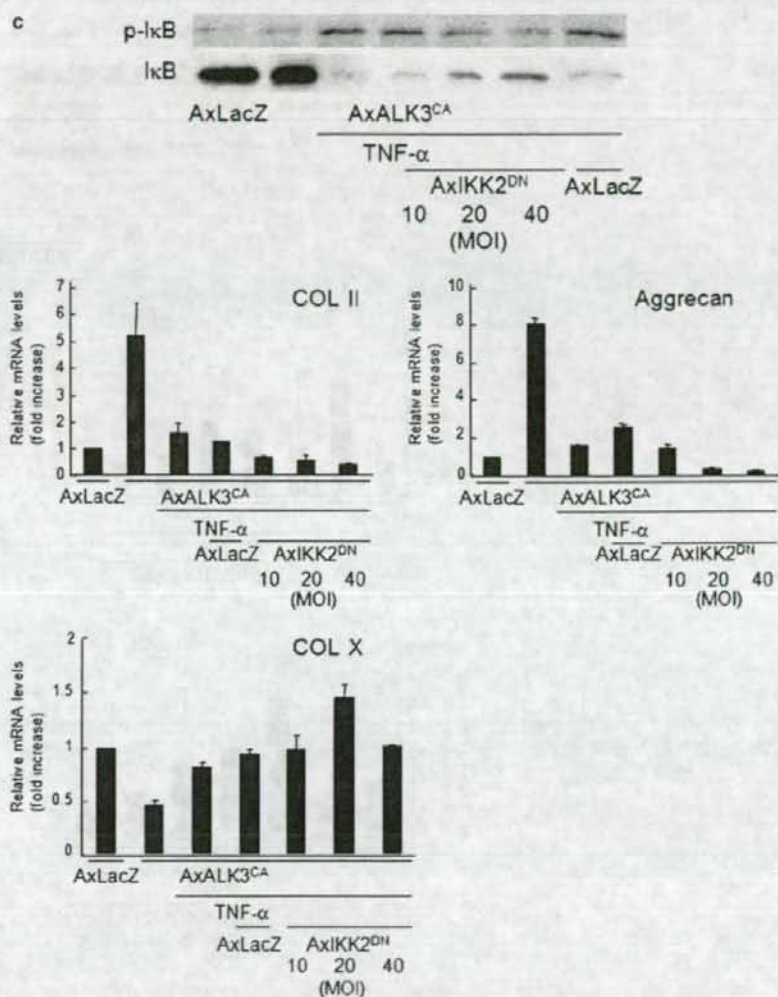
II collagen and aggrecan genes expression at doses of less than 0.3 μ M (Fig. 2d, middle panels). However, such recovery effects were not observed at doses higher than 1 μ M (Fig. 2d, middle panels). In contrast, the type X collagen expression induced by TNF- α was suppressed by SB203580 in a dose-dependent manner (Fig. 2d, lower panels). SB203580 at 3 μ M completely suppressed the p38 MAPK activation, as determined by the molecular weight shift of MAPKAPK-2, while 0.3 μ M SB203580 reduced it by about 50% (Fig. 2d, upper panels). It should be noted that SB203580 rather stimulated JNK activation in SFs (Fig. 3) [31]. To exclude the possibility that SB203580 acts on SFs by stimulating JNK pathway, SFs were co-treated with SB203580 and SP600125. Co-treatment completely suppressed both p38 and JNK activation, but had no additive or negative effects on the effect of SB203580 (Fig. 3).

The effect of SB203580 was further confirmed by histological analysis. The suppressive effect of TNF- α on cartilage matrix production, as shown by Alcian Blue staining and anti-type II collagen immunostaining, was restored by the addition of SB203580 at 0.3 μ M (Fig. 4). SB203580 also inhibited the type X collagen staining induced by TNF- α (Fig. 4).

Discussion

We previously reported that adenovirus vector-mediated overexpression of ALK3^{CA} effectively induced chondrocyte-specific gene expression in SFs [14]. ALK3 is a BMP type IA receptor that binds BMP-2 [32], and therefore, we examined the effect of rhBMP-2 on SFs. As expected, rhBMP-2 treatment effectively induced the expression of

