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RANKL in POB stimulated with IL-6 and sIL-6R. Taken together, our results clearly indicate that PIAS3 negatively regulates RANKL-mediated osteoclastogenesis directly in osteoclast precursors and indirectly via osteoblasts.

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extracellular stimuli, including the IL-6 cytokine family. IL-6 cytokine family members (IL-6, IL-11, leukemia inhibitory factor (LIF), Oncostatin M (OSM), and cardiotropin-1 (CT-1)) promote osteoclastogenesis and bone resorption in bone marrow cultures.[13,14] STAT3 is tyrosine phosphorylated by Jak kinase (JAK) and translocates as a dimer into the nucleus, where it activates specific genes.[15] Activated STAT3 is involved in the regulation of cell growth, differentiation, and survival, and is essential for gp130-mediated osteoclast formation, during which it induces the expression of RANKL.[16,17]

Protein inhibitor of activated STAT3 (PIAS3) was originally isolated as a molecule that binds to signal transducer and activator of transcription 3 (STAT3) and blocks its DNA-binding ability, thereby inhibiting STAT3-mediated gene activation.[18] In addition, PIAS3 binds directly to MITF and blocks MITF's binding and transcription of target genes in mast cells and melanocytes.[19] PIAS proteins mediate their functions in either a sumoylation-dependent (as an E3 ligase) or independent manner. Sumoylation has emerged as an important regulatory mechanism, especially in transcription and signal transduction.[20,21] However, the effect of PIAS3-induced MITF sumoylation on MITF transcriptional activity is unclear. [22]

In this study, we further examined the role of PIAS3 in RANKL-mediated osteoclastogenesis. It was recently reported that PIAS3 acts as a negative regulator of RANKL-mediated osteoclastogenesis by down-regulating NFTAc1 and OSCAR, in part by recruiting the histone deacetylase 1 (HDAC1)

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promoter reporter plasmid,[25] pEGFP-PIAS3,[26] and pCI-PIAS3(C334S).[27] Anti-PIAS3, STAT3, pSTAT3, MITF, c-Fos, and NFATc1 Ab were from Santa Cruz (Santa Cruz, CA). Anti-β-actin Ab was from Sigma-Aldrich. Anti-myc Ab was from MBL (Nagoya, Japan). Anti-GFP Ab was from Nacalai tesque (Kyoto, Japan).

Generation of transgenic mice

Mouse PIAS3 cDNA was fused to 1.8 kb of the mouse TRAP gene promoter.[28-30] The transgenic mice were generated by pronuclear injection methods into C57BL/6 mice. Genomic DNA from the tail was analyzed by polymerase chain reaction (PCR) and Southern blot analysis. For PCR analysis, specific primers (P1: 5'-CACTTCGCTAGATGAACAGGAC-3' P2: 5'-TGAGTTTGGACAAACCACAAC-3') were used. For Southern blot analysis, tail DNA were digested NheI and HpaI, fractionated on 0.8% agarose gel, transferred to Hybond-N+ membrane (GE healthcare, Tokyo, Japan) and probed with a 403 bp, alkaline phosphatase labeled PCR fragment of mouse PIAS3 cDNA. Southern blot probe was generated by PCR reaction with specific primers (P3: 5'-ACTGCCCTTCTATGAAGTCTATGG-3' P4: 5'-CTCAGATGACCAATTAACTACGATG-3'). All experiments were performed with sex- and age- matched mice according to the protocol approved by the

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Glycolmethacrylate. Three micrometer thick section was cut longitudinally in the proximal region of the tibia, and stained for toluidine blue O and TRAP. We analyzed the total 28-33 fields to generate the bone histomorphometrical data by the semiautomatic image analyzing system (Osteoplan II, Carl Zeiss, Thornwood, NY). The nomenclature and units used were according to the recommendation of the Nomenclature Committee of the American Society for Bone and Mineral research.[31]

Retroviral gene transfer

In order to generate retroviral stocks, pMX-PIAS3 was transfected into the packaging cell line Plat E. Viral supernatant was collected from culture media 48 h after transfection. BMMs and POBs were incubated with viral supernatant for 48 h, selected by 1μg/ml blasticidine for additional 48 h, and used for following experiments. The transducing efficiency was approximately 10 percents. RAW264.7 cells were also incubated with viral supernatant for 48 h and were selected by culturing for 2 weeks at the presence of 10μg/ml blasticidine.

Osteoclast formation

Osteoclasts were induced from RAW264.7 cells by plating at 5×10^3 cells/48-well plate in α-MEM

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Vascular endothelial growth factor-A is a survival factor for nucleus pulposus cells in the intervertebral disc

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ABSTRACT

The intervertebral disc (IVD) is composed of two avascular tissue types, the nucleus pulposus (NP) and the annulus fibrosus (AF). IVDs is the largest avascular tissue in the human body, however, how these tissues are maintained without a blood supply is poorly understood. Here we show that vascular endothelial growth factor-A (VEGF-A) is highly expressed in NP and that VEGF-A plays a role in NP survival. High VEGF-A expression in NP was detected by microarray analysis, and NP was positive for the hypoxic probe pimonidazole and hypoxia-responsive genes. VEGF-A expression in NP was promoted by hypoxic conditions *in vitro*. NP cells also expressed the membrane-bound VEGF receptor-1 (VEGFR-1), and the number of apoptotic cells in cultured cell model of NP increased following treatment with VEGFR-1-Fc, which traps VEGF-A in NP. These results indicate that NP is a hypoxic tissue, and that VEGF-A functions in NP survival in an autocrine/paracrine manner.
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The intervertebral disc (IVD) is located between vertebral bodies and is composed of two distinct tissues: a gelatinous center known as the nucleus pulposus (NP) and surrounding coaxial lamellae that form the annulus fibrosus (AF). NP is an avascular tissue, and NP avascularity is crucial for NP homeostasis and function. NP plays essential roles in flexibility and stability of the spine, which decrease with aging, suggesting that NP homeostasis may be downregulated in an age-dependent manner or that the NP undergoes degeneration with aging.