Fig. 2 continued

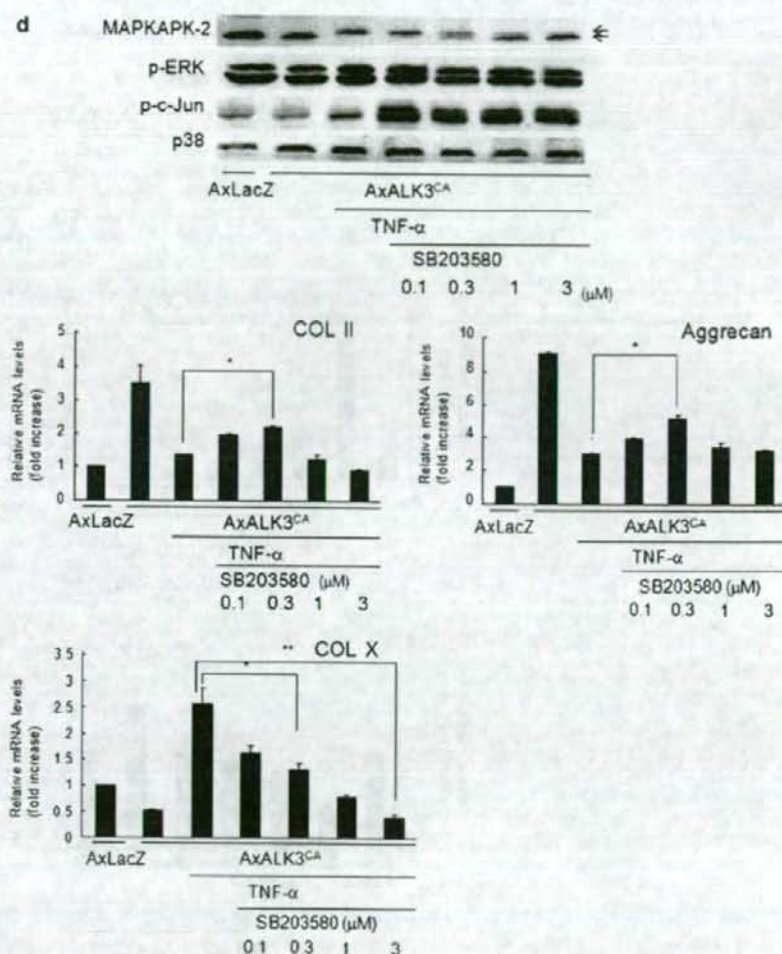


chondrocyte-specific genes such as type II collagen and aggrecan in a dose-dependent manner. On the other hand, the expression of type X collagen was significantly suppressed by rhBMP-2 treatment, consistent with the results obtained from ALK3^{CA} overexpression. Type X collagen is a marker of hypertrophic chondrocytes and its expression is upregulated in degenerative cartilage as previously reported [33]. Therefore, rhBMP-2 has dual functions in chondrogenesis: it not only promotes chondrogenic differentiation of SFs, but also protects them from terminal hypertrophic differentiation and degeneration.

Recent clinical experience has revealed that anti-TNF- α agents such as infliximab and etanercept not only retard

joint destruction in RA by ameliorating synovitis, but also promote the repair of previously existing joint damage [15–17]. TNF- α stimulates cellular infiltration into synovium, and exacerbates joint damage by stimulating the production of various proteinases which directly degrade articular cartilage [34]. Our results suggest that TNF- α antagonized the anabolic function of the BMP-2-ALK3 signaling pathways, strongly reduced type II collagen and aggrecan gene expression, and increased type X collagen expression in both rhBMP-2-treated and ALK3^{CA}-transduced SFs. IL-1 β also exhibited a similar tendency, but its effect was much milder than that of TNF- α (data not shown). In addition, histological analysis revealed that TNF- α

Fig. 2 continued



suppressed glycosaminoglycan production, as shown by Alcian Blue staining, type II collagen immunostaining, and increased type X collagen staining. These results suggest that TNF- α deteriorates joint integrity not only by directly degenerating articular cartilage, but also indirectly, by promoting hypertrophic differentiation and the degradation of cartilaginous matrix in SFs. This finding may explain the molecular mechanism how anti-TNF- α agents restored the joint damage in RA patients.

To analyze the signal transduction pathways of TNF- α that leads to hypertrophic differentiation of SFs, we conducted inhibitor experiments. rhBMP-2 treatment or the introduction of ALK3^{CA} induced phosphorylation of Smad1/5/8 and p38 MAPK. Treating the cells with TNF- α

markedly stimulated the p38 MAPK, ERK, JNK and NF- κ B pathways. A specific inhibitor of ERK, PD98059, suppressed the ERK activation induced by TNF- α without affecting other MAPK activation, but did not restore the suppressive effect of TNF- α on chondrogenic differentiation of SFs at any of the doses examined. A specific inhibitor of JNK, SP600125, suppressed the JNK activation induced by TNF- α , but did not restore the suppressive effect of TNF- α on chondrogenic differentiation of SFs at any of the doses examined. Adenovirus vector-mediated IKK2^{DN} suppressed the NF- κ B activation induced by TNF- α , but had no effect on type II collagen or aggrecan expression. Interestingly, however, a p38 MAPK inhibitor SB203580 displayed a dual function, and partially

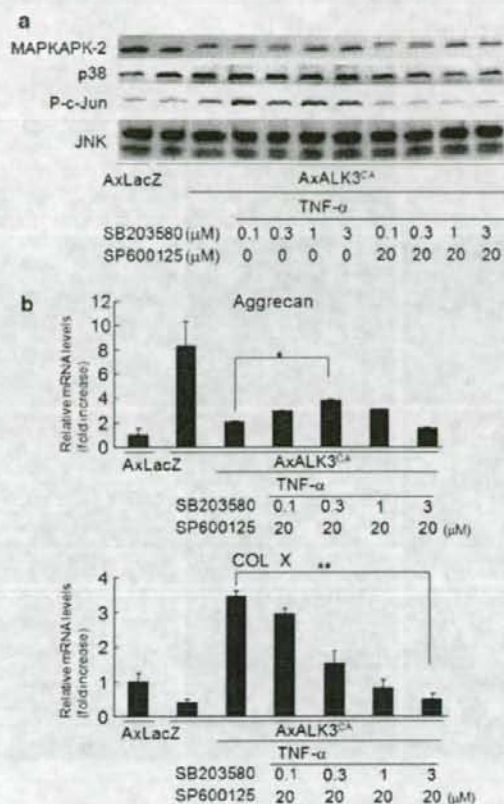


Fig. 3 Dual suppression of the p38 MAPK and JNK pathways had no additive effects. **a** Co-treatment of SFs with SB203580 and SP600125 completely abolished TNF- α -induced p38 MAPK and JNK activation, as shown by the molecular weight shift of MAPKAPK-2 and phosphorylation of c-Jun. **b** SB203580 at 0.3 μ M partially restored the TNF- α -induced reduction of aggrecan expression and suppression in type X collagen expression. SP600125 had no additive effect to that of SB203580 at any of the doses examined. Single asterisk and double asterisks are significantly different, * $P < 0.01$, ** $P < 0.005$