NP is rich in chondrogenic extracellular-matrix (ECM) proteins, which maintain tissue integrity. Recent studies indicate that several genes encoding ECM proteins function in the etiology and pathogenesis of IVD degeneration, and that these proteins maintain IVD homeostasis in humans and mice [1–3].

VEGF-A has strong angiogenic activity and specific mitogenic and chemotactic actions on endothelial cells [4]. VEGF-A synthesis is increased several fold by hypoxia [5]. Vascular endothelial cells highly express both VEGF receptor-1 (VEGFR-1) and VEGFR-2 [6]. Non-endothelial cells, including monocytes and hematopoietic stem cells, reportedly express VEGFR-1 or VEGFR-2, respectively [7–9]. A soluble form of VEGFR-1 (sVEGFR-1), a VEGFR-1 splice variant, has been shown to have potent anti-angiogenic activity in cornea [10]. On the other hand, VEGFR-1-mediated signaling through the membrane-bound form of VEGFR-1 (mbVEGFR-1) reportedly plays a role in pathological conditions, including carcinogenesis and inflammatory disease [11,12].

Here, we demonstrate that use microarray, RT-PCR and immunohistochemical analysis to show that NP cells express both VEGF-A and mbVEGFR-1, and that VEGF-A acts as a survival factor in NP in an autocrine/paracrine manner. NP is a hypoxic probe, pimonidazole, positive and hypoxia-responsive gene expressing hypoxic tissue, and VEGF-A expression is promoted by hypoxia in NP.

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Materials and methods

Animals. All animals were born and kept under pathogen-free conditions and cared for in accordance with guidelines of Keio University School of Medicine. *Vegfr-1*$^{lacZ/+}$ and *Vegfr-2*$^{lacZ/+}$ mice were gifts of J. Rossant (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ont., Canada).

Microarray analysis. Microarray analysis was performed as described [13]. Total RNA was extracted from tissues of 8-week-old male Wistar rats using TRIzol Reagent (Invitrogen). To ensure a sufficient amount of Poly $(A)^+$ RNA for screening, we individually pooled tissues from NP, AF, patella tendon and articular cartilage of the femoral head from 50 rats. For the remaining tissues, we pooled tissues from 10 rats.

RT-PCR and real-time quantitative RT-PCR analysis. Total RNA was extracted from NP and AF of 12-, 40- and 80-week-old Wistar male rats or from cultured rat NP cells. Total RNA was also extracted from human IVD tissues dissected from scoliosis patients as normal IVD using an RNeasy mini kit (Qiagen GmbH) or Trizol (Invitrogen). First strand cDNA was prepared using a first strand synthesis kit (Invitrogen) according to the manufacturer's instruction. PCR and real-time PCR were performed as described [13]. mRNA expression levels are normalized to *β-actin* expression. The primers were as follows or as described [13]. Real-time quantitative RT-PCR analysis:

5′-rat *Vegf-A* 5′-GGAGAAAGCATTTGTTTGTCCAAG-3′
3′-rat *Vegf-A* 5V-TCACATCTGCAAGTACGTTCGTTT-3′
5′-human *Vegf-A* 5′-CGAGTACATCTTCAAGCCATCCTGT-3′
3′-human *Vegf-A* 5′-CTTGGTGAGGTTTGATCCGCATAAT-3′

RT-PCR:

5′-rat *Vegf-A* splice variant 5′-GATCATGCGGATCAAACCTCACCA A-3′
3′-rat *Vegf-A* splice variant 5′-CCGGTGAGAGGTCTAGTTCCCG AAA-3′
5′-rat *Vegfr-2* 5′-AGAAGATTGTAAACCGGGATGTGA-3′
3′-rat *Vegfr-2* 5′-GCAGGTGTATTCTCCTTGGTCACT-3′
5′-rat soluble *Vegfr-1* 5′-TGCACCATAGCATCAGTAAGCAAAA-3′
3′-rat soluble *Vegfr-1* 5′-TGACATTACTTTGTGTGGCACAACC-3
5′-rat membrane-bound *Vegfr-1* 5′-TGATCGTGGAATACTGCAAAT ATGG-3′
3′-rat membrane-bound *Vegfr-1* 5′-ACACGTCACTCTTGGTGCTG TAGAC-3′

Immunohistochemical analysis. Frozen IVD sections that had not been decalcified were obtained as described [13]. Cryosections were stained with anti-VEGF-A (Santa Cruz, 1:50) and anti-GLUT-1 (Abcam, 1:100) followed by Alexa Fluor488-conjugated anti-rabbit IgG (molecular Probes, 1:200) and TOTO3 (Invitrogen, 1:750) for nuclear staining. For GLUT-1 staining, specimens were fixed with acetone on ice for 5 min before staining. Immunoreactivity was detected by confocal microscopy (Olympus).

Histochemical analysis. Frozen IVD sections from 12-, 40- and 80-week-old Wistar male rats were stained by hematoxylin (Muto Pure Chemicals) and eosin (Muto Pure Chemicals). For LacZ staining, NP tissues were dissected macroscopically from *Vegfr-1*$^{lacZ/+}$ and *Vegfr-2*$^{lacZ/+}$ mice and fixed with 2% paraformaldehyde plus 0.2% glutaraldehyde for 1 h at 4 °C. After washing three times with PBS, they were incubated in staining solution [1 mg/ml X-gal (Nacalai tesque), 4 mM $K_4Fe(CN)_6$, 4 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$ in PBS] at 37 °C overnight and then observed by stereoscopic microscopy (Olympus).

Human samples. The human study was approved by an Institutional Review Board, and informed consent was obtained from all the patients according to the Hospital Ethical Guideline (Keio Hospital #15-52).

Cell preparation and culture. NP tissues were isolated and cultured as described [13,14]. For hypoxic culture, NP cells were exposed to 100 μM $CoCl_2$ (WAKO) for 24 h before harvest. For the apoptosis assay, 10 μg/ml sVEGFR-1-Fc (R&D systems) or CD4-Fc was added to the culture medium. CD4-Fc was used as control-Fc. After 48 h of culture, cells were stained with propidium iodide (PI) and FITC-conjugated annexin-V according to the manufacturer's instructions (Pharmingen) and analyzed by flow cytometry using FACS Calibur (Becton-Dickinson Immunocytometry Systems).

Hypoxic probe injection. Adolescent mice were injected in the tail vein with 10 mM pimonidazole (Molecular Probes). Three hours later, IVD were dissected to prepare frozen sections. Cryosections were stained with anti-pimonidazole antibody (Molecular Probes, 1:200) followed by Alexa Fluor488-conjugated anti-mouse IgG (Molecular Probes, 1:200). Immunoreactivity was detected by confocal microscopy (Olympus).

MRI analysis. All MRI data were acquired using a horizontal 7T animal MR imager equipped with magnetic field gradients of up to 300 mT/m (PharmaScan 70/16, Bruker BioSpin). A 60 mm inner diameter integrated coil was used for RF transmission and signal reception. Sagittally sectioned T2 weighted images (WI) of the rat lumbar spine were obtained using 2D-RARE pulse sequence with the following parameters: repetition time (TR) = 2500 ms, echo time (TE) = 60 ms, flip angle (FA) = 90°, echo train length (ETL) = 16, matrix size = 192 × 160, field of view (FOV) = 40 × 33 mm, slice thickness = 2 mm (gapless), slices = 3, and total acquisition time = 100 s.

Results

VEGF-A is highly expressed in avascular NP tissue

To investigate molecular mechanisms underlying survival of NP cells, we performed microarray and cluster analysis in 13 tissues including five avascular tissues (NP, AF, cartilage, lens and tendon). Microarray analysis in 10 of the 13 tissues was previously performed [13]. Here, we added data from two avascular tissues, lens and cartilage, and from a vascularized tissue, white adipose tissue (WAT). Surprisingly, we found that *Vegf-A* expression in NP was highest among the 13 tissues examined (Fig. 1A). VEGF-A has various splice variants: VEGF-A_{120} is diffusible through tissues, VEGF-A_{188} binds to the cell surface and extracellular matrix, and VEGF-A_{164} combines these properties. All three *Vegf-A* splice variants were detected in NP by RT-PCR analysis (Fig. 1B). VEGF-A protein expression in NP was evaluated by immunohistochemistry, and analysis indicated that VEGF-A was specifically expressed in NP but not in AF (Fig. 1C). *Vegf-A* mRNA expression was also significantly high in NP compared with AF in human samples (Fig. 1D). These results indicate that the angiogenic factor VEGF-A is highly expressed in NP, even though NP is an avascular tissue.

VEGF-A expression in NP is promoted by hypoxic conditions

VEGF-A expression is reportedly regulated mainly by hypoxia-inducible factor-1α (HIF-1α), which is activated under hypoxic conditions [5]. Microarray analysis showed that expression of *glucose-transporter-type-1* (*glut-1*) and *phosphoglycerate-kinase-1* (*pgk-1*), which are representative hypoxic responsive genes, was high in NP compared with other tissues (Fig. 2A). Tissue-specific expression of GLUT-1 protein in NP was also detected by immunohistochemistry (Fig. 2B). To further evaluate the hypoxic state of NP, we utilized pimonidazole, a hypoxic probe, which is reductively activated in an oxygen-dependent manner and covalently bound to thiol-containing proteins specifically in hypoxic cells [15].

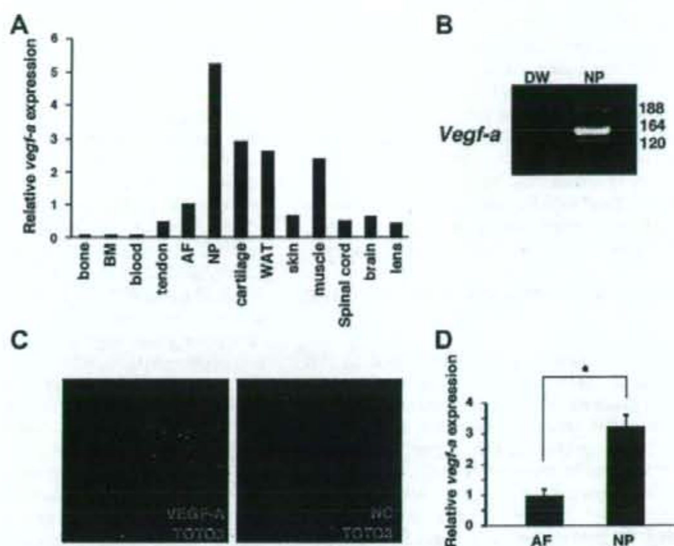

Fig. 1. VEGF-A expression is high in NP of the intervertebral disc (IVD). (A) Microarray analysis of 13 tissues including NP from Wistar rats. AF, annulus fibrosis; NP, nucleus pulposus; BM, bone marrow; WAT, white adipose tissue. (B) *Vegf-A* splice variants VEGF120, 164 and 188 were detected in rat NP by RT-PCR analysis. (C) IVD dissected from 8-week-old rats was stained with rabbit anti-VEGF antibody (VEGF-A) followed by Alexa Fluor488-conjugated anti-rabbit IgG antibody and observed by confocal microscopy. TOTO3 was used as a counter-stain for nuclei. NC, no primary antibody control. (D) Semi-quantitative real-time PCR analysis of *Vegf-A* mRNA expression in human samples. Data are mean relative ratios ± SD of *Vegf-A*/*β-actin* mRNA expression in NP compared with that of AF ($^*P < 0.01$).