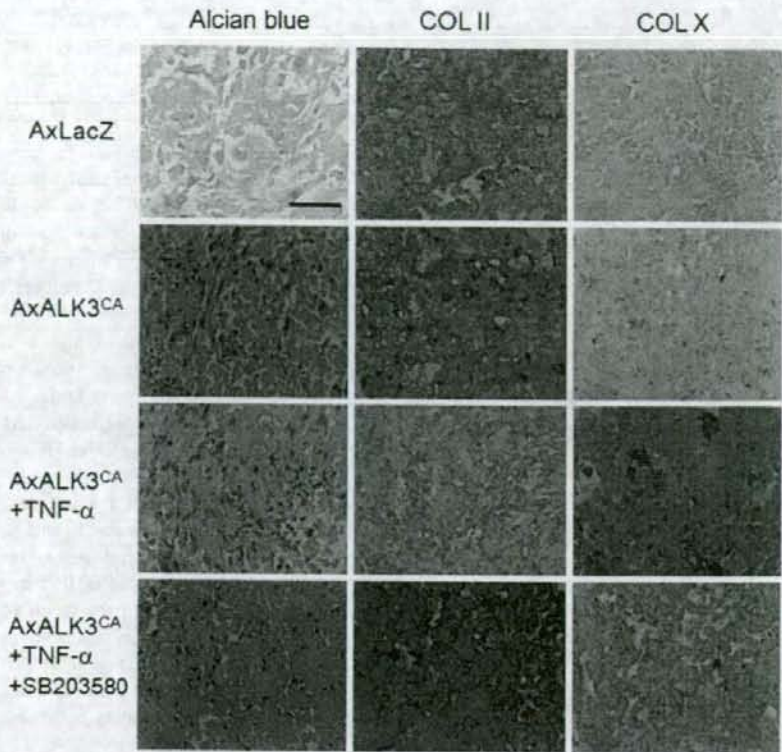
restored type II collagen and aggrecan gene expression at lower doses, but the effect was not observed at higher doses. SB203580 suppressed type X collagen expression in SFs dose-dependently. Histological analysis also demonstrated the restoration potential of SB203580 on the cartilaginous matrix production by SFs. Although SB203580 displayed stimulatory effects on JNK pathways, co-treatment of the cells with SP600125 had neither an additive nor antagonistic effect on the SB203580 activity (Fig. 3). Treatment of SB203580 alone had no stimulatory effects on ALK3^{CA}-induced chondrogenesis (data not shown). It was previously reported that the

inhibition of p38 pathways by SB203580 suppressed the chondrogenic differentiation of ATDC5 cells induced by growth differentiation factor-5 [35, 36]. On the other hand, Zhen et al. [37] reported that parathyroid hormone inhibits type X collagen expression in hypertrophic chondrocytes by suppressing p38 pathways. These findings, combined with our present observation, suggest that although activation of the p38 MAPK pathways is necessary for the chondrogenic differentiation of SFs, its hyperactivation by TNF- α leads to hypertrophic differentiation and reduces type II collagen and aggrecan expression.

In this study, it was demonstrated that the BMP-2 signaling pathways promote the chondrogenic differentiation of SFs. Lories RJ et al. [38] reported that BMP-2 and BMP-6 were detected in the hyperplastic lining and the sublining layer of synovium from RA patients, and yet, neochondrogenesis is hardly observed in the inflamed joints of RA patients. We speculate that this is because TNF- α strongly antagonizes the anabolic action of BMP-2, and therefore, combination therapy with anti-TNF- α agents and BMP-2 may be a useful therapeutic strategy to stimulate the repair of damaged joints in RA. Moreover, since our results suggest that the anti-anabolic activity of TNF- α is at least partially mediated by the p38 MAPK pathways, therapeutics targeting p38 MAPK may be able to stimulate neochondrogenesis in the damaged joints as well as to inhibit joint destruction. In fact, several p38 inhibitors are currently in clinical trials for RA [39–41]. However, it should be noted that SB203580 only partially restored the catabolic effects of TNF- α at 0.3 μ M, and that higher doses of the compound rather abrogated its anti-catabolic effect. Since p38 MAPK is required for the differentiation of chondrocytes, excessive suppression of the pathways is not favorable for chondrogenesis. Other signaling pathways may be involved, or more specific inhibitors may be required to establish a complete recovery.

We here demonstrated the chondrogenic potential of SFs obtained from RA patients. Sekiya and co-workers recently reported that SFs from patients with anterior cruciate ligament injuries or osteoarthritis exhibited chondrogenesis potential in the presence BMP-2 [42]. They also reported that mesenchymal stem cells obtained from synovium had higher chondrogenic activity than those from other tissues. These observations suggest the high chondrogenic potential of SFs [43] although the molecular mechanism underlying remains unclear. In addition, the difference of chondrogenic potential of SFs from RA and other diseases has not been clarified yet, and should be analyzed in future.

Fig. 4 Histological analysis. SFs pellets after 4 weeks of culture were fixed and subjected to Alcian Blue staining and immunostaining with anti-type II collagen (COL II) or anti-type X collagen (COL X) antibody. The Alcian Blue staining and type II collagen immunostaining suppressed by TNF- α treatment were restored by adding 0.3 μ M SB203580. TNF- α -induced type X collagen staining was inhibited by the addition of SB203580 at 0.3 μ M. No positive staining was observed in the untreated cultures. Bar 50 μ m



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The Efficacy of Prostaglandin E1 Derivative in Patients With Lumbar Spinal Stenosis

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Study Design. Randomized controlled trial.

Objective. To examine the effect of limaprost, an oral prostaglandin (PG) E1 derivative, on health-related quality of life (HRQOL) in patients with symptomatic lumbar spinal stenosis (LSS), compared to etodolac, a NSAID.

Summary of Background Data. Limaprost, an oral PGE1 derivative, was developed in Japan to treat numerous ischemic symptoms of thromboangiitis obliterans (TAO) and LSS. Previous studies have demonstrated the effectiveness of limaprost in the symptoms in patients with LSS. However, the evidence for effect on patient-reported outcomes, such as patient's HRQOL or satisfaction, is limited.

Methods. This study was conducted at 4 study sites in Japan. Briefly, inclusion criteria were: age between 50 and 85 years; presence of both neurogenic intermittent claudication (NIC) and cauda equina symptoms (at least presence of bilateral numbness in the lower limbs); and MRI-confirmed central stenosis with acquired degenerative LSS. Limaprost (15 µg/d) or etodolac (400 mg/d) was administered for 8 weeks. The primary outcome was Short Form (SF)-36, and the secondary outcomes were the verbal rating scale of low back pain and leg numbness, walking distance, subjective improvement, and satisfaction.

Results. A total of 79 participants were randomized (limaprost:etodolac = 39:40). Thirteen participants withdrew from the study (limaprost:etodolac = 5:8) and 66 completed the study (limaprost:etodolac = 34:32). Comparisons showed that limaprost resulted in significantly greater improvements in the SF-36 subscales of physical functioning, role physical, bodily pain, vitality, and mental health. Limaprost was also significantly better than etodolac for leg numbness, NIC distance, and subjective improvement and satisfaction. In the subgroup analysis stratified by symptom severity, limaprost seemed more effective for milder symptoms. No serious adverse effects were reported in either treatment group.

Conclusion. In this study, limaprost was found to be efficacious on most outcome measures, such as HRQOL, symptoms and subjective satisfaction, in LSS patients with cauda equina symptoms.

Key words: lumbar spinal stenosis, limaprost, neurogenic intermittent claudication. *Spine* 2009;34:115-120

Lumbar spinal stenosis (LSS) is a neurologic syndrome caused by vertebral degeneration.^{1,2} Patients usually present with low back pain, leg pain, and leg numbness. These symptoms are exacerbated by lumbar extension but relieved by flexion. The cardinal symptom of LSS is neurogenic intermittent claudication (NIC).³ Symptoms associated with LSS generally decrease the quality of life of patients, which may cause them to seek treatment. Initial treatment of LSS is not surgical intervention but conservative management because rapid neurologic progression and cauda equina syndrome are rarely seen⁴ and also because patients are often elderly. Drug therapy is usually used in patients with mild to moderate symptoms. As far as pharmacotherapy is concerned, nonsteroidal anti-inflammatory drugs (NSAIDs) have primarily been administered.^{4,5}

Although the pathogenesis of LSS symptoms remains controversial, mechanical compression of the cauda equina and nerve roots by the degenerative lumbar spine or the related soft tissues including thickened ligamentum flavum disturb vascular circulation of neural elements in the spinal canal. In other words, a state of relative ischemia in nerve tissues is a potential mechanism causing NIC.⁶⁻¹⁷

Prostaglandin (PG) E1 is a vasodilator that increases blood flow and inhibits platelet aggregation. Intravenous PGE1 is primarily used for chronic peripheral arterial occlusive diseases (PAOD) and erectile dysfunction (ED) in the United States and Europe. Limaprost is an oral PGE1 derivative that was developed in Japan to treat ischemic symptoms of thromboangiitis obliterans (TAO) and LSS because of the well-known vasodilatory and antiplatelet properties. The efficacy of oral limaprost was evaluated in adult Japanese patients in 3 randomized, double-blind, 6-week trials.¹⁸ One study included patients with TAO and 2 trials included patients with LSS.

Previous studies have demonstrated the effectiveness of limaprost in the symptoms of patients with LSS. However, the evidence of the effect on patient-based outcomes, such as patient's HRQOL or satisfaction, is limited. As well as symptomatic assessment, HRQOL

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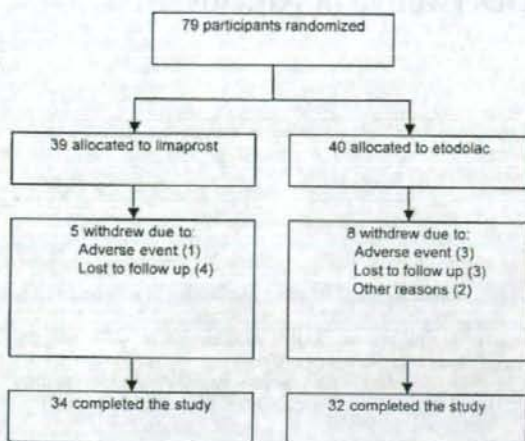


Figure 1. Flow chart showing flow of participants through the study.

assessment is also crucial to assess the treatment of a disabling chronic condition like LSS. The primary objective of this study was to examine the effect of limaprost on health-related quality of life (HRQOL), compared to etodolac,¹⁹ a NSAID, which is commonly used in drug therapy for LSS.