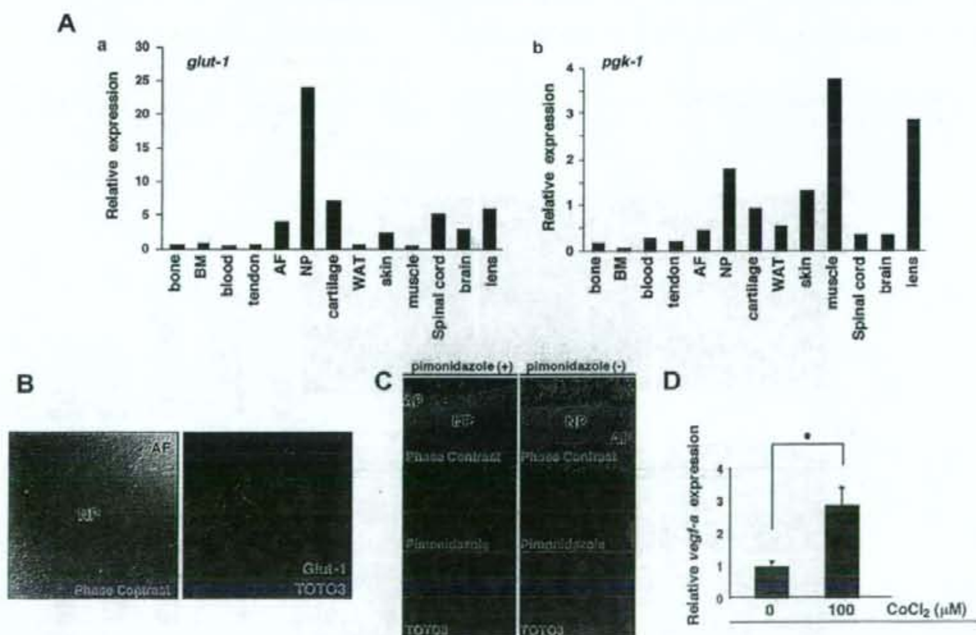

Fig. 2. VEGF-A expression in NP is promoted by hypoxic conditions. (A) Microarray analysis of hypoxia-responsive genes (a) *glut-1* and *pgk-1* (b). (B) IVD sections of 8-week-old rats were stained by rabbit anti-Glut-1 antibody followed by Alexa Fluor488-conjugated anti-rabbit IgG and observed under confocal microscope. TOTO3 served as counter-stain for nuclei. (C) The hypoxic probe, pimonidazole, was injected intravenously into 8-week-old mice and 3 h later IVDs were dissected from pimonidazole-injected (left panels) or -uninjected (right panels) mice. Sections were stained with mouse anti-pimonidazole antibody, followed by Alexa Fluor488-conjugated anti-mouse IgG, and observed by confocal microscopy. (D) NP cells were isolated from 8-week-old rats and cultured in the presence or absence of 100 μM $CoCl_2$. After 24 h, *Vegf-A* mRNA expression in NP was analyzed by semi-quantitative real-time PCR. Data are mean relative ratios ± SD of *Vegf-A*/*β-actin* mRNA expression in $CoCl_2$ (100 μM)-treated NP compared with non-treated (0 μM) NP ($^*P < 0.01$).

Pimonidazole was injected into mouse tail veins, and the hypoxic state of NP evaluated by immunohistochemistry against pimonidazole, which remained at the hypoxic area. As expected, NP cells were pimonidazole-positive (Fig. 2C). To investigate the effect of hypoxia on VEGF-A expression in NP cells, cells were cultured under hypoxic conditions generated by addition of $CoCl_2$, which chelates oxygen [16]. After 24 h of cultivation, we examined *Vegf-A* expression in NP cells by real-time PCR. *Vegf-A* expression in NP cells was significantly upregulated in hypoxic rather than in normoxic conditions (Fig. 2D). These results suggest that NP is a hypoxic tissue, and that VEGF-A expression in NP is promoted by hypoxia.

IVD degeneration correlates with decreased VEGF-A expression in NP seen with aging

Lumbar disc disease associated with aging is caused by IVD degeneration in the lumbar spine. Magnetic resonance imaging (MRI) analysis is used to diagnose IVD degeneration clinically. We performed MRI analysis of the lumbar spine in 12-, 40- and 80-week-old rats to correlate IVD degeneration with aging. In this analysis, T2 high signal intensity in NP detected by MRI indicates high water content, whereas low signal intensity suggests low water content resulting from degenerative changes. T2 signal intensity in the NP decreased with aging, suggesting that, as in humans, degenerative changes in rat NP can be followed by MRI analysis (Fig. 3A). As NP degenerated with age, the size of NP cells decreased accompanied by a reduction in the number of characteristic vesicles (Fig. 3B). Interestingly, NP *Vegf-A* expression was downregulated with age, while *CD24*, an NP-specific marker [13], remained constant (Fig. 3C). These results suggest that NP degeneration is closely associated with downregulation of VEGF-A expression in NP with aging.

Membrane-bound Vegfr-1 (mbVegfr-1) is predominantly expressed in NP

To investigate a role of VEGF-A in NP, we asked whether VEGF-A receptors, VEGFR-1 and VEGFR-2, were expressed in NP. Microarray analysis indicated high *Vegfr-1* expression compared with that of *Vegfr-2* in NP (Fig. 4A). To confirm this observation, we undertook LacZ staining of NP in *Vegfr-1*$^{lacZ/+}$ and *Vegfr-2*$^{lacZ/+}$ knock-in mice. LacZ expression indicative of VEGFR-1 and VEGFR-2 expression was detected in these mice. Expression of VEGFR-1 but not VEGFR-2 was detected as LacZ-positive staining in the center of the NP (Fig. 4B). Two VEGFR-1 isoforms generated by alternative splicing have been reported: soluble VEGFR-1 (sVEGFR-1), a secreted protein, and membrane bound, signaling VEGFR-1 (mbVEGFR-1). sVEGFR-1 lacks the transmembrane and tyrosine kinase domains of mbVEGFR-1 but has a high binding affinity to VEGF-A, thereby acting as VEGF-A antagonist. It has been reported that sVEGFR-1 is expressed in placenta and cornea and is essential to preserve an avascular state in cornea [10]. To determine which *Vegfr-1* isoform is expressed in NP, RT-PCR analysis was performed in AF, NP and bone (Fig. 4C). Bone served as a positive control for *Vegfr-2*. *mbVegfr-1* was predominantly expressed in NP compared with *sVegfr-1* (Fig. 4C). *Vegfr-2* was not detected in NP (Fig. 4C). Both VEGF-A and mbVEGFR-1 were expressed in NP (Figs. 1 and 4C), suggesting that VEGF-A signals through mbVEGFR-1 in an autocrine or paracrine manner in NP cells.