Materials and Methods

This randomized, open-label, active-controlled trial was conducted at 4 study sites in Japan as follows: the University of Tokyo Hospital, Tokyo Metropolitan Geriatric Hospital, Japanese Red Cross Medical Center, and Musashino Red Cross Hospital. The study protocol was reviewed and approved by the ethics committees of all the study sites. All participants provided written informed consent before study procedures were initiated.

Patients who met the following eligibility criteria were enrolled in the study. Briefly, inclusion criteria were: age between 50 and 85 years; presence of both NIC and cauda equina symptoms (at least presence of bilateral numbness in the lower limbs); and MRI-confirmed central stenosis with acquired degenerative LSS.²⁰ Exclusion criteria were: presence of radicular leg pain only; positive straight leg raising (SLR) test with suspected lumbar disc herniation; presenting lower extremity peripheral arterial occlusive diseases PAOD (arteries of foot palpable and ankle brachial pressure index of <0.9); history of spinal surgery, myelopathy, peripheral neuropathy such as diabetic neuropathy of the leg; disorders potentially hindering gait besides LSS, *i.e.*, severe visual impairment, cerebral infarction, parkinsonism, rheumatoid arthritis, reduced cardiopulmonary function, or digestive, hematological, hepatic and/or renal disorders; or use of cardiac medication, *i.e.*, vasodilators or antiplatelet agents, or antidepressant medication.

Eligible patients were randomly allocated either to the limaprost or the etodolac group. Allocation to the groups was performed by the University Hospital Medical Information Network (UMIN), which was completely independent from the study sites, through a central web-based registration system. The minimization technique was used in random allocation to ensure balance on the following 4 factors: age (<75 years/ \geq 75

years), gender, severity (mild/severe; definition of severe symptoms is the existence of perineal symptoms including urinary disturbance, and/or leg numbness while resting), and study site. Participants were randomly allocated to receive 15 μ g of limaprost 3 times a day, or 400 mg of etodolac, NSAID, twice a day. Both treatments were administered for 8 weeks in an open-label fashion. Analgesics, anti-inflammatory agents, muscle relaxants, calcitonin, and methycobalamin were prohibited. If any of these drugs had been used, a 2-week washout period was established. During the study period, injection therapy, such as root block, epidural block, trigger point injection, physical therapy, or the use of a flexion brace, was also not allowed.

The primary efficacy outcome was SF-36^{21,22} version 2 score, ranging from 0 to 100. The secondary efficacy outcomes were the verbal rating scales (rating score of 6: none, slight, mild, moderate, strong, and severe) of low back pain, leg pain and leg numbness, walking distance (rating score of 5: \geq 1000 m, \geq 500 m, \geq 100 m, <100 m, and a few gait), and subjective improvement (rating score of 4: improved, slightly improved, unchanged, and became worse) and satisfaction (4 rating score: satisfied, slightly satisfied, slightly dissatisfied and dissatisfied). These outcomes were measured at baseline and then at week 8 after administration. All reported or observed adverse events were recorded at each visit.

Statistical Analysis

Assessments were conducted of all efficacy outcomes for participants who received at least 1 dose of study drug and had had a baseline assessment and a final assessment. Between-group differences in mean changes from baseline on the SF-36 subscales were analyzed by the independent samples *t* test unless its assumptions were violated, defined in the protocol as the primary efficacy analysis. Secondary efficacy outcomes, which all were ordered categorical response variables, were analyzed by the Cochran-Mantel-Haenszel statistics to test for association between study drug and response. Baseline adjustments were made for all the outcomes except patient-reported improvement and satisfaction. In addition, both efficacy outcomes were also analyzed in relation to the degree of symptom severity. Safety assessments were based on all participants who received at least 1 dose of study drug. Adverse events were summarized descriptively. All statistical tests were 2-tailed, and the significance level was fixed at 0.05 throughout. All computations were performed using SAS version 9.1 and JMP 7.0.

Table 1. Baseline Characteristics of the Participants

Characteristic	Limaprost Group (n = 34)	Etodolac Group (n = 32)
Age (yrs)		
\geq 75	12	12
<75	22	20
Mean \pm SD	69.6 \pm 9.0	72.2 \pm 8.2
Sex		
Male:Female	20:14	22:10
Symptom severity		
Mild	17	16
Severe	17	16
Study center		
University of Tokyo Hospital	24	22
Japanese Red Cross Medical Center	5	4
Musashino Red Cross Hospital	2	2
Tokyo Metropolitan Geriatric Hospital	3	4

Values are n unless otherwise stated.

Table 2. Primary Efficacy Outcomes: SF-36 Subscale Scores

SF-36 Subscales	Limaprost Group (n = 34)		Etodolac Group (n = 32)		Limaprost Group Change From Baseline	Etodolac Group Change From Baseline	P*
	Baseline	Wk 8	Baseline	Wk 8			
Physical functioning	56.9 ± 20.0	67.9 ± 19.1	55.7 ± 21.1	57.3 ± 22.4	11.0 ± 14.6	1.6 ± 14.9	0.01
Role physical	61.8 ± 26.6	73.0 ± 24.5	62.9 ± 24.3	60.4 ± 32.7	11.2 ± 22.0	-2.5 ± 27.0	0.03
Bodily pain	42.3 ± 15.0	56.0 ± 18.7	47.0 ± 18.3	45.2 ± 18.3	13.7 ± 20.6	-1.8 ± 12.1	<0.01
General health	53.5 ± 17.5	57.8 ± 17.5	58.7 ± 17.7	56.4 ± 17.1	4.3 ± 11.2	-2.3 ± 18.1	0.08
Vitality	57.5 ± 23.1	64.7 ± 19.2	64.8 ± 23.4	60.7 ± 24.2	7.2 ± 19.9	-4.1 ± 17.4	0.02
Social functioning	70.2 ± 25.9	79.8 ± 25.7	69.1 ± 22.9	70.7 ± 23.2	9.6 ± 24.0	1.6 ± 22.6	0.17
Role emotional	68.1 ± 27.0	75.7 ± 20.9	65.6 ± 22.4	63.0 ± 31.0	7.6 ± 19.3	-2.6 ± 25.7	0.07
Mental health	66.3 ± 20.6	73.7 ± 17.2	71.1 ± 23.3	65.8 ± 23.0	6.9 ± 16.0	-5.3 ± 16.6	<0.01

Values are mean ± SD.

*The independent samples t test.

Results

A total of 79 participants were enrolled and randomized to receive study treatment between June 2002 and January 2005. Of these, 39 participants received limaprost, and 40 received etodolac (Figure 1). Thirteen participants withdrew during the study: 5 from the limaprost group (1 adverse event and 4 lost to follow-up) and 8 participants from the etodolac group (3 adverse events, 3 lost to follow-up and 2 for another reason). Thus, a total of 34 participants in the limaprost group and 32 in the etodolac group were assessed after the completion of the study. Baseline characteristics were similar between treatment groups (Table 1).

In the primary efficacy assessment, compared to the etodolac group, the limaprost group had a significantly greater improvement in the SF-36 subscales of physical functioning (PF) (mean change of limaprost *vs.* etodolac: 11.0 *vs.* 1.6, $P = 0.01$), role physical (RP) (11.2 *vs.* -2.5, $P = 0.03$), bodily pain (BP) (13.7 *vs.* -1.8, $P < 0.01$) for the physical health component summary and vitality

(VT) (7.2 *vs.* -4.1, $P = 0.02$), and mental health (MH) (6.9 *vs.* -5.3, $P < 0.01$) for the mental health component summary (Table 2). Improvement trends for other subscales, such as general health (GH) for the mental health component summary and social functioning (SF) and role emotional (RE) for the mental health component summary, were also impressive in limaprost, but they did not reach statistical significance (mean change of limaprost *vs.* etodolac, P value: 4.3 *vs.* -2.3, $P = 0.08$; 9.6 *vs.* 1.6, $P = 0.17$; and 6.9 *vs.* -5.3, $P = 0.07$, respectively). In the additional analysis stratified by symptom severity, the subscales that demonstrated significantly higher scores in limaprost were: PF, BP, VT, and MH among participants with mild symptoms ($P = 0.04$, $P < 0.01$, $P = 0.03$ and $P < 0.01$, respectively); and BP among those with severe symptoms ($P = 0.02$) (Table 3). Overall, limaprost showed better results in the mild group, compared to the severe group. In addition, the results of the stratified analysis indicated the same directional effects as the overall results, favoring limaprost instead of

Table 3. Primary Efficacy Outcomes: SF-36 Subscale Scores by Symptom Severity

SF-36 Subscales	Severity	Limaprost Group (n = 34)*		Etodolac Group (n = 32)*		Limaprost Group Change From Baseline	Etodolac Group Change From Baseline	P†
		Baseline	Wk 8	Baseline	Wk 8			
Physical functioning	Mild	61.8 ± 18.1	77.5 ± 12.9	61.4 ± 19.3	64.4 ± 22.7	15.7 ± 17.6	3.0 ± 15.4	0.04
	Severe	52.1 ± 21.1	58.3 ± 19.7	50.0 ± 21.8	50.2 ± 20.3	6.3 ± 9.0	0.2 ± 14.8	0.16
Role physical	Mild	60.7 ± 31.2	74.6 ± 28.0	66.0 ± 25.2	68.5 ± 26.6	14.0 ± 19.3	3.5 ± 19.4	0.13
	Severe	62.9 ± 21.9	71.3 ± 21.5	59.8 ± 23.8	51.2 ± 36.3	8.5 ± 24.6	-8.6 ± 32.4	0.10
Bodily pain	Mild	43.5 ± 17.2	62.2 ± 20.3	49.6 ± 19.1	48.4 ± 15.7	18.6 ± 24.9	-1.2 ± 13.0	<0.01
	Severe	41.0 ± 12.9	49.8 ± 15.0	44.4 ± 17.8	41.9 ± 20.5	8.8 ± 14.3	-2.4 ± 11.6	0.02
General health	Mild	54.1 ± 19.0	59.9 ± 18.5	64.4 ± 16.8	62.2 ± 16.6	5.8 ± 11.3	-2.2 ± 17.6	0.13
	Severe	52.8 ± 16.5	55.6 ± 16.8	53.1 ± 17.2	50.6 ± 16.0	2.8 ± 11.2	-2.5 ± 19.3	0.34
Vitality	Mild	61.8 ± 22.0	69.1 ± 18.2	74.2 ± 16.0	69.1 ± 17.1	7.4 ± 19.2	-5.1 ± 11.7	0.03
	Severe	53.3 ± 24.1	60.3 ± 19.8	55.5 ± 26.2	52.3 ± 27.8	7.0 ± 21.2	-3.1 ± 22.1	0.19
Social functioning	Mild	71.3 ± 27.9	80.1 ± 28.7	71.1 ± 22.7	71.1 ± 22.7	8.8 ± 16.4	0.0 ± 25.0	0.24
	Severe	69.1 ± 24.7	79.4 ± 23.4	67.2 ± 23.7	70.3 ± 24.5	10.3 ± 30.4	3.1 ± 20.7	0.44
Role emotional	Mild	70.1 ± 30.5	77.0 ± 22.9	71.9 ± 19.0	69.8 ± 29.2	6.9 ± 13.6	-2.1 ± 22.5	0.17
	Severe	66.2 ± 23.8	74.5 ± 19.2	59.4 ± 24.3	56.3 ± 32.3	8.3 ± 24.1	-3.1 ± 29.3	0.23
Mental health	Mild	66.8 ± 21.9	75.6 ± 16.8	80.9 ± 14.5	74.7 ± 15.0	8.8 ± 15.9	-6.3 ± 14.0	<0.01
	Severe	65.9 ± 19.8	70.9 ± 17.9	61.3 ± 26.5	56.9 ± 26.4	5.0 ± 16.4	-4.4 ± 19.3	0.14