VEGFR-1 is expressed in monocytes/macrophages, and VEGF-A transduces signaling through VEGFR-1 to promote migration and differentiation [8] [17]. To clarify the role of VEGF-A in NP cells, we examined the effect of loss of VEGFR-1 signaling on NP cells following treatment with VEGFR-1-Fc, a VEGF-A antagonist. The number of apoptotic cells increased following VEGFR-1-Fc treatment compared with control CD4-Fc (Fig. 4D), indicating that VEGF-A functions in NP survival.

Discussion

Avacsularity is required for NP function, since compressive loading on NP may disrupt the blood vessels, leading to a hypoxic state. Hypoxic conditions are known to induce local VEGF-A expression resulting in vascular invasion. Thus NP must both prevent vascular invasion induced by VEGF-A and survive without a

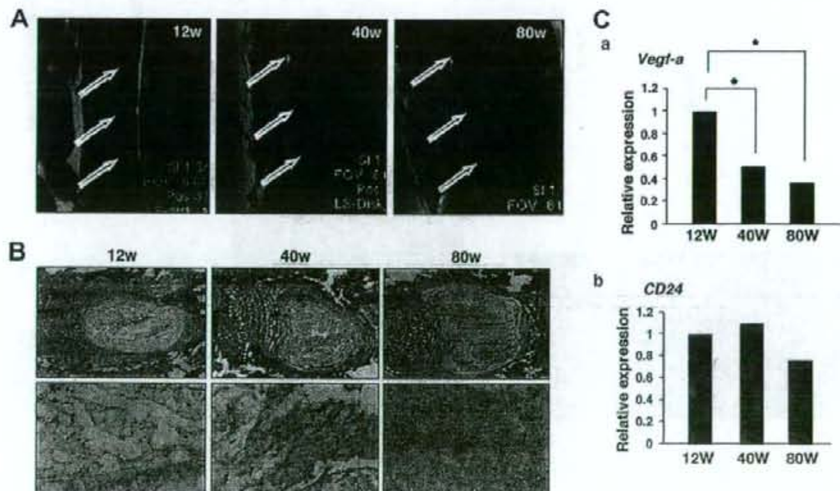

Fig. 3. IVD degeneration correlates with decreased VEGF expression and aging in NP. (A) MRI analysis showing T2 signal intensity of intervertebral disc of 12-, 40- and 80-week-old rats. Arrows indicate NP. (B) H/E staining of IVD sections of 12-, 40- and 80-week-old rats. Upper panels, low magnification; upper panels, high magnification of images shown in lower panels. (C) Expression of *Vegf-A* (a) and *CD24* (b) relative to *β-actin* in NP from 12-, 40- and 80-week-old rats was evaluated by semi-quantitative real-time PCR. Data are mean relative ratios ± SD of *Vegf-A/β-actin* (a) or *Vegf-A/β-actin* (b) mRNA expression in NP of 40- or 80-week-old rats compared with that of 12-week-old animals ($^*P < 0.01$).

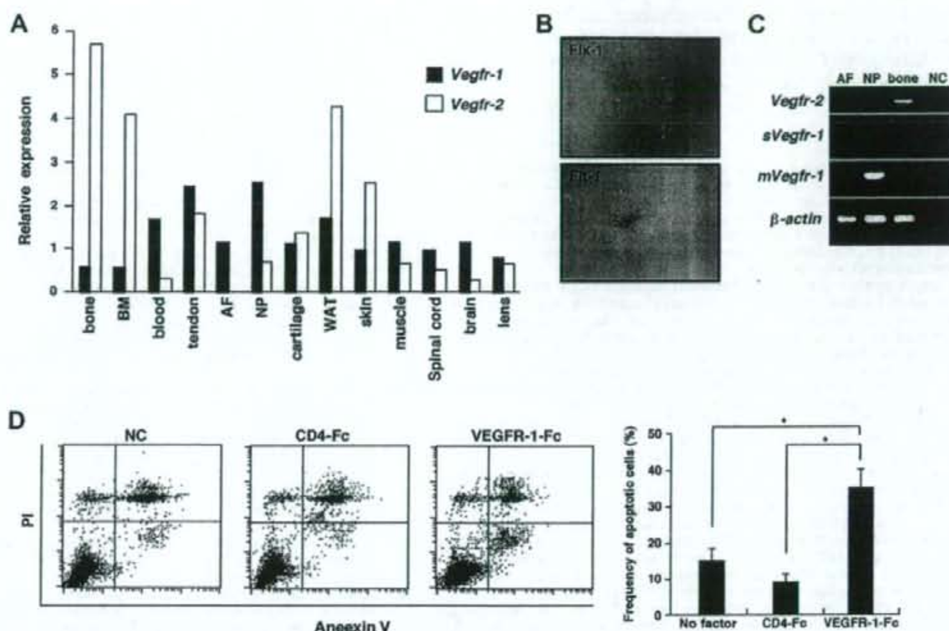

Fig. 4. Membrane-bound VEGFR-1 (mbVEGFR-1) is expressed in NP. (A) *Vegfr-1* and *Vegfr-2* expression was analyzed by microarray analysis. AF, annulus fibrosis; NP, nucleus pulposus; BM, bone marrow; WAT, white adipose tissue. (B) LacZ staining of NP derived from *Vegfr-2*$^{lacZ/+}$ (left panel) and *Vegfr-1*$^{lacZ/+}$ (right panel) mice. (C) *Vegfr-2*, soluble *Vegfr-1* (*sVegfr*-1) and membrane-bound *Vegfr-1* (*mbVegfr-1*) expression was analyzed in AF, NP and bone by RT-PCR. (D) NP cells isolated from 8-week-old rats were suspended in sodium alginate and cultured in serum free medium with or without 10 μg/ml sVEGFR-1-Fc or CD4-Fc for 48 h. Cells were then stained with FITC-conjugated annexin-V and propidium iodide (PI) and analyzed by flow cytometry using FACS Calibur. Apoptotic cells were detected as annexin-V-positive/PI-negative. Upper panels, representative data; lower panels, mean frequency ± SD of apoptotic cells ($^{*}P < 0.01$).