Values are mean ± SD.

*For limaprost, mild/severe = 17/17; and for etodolac, mild/severe = 16/16.

†The independent samples t test.

Table 4. Secondary Efficacy Outcomes: Low Back Pain, Leg Pain, and Leg Numbness

Outcome	Severity	Limaprost Group (n = 34)		Etodolac Group (n = 32)		P*
		Baseline	Wk 8	Baseline	Wk 8	
Low back pain	None	6	7	4	7	0.77
	Slight	1	8	5	5	
	Mild	8	9	6	6	
	Moderate	16	7	11	9	
	Strong	3	2	4	3	
	Severe	0	1	2	2	
Leg pain	None	2	2	4	2	0.08
	Slight	2	4	0	4	
	Mild	1	15	5	6	
	Moderate	19	8	13	11	
	Strong	9	4	7	7	
	Severe	1	1	3	2	
Leg numbness	None	0	3	0	0	<0.01
	Slight	3	8	0	1	
	Mild	5	10	4	2	
	Moderate	15	9	14	17	
	Strong	7	3	13	9	
	Severe	4	1	1	3	

Values are n.

*The Cochran-Mantel-Haenszel test, adjusting for baseline value.

etodolac; however, the magnitudes of the effects were changed, and some of the effects failed to reach statistical significance (Table 3).

Secondary efficacy outcomes significantly favoring limaprost over etodolac included leg numbness ($P < 0.01$) (Table 4), walking distance ($P < 0.01$) (Table 5), and subjective improvement and satisfaction ($P < 0.01$ for both) (Table 6). The secondary outcomes of low back pain and leg pain tended to be better for the limaprost group without achieving statistical significance ($P = 0.77$ and $P = 0.08$, respectively) (Table 4). The stratified analysis did not alter the overall results. In addition, the secondary outcomes were stratified by symptom severity and analyzed in the same manner. The stratified analysis did not alter the overall results, with the exception of walking distance not being statistically significant in the mild group (limaprost *vs.* etodolac: for low back pain, $P = 0.26$ in mild and $P = 0.52$ in severe; leg pain, $P = 0.12$ mild and $P = 0.78$ severe; leg numbness, $P < 0.01$ mild and $P < 0.01$ severe; walking distance, $P = 0.03$

Table 5. Secondary Efficacy Outcomes: Neurogenic Intermittent Claudication (NIC) Distance

Distance	Limaprost Group (n = 34)		Etodolac Group (n = 32)		P*
	Baseline	Wk 8	Baseline	Wk 8	
≥1000m	2	9	5	4	<0.01
≥500m	12	9	8	7	
≥100m	11	10	11	9	
<100m	8	6	8	12	
A few gait	1	0	0	0	

*The Cochran-Mantel-Haenszel test, adjusting for baseline value.

mild and $P = 0.07$ severe; subjective improvement, $P < 0.01$ mild and $P < 0.01$ severe; and subjective satisfaction, $P < 0.01$ mild and $P = 0.01$ severe).

During the study, 3 participants in the limaprost group and 3 in the etodolac group reported a minor adverse event. In the limaprost group, 1 participant experienced hot flashes/flushing and anorexia a few days after administration, leading to withdrawal from the study, and 2 complained of stomach discomfort (digestive tract symptoms) at the start of administration and 4 weeks after administration, respectively. However, both of them completed the study because the reported event disappeared a few days later. In the etodolac group, 2 participants reported gastric pain and 1 diarrhea and all of them withdrew from the study at week 2, week 4, and week 1 after administration, respectively. No serious adverse events were reported in either treatment group.

Discussion

The results of this study suggested that limaprost might be more efficacious than etodolac in LSS patients with cauda equina symptoms. In the comparison of SF-36 subscales between the 2 drug groups, limaprost proved significantly better than etodolac in improving both the physical (PF, RP, and BP) and mental health (VT and MH) component summaries. In the secondary efficacy assessment, leg numbness, walking distance, and patient-reported improvement and satisfaction were significantly improved in the limaprost group over the etodolac group. In the subgroup of patients with milder symptoms, not consisting of perineal symptoms including urinary disturbance and/or leg numbness while resting, limaprost seemed more effective. Increasing physical activities resulting from symptom improvement promote sequential improvements in mental and emotional factors. This might be part of the reason for improvements of the mental component summary, such as VT and MH.