blood supply. Here we demonstrate that VEGF-A is induced by hypoxia in NP and that VEGF-A functions in NP as a survival factor. Since mbVEGFR-1, a VEGF-A receptor with 10-fold higher affinity for VEGF-A compared with VEGFR-2, is also expressed in NP, vascular invasion may be inhibited in part by an autocrine trap consisting of VEGF-A-expressing cells that also express VEGFR-1.

Here we performed microarray analysis in 13 tissues including five avascular ones, namely, NP, AF, cartilage, tendon and lens. Interestingly, we did not identify common molecules highly expressed in avascular tissues, suggesting that mechanisms underlying maintenance of avascularity are tissue-specific. Recently Yoshioka et al. reported that chondromodulin-I functions to prevent angiogenesis in avascular cardiac valves [18], although such a function has not been detected in cartilage, which also expresses chondromodulin-I [19,20]. On the other hand, Ambati et al. report that cornea, another avascular tissue, exclusively expresses sVEGFR-1 [10]. In NP, aggrecan reportedly inhibits endothelial cell adhesion and cell migration [21]. *Aggrecan* expression is high in NP, AF and cartilage but low in tendon and lens among avascular tissues that we analyzed (data not shown), further supporting the idea that mechanisms underlying maintenance of avascularity are tissue-specific.

VEGF-A expression is promoted by hypoxic conditions in NP. In the hypoxic lens, expression of hypoxic response genes such as *glut-1* and *pgk-1* was observed [22]. However, *Vegf-A* expression in lens is much lower than that of NP (data not shown). These results suggest that VEGF-A expression is not simply promoted by hypoxic conditions but is regulated by tissue-specific factors other than HIF-1α. Further studies are needed to clarify how VEGF-A expression is specifically regulated in NP.

There are two types of tyrosine kinase VEGF-A receptors: VEGFR-1 and VEGFR-2. We found that both VEGF-A and mbVEGFR-1 were expressed in NP, while VEGFR-2 was not (Figs. 1 and 2). These results suggest that VEGF-A signaling can be transduced through VEGFR-1 in an autocrine or a paracrine manner in NP. VEGF-A is known to transduce a migration signal in macrophages and a survival signal in tumors and hematopoietic stem cells [9,12]. We found that NP cells express both VEGF-A and VEGFR-1 and that treatment of NP cells with the VEGFR-1-Fc, a VEGF-A antagonist, induced apoptosis in NP cells, suggesting that the VEGF-A/VEGFR-1 cascade mediates an anti-apoptotic function in NP. Similar to NP, several tumor cells express both VEGF-A and VEGFR-1, and autocrine interactions of VEGF-A and VEGFR-1 function to resist apoptosis under hypoxic conditions [12]. On the other hand, hematopoietic stem cells express both VEGF-A and VEGFR-2, and genetic ablation of VEGF-A in hematopoietic stem cells reduces cell survival, suggesting that autocrine interaction of VEGF-A and VEGFR-2 is crucial for cell survival [9]. Apoptotic cells in NP reportedly increase with aging [23], suggesting that NP degeneration is associated with aging. We have shown here that VEGF-A expression in NP is downregulated with age. Thus regulation of VEGF-A expression in NP may be a good target to prevent age-associated degeneration.

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Original article

Japanese Orthopaedic Association Back Pain Evaluation Questionnaire. Part 3. Validity study and establishment of the measurement scale

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Abstract

Background. The Japanese Orthopaedic Association decided to revise the JOA score for low back pain and to develop a new outcome measure. In February 2002, the first survey was performed with a preliminary questionnaire consisting of 60 evaluation items. Based on findings of that survey, 25 items were selected for a draft of the JOA Back Pain Evaluation Questionnaire (JOABPEQ). The second survey was performed to confirm the reliability of the draft questionnaire. This article further evaluates the validity of this questionnaire and establishes a measurement scale.

Methods. The subjects of this study consisted of 355 patients with low back disorders of any type (201 men, 154 women; mean age 50.7 years). Each patient was asked to fill in a self-administered questionnaire. Superficial validity was checked in terms of the completion rate for filling out the entire questionnaire. Factor analysis was then performed to evaluate the validity of the questionnaire and establish a measurement scale.

Results. As a result of the factor analysis, 25 items were categorized into five factors. The factors were named based on the commonality of the items: social function, mental health, lumbar function, walking ability, and low back pain. To establish a measurement scale for each factor, we determined the coefficient for each item so the difference between the maximum factor scores and minimum factor scores was approximately 100. We adjusted the formula so the maximum for each factor score was 100 and the minimum was 0.

Conclusions. We confirmed the validity of the JOA Back Pain Evaluation Questionnaire and est ablished a measurement scale.

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Introduction

The evaluation criteria were based on physiological, biological, and anatomical outcome measure results of the Japanese Orthopaedic Association (JOA) score for low back pain.[1] The criteria include laboratory values, physiological findings, and imaging findings. These findings are significant for doctors but have little meaning for patients. From a patient's perspective, the presence of a symptom or its degree and functional condition

must have real meaning. This means that outcome measures need to be translatable from an objective evaluation to a subjective one, or from the doctor's perspective to the patient's perspective. The JOA decided to revise the JOA score for low back pain and develop a new scientific, patient-oriented outcome measure.

The first committee meeting was held in June 2000, and the first survey was initiated in February 2002 using a preliminary questionnaire consisting of 60 items. It was a self-administered, disease-specific measure developed with reference to the Japanese editions of SF-36[2,3] and the Roland-Morris Disability Questionnaire (RDQ)[4,5] to assess health-related quality of life. Based on findings of the survey, 25 items were selected for a draft of the JOA Back Pain Evaluation Questionnaire (JOABPEQ) (see Appendix 1).

The second survey was started in January 2004 to evaluate the reliability of the 25 items selected for the draft JOABPEQ. We successfully confirmed the reliability, and these details have been described in previous reports of Part 1[6] and Part 2.[7] Part 3 of this study involves further development of the new JOA questionnaire, evaluation of the validity of the draft JOABPEQ, and establishment of a measurement scale.

Materials and methods

Recruitment of patients

A total of 369 of the 829 Japanese board-certified spine surgeons were randomly selected and asked to recruit at least three patients each to participate in evaluating the JOABPEQ during February 2004. The inclusion criterion was any type of lumbar spine disorder. Exclusion criteria were patients who had:

- Other musculoskeletal diseases requiring medical treatment
- Psychiatric disease, potentially leading to inappropriate answers
- Postoperative condition
- Participation in previous surveys related to this study

Testing the questionnaire

Each patient was asked to fill in the self-administered questionnaire. The attending surgeon filled out information on the diagnosis, presence or absence of concomitant diseases, and a judgment regarding the severity of symptoms using a three-step rating scale (mild, moderate, severe). The severity of the symptoms was determined subjectively by the attending surgeon, who was asked not to select a similar patient based only on the severity. This study was approved by the Ethics Committee of the Japanese Society for Spine Surgery and Related Research, and informed consent was obtained from each patient.

Factor analysis was used to check the statistical validity of the questionnaire and establish the measurement scale. All statistics were calculated using SPSS software (version 12; SPSS, Chicago, IL, USA).

Results

Patient characteristics

Of the 452 patients selected for participation in this survey, 1 patient who was judged inappropriate by the attending doctor and 60 patients with other musculoskeletal diseases requiring medical treatment were excluded. The responses from 36 patients who answered incompletely were also excluded, leaving 355 patients available for analysis: 201 men and 154 women, with a mean ± SD age of 50.7 ± 18.0 (Table 1). The diagnosis was lumbar disc herniation in 167, lumbar spinal canal stenosis in 103, and spondylolisthesis in 37.

According to the judgment of the attending doctor, there were 115 mild, 142 moderate, and 98 severe cases. Table 2 summarizes the severity of low back pain evaluated by the current JOA scoring system and shows that the characteristics of the recruited patients were not

Table 1. Distribution of age and severity of symptoms ($n = 355$)

	Severity of symptoms			
Age (years)	Mild	Moderate	Severe	Total
Males				
10–19	3	4		7
20–29	8	4	9	21
30–39	12	14	6	32
40–49	12	14	8	34
50–59	12	9	11	32
60–69	10	12	12	34
70–79	11	16	8	35
80+	4	2		6
Total	72	75	54	201
Females				
10–19	0	1	3	4
20–29	9	5	5	19
30–39	7	13	10	30
40–49	5	14	7	26
50–59	4	9	5	18
60–69	7	12	7	26
70–79	11	10	4	25
80+		3	3	6
Total	43	67	44	154
Total no.	115	142	98	355

Table 2. Distribution of the severity evaluated by the current JOA scoring system and finger-floor distance ($n = 355$)

Parameter	No.
Straight-leg raising (SLR) test	
Normal	183
30°–70°	130
<30°	42
Motor function	
Normal	182
Slight weakness (MMT good)	126
Severe weakness (MMT less than good)	47
Sensory function	
Normal	127
Slight disturbance	162
Severe disturbance	66
Bladder function	
Normal	315
Mild dysuria	36
Severe dysuria	4
Finger-to-floor distance (cm)	
to −15	1
−14 to −5	12
−4 to 4	69
5 to 14	73
15 to 24	69
25 to 34	43
35 to 44	25
45 to 54	30
55 to 64	6
65 to 74	4
Not measurable	14
Total number	355

JOA, Japanese Orthopaedic Association; MMT, manual muscle testing

specific. There was no marked difference in the distribution of the severity levels between the 451 patients who were initially recruited and the 355 who were finally analyzed.

Superficial validity

Superficial validity was checked in terms of the completion rate for filling out the questionnaire. Regarding the distribution of responses for each item, it was judged that none of the questions was too difficult to answer because the highest rate of nonrespose was 1.8%. With regard to deflection of an answer, the highest rate (78.3%) was concentrated on "yes" responses to question 1–14, although this was judged not to be inappropriate. Therefore, the distribution was not skewed, which would indicate "floor and ceiling" effects (Table 3).

Factor analysis

First, we tried to extract some observed variables from 25 items by the Maximum Likelihood Method. It was found that the eigenvalue was >1.0 for five items, and the accumulative contribution ratio until the fifth factor was 53.1% (Table 4).

Next, we performed orthogonal rotation by the direct oblimin method. The results are shown in Table 5. Each item was categorized into five factors: Four items (Q2-6, Q2-5, Q1-2, Q2-4) related to factor 1; seven items (Q2-8, Q2-7, Q2-11, Q1-13, Q2-9, Q2-10, Q2-1) related to factor 2; six items (Q1-9, Q1-6, Q2-3, Q1-8, Q1-5, Q1-4) related to factor 3; five items (Q1-10, Q2-4, Q1-12, Q1-14, Q2-2) to factor 4; and the last four items to factor 5. Although factor loading was <0.30 in three items (Q1-4 to factor 3, Q2-2 to factor 4, Q1-11 to factor 5), we adopted all of them for the reason that the question itself was important for the factor or the number of questions in each factor needed to be more than four.