In Japan, limaprost was approved in 1988 with an indication for the "improvement of various ischemic symptoms, such as ulcer, pain, and feeling of coldness associated with TAO." It was also approved in 2001 with an indication for "the improvement of subjective symptoms (pain and numbness of lower legs) and gait ability associated with acquired LSS." In a phase III trial in LSS patients, limaprost was considered by investigators to provide an overall improvement from baseline to study end and to improve an overall drug usefulness.¹⁸ Assessment of overall improvement considered symptoms (e.g., leg pain or numbness, walking distance), whereas overall usefulness also considered safety issues. Our study provides significant improvements in leg numbness and walking distance, which was consistent with the above phase III trial. However, there was no significant improvement in low back pain and leg pain. According to previous studies, including postmarketing studies, limaprost was safely administered and well tolerated. In the postmarketing surveillance where 1930 patients with LSS took limaprost for a mean duration of

Table 6. Secondary Efficacy Outcomes: Patient's Subjective Improvement and Satisfaction

Outcome	Subjective Rating	Limaprost Group (n = 34) n (%)	Etodolac Group (n = 32) n (%)	P*
Patient's subjective improvement	Improved	11 (32.4)	0 (0)	<0.01
	Slightly improved	14 (41.2)	6 (18.8)	
	Unchanged	8 (23.5)	19 (59.4)	
	Became worse	1 (2.9)	7 (21.9)	
Patient's subjective satisfaction	Satisfied	14 (41.2)	1 (3.1)	<0.01
	Slightly satisfied	17 (50.0)	13 (40.6)	
	Slightly dissatisfied	2 (5.9)	16 (50.0)	
	Dissatisfied	1 (2.9)	2 (6.3)	

Values are n (%).

*The Cochran-Mantel-Haenszel test.

approximately 4 months, the overall incidence of treatment-related adverse effects occurred in 5.2% of patients. One serious adverse event (bleeding duodenal ulcer) was reported (<0.001% in total). The common adverse effects included gastrointestinal (GI)-related effects and hot flushes/flushing. These effects were usually not serious. The safety findings of our study were in general agreement with the findings in other studies.

Elderly people sometimes have comorbidities, and patients, as well as physicians, seek effective conservative treatment. However, nonoperative approaches have been limited to NSAIDs and physical treatment, and the actual effectiveness of these has not been sufficiently studied. NSAIDs are usually administered for pain control,^{4,5} but no RCTs have been conducted to assess the efficacy of NSAIDs. Although several RCTs assessing the efficacy of calcitonin have been performed since Porter and Miller's report,²³ sufficient evidence to support the efficacy of this drug has not been well established because contradictory findings were reported.²⁴⁻²⁶ Recently, Yaksi *et al*²⁷ suggested that adding gabapentin to conventional therapy, such as physical therapy, brace therapy, and NSAIDs, may improve walking disturbance and pain. However, they concluded that the further evaluation for gabapentin was necessary because it was administered as an additional therapy. Thus, the investigations to assess pharmacotherapeutic options for LSS are still insufficient.

It has been reported that limaprost improved walking distance and blood flow of cauda equina tissue in a rat model with compressed cauda equina.²⁸ No such effects were observed with loxoprofen sodium (NSAID), ticlopidine (an antiplatelet), nifedipine (a vasodilator), or cilostazol (an antiplatelet and vasodilator) in the same model.^{29,30} These findings may support limaprost's expected effectiveness for NIC by increasing blood flow in the cauda equina.

Several limitations of this study should be considered. First, owing to the absence of a placebo group, the placebo effect was not eliminated, which may have weakened the results of this study. Second, because the duration of treatment was 8 weeks, the results may not be generalizable to longer treatment periods. Third, the sample size may have been insufficient for a rigorous

statistical analysis to detect true difference between the groups. Finally, the results may generalize to patients only if they are similar in patient characteristics of this study. The fact that LSS patients generally have various comorbidities may limit the generalizability of the study results.

■ Conclusion

In this randomized controlled trial, limaprost was found to be efficacious on most outcome measures, such as HRQOL, symptoms, and subjective satisfaction, in LSS patients with cauda equina symptoms. Limaprost also seemed more effective for patients with milder symptoms, compared to those with more severe symptoms. Together with previous findings, limaprost can be considered to a likely candidate for drug therapy in LSS with cauda equine symptoms. Further studies with placebo-controls, longer treatment periods, larger sample sizes, and a variety of study populations are warranted to learn more about clinical benefits are recommended.

■ Key Points

- In this randomized controlled trial, the efficacy of limaprost, an oral PGE1 derivative, on the HRQOL and the clinical symptoms in patients with cauda equina symptoms of LSS, was examined, compared to etodolac, a NSAID. Thirty-two etodolac patients and 34 limaprost patients were analyzed.
- In terms of differences between baseline and 8-week SF-36 scores, the limaprost group showed significant improvement not only in PF, RP, BP, VT, and MH but also in leg numbness and walking distance, compared to the etodolac group. In the subgroup analysis stratified by symptom severity, limaprost seemed more effective for milder symptoms. No serious adverse events were reported in either treatment group.
- Limaprost can be considered to a likely candidate for drug therapy in LSS with cauda equine symptoms.

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