Factor names were determined based on the commonality of the items that showed a large value on factor loading: factor 1, social function (four items); factor 2, mental health (seven items); factor 3, lumbar function (six items); factor 4, walking ability (five items); and factor 5, low back pain (four items).

Measurement scale

To establish a measurement scale for each factor, we determined the size of the coefficient for each item so the difference between the maximum factor scores and minimum factor scores was approximately 100 (Table 6). When a coefficient became a negative numerical value, we changed the coefficient to a positive numerical value by reversing the order of the answer choice. We adjusted the formula so the maximum for each factor score was 100 and the minimum was 0 (see Appendix 2).

Discussion

It is considered ideal for the outcome measure to evaluate patients from various perspectives, such as dysfunction, disability, handicap, and psychological problem. The outcome measure should be patient-oriented, and its reliability and validity should be confirmed by statistical analysis. However, the current JOA score does not include subjective evaluations and does not meet such requirements. We developed a new questionnaire, JOABPEQ, specifically to evaluate low back pain. It is patient-oriented and mainly based on recognizing problems with activities of daily living. We categorized 25 questions into five factors; each factor is then scored up to 100 points using the measurement scale. The factors are then evaluated separately. The point is to be aware that it is meaningless and inadequate to total

Table 3. Distribution of answers for each item in the questionnaire ($n = 451$)

Item	Choices for answer 1	2	3	4	5	No answer
Q1-1	336 (74.5%)	114 (25.3%)				1 (0.2%)
Q1-2	152 (33.7%)	297 (65.9%)				2 (0.4%)
Q1-3	302 (67.0%)	146 (32.4%)				3 (0.7%)
Q1-4	157 (34.8%)	291 (64.5%)				3 (0.7%)
Q1-5	242 (53.7%)	209 (46.3%)				0
Q1-6	167 (37.0%)	281 (62.3%)				3 (0.7%)
Q1-7	215 (47.7%)	236 (52.3%)				0
Q1-8	240 (53.2%)	208 (46.1%)				3 (0.7%)
Q1-9	272 (60.3%)	177 (39.2%)				2 (0.4%)
Q1-10	288 (63.9%)	160 (35.5%)				3 (0.7%)
Q1-11	158 (35.0%)	292 (64.7%)				1 (0.2%)
Q1-12	156 (34.6%)	286 (63.4%)				9 (2.0%)
Q1-13	116 (25.7%)	333 (73.8%)				2 (0.4%)
Q1-14	353 (78.3%)	90 (20.0%)				8 (1.8%)
Q2-1	4 (0.9%)	27 (6.0%)	155 (34.4%)	185 (41.0%)	79 (17.5%)	1 (0.2%)
Q2-2	103 (22.8%)	233 (51.7%)	113 (25.1%)			2 (0.4%)
Q2-3	126 (27.9%)	253 (56.1%)	67 (14.9%)			5 (1.1%)
Q2-4	181 (40.1%)	175 (38.8%)	95 (21.1%)			0
Q2-5	62 (13.7%)	111 (24.6%)	206 (45.7%)	48 (10.6%)	23 (5.1%)	1 (0.2%)
Q2-6	113 (25.1%)	124 (27.5%)	138 (30.6%)	50 (11.1%)	23 (5.1%)	3 (0.7%)
Q2-7	53 (11.8%)	66 (14.6%)	225 (49.9%)	72 (16.0%)	35 (7.8%)	0
Q2-8	52 (11.5%)	76 (16.9%)	224 (49.7%)	75 (16.6%)	23 (5.1%)	1 (0.2%)
Q2-9	11 (2.4%)	57 (12.6%)	190 (42.1%)	132 (29.3%)	60 (13.3%)	1 (0.6%)
Q2-10	64 (14.2%)	125 (27.7%)	114 (25.3%)	102 (22.6%)	45 (10.0%)	1 (0.2%)
Q2-11	48 (10.6%)	149 (33.0%)	141 (31.3%)	89 (19.7%)	23 (5.1%)	1 (0.2%)

Table 4. Results of factor analysis: eigenvalue of each item

Factor	Eigenvalue	Cumulative contribution rate (%)
1	**7.600**	**30.4**
2	**1.795**	**37.6**
3	**1.556**	**43.8**
4	**1.217**	**48.7**
5	**1.095**	**53.1**
6	0.996	57.0
7	0.942	60.8
8	0.893	64.4
9	0.783	67.5
10	0.756	70.5
11	0.728	73.4
12	0.680	76.2
13	0.656	78.8
14	0.643	81.4
15	0.617	83.8
16	0.584	86.2
17	0.505	88.2
18	0.482	90.1
19	0.433	91.9
20	0.427	93.6
21	0.387	95.1
22	0.361	96.6
23	0.320	97.8
24	0.302	99.0
25	0.239	100.0

Bold typeface indicates eigenvalues over 1.0

Table 5. Results of factor analysis: factor loading of each item

Item	Factors 1	2	3	4	5
Q2-6	**0.81**	0.04	0.04	−0.04	0.14
Q2-5	**0.71**	0.01	0.08	0.14	0.06
Q1-2	0.33	0.16	0.21	0.34	−0.21
Q2-8	0.07	**0.68**	0.08	−0.08	0.10
Q2-7	0.15	**0.62**	−0.07	0.12	0.15
Q2-11	−0.03	**0.62**	−0.12	−0.02	0.06
Q1-13	−0.04	**0.35**	0.08	−0.01	0.14
Q2-9	−0.23	**−0.52**	−0.11	0.05	0.05
Q2-10	0.06	**−0.55**	−0.06	−0.10	0.15
Q2-1	0.03	**−0.55**	−0.14	−0.11	−0.02
Q1-9	0.02	−0.07	**0.69**	−0.07	0.10
Q1-6	−0.01	0.12	**0.56**	0.08	−0.10
Q2-3	0.23	0.05	**0.56**	−0.04	0.07
Q1-8	−0.03	−0.09	**0.38**	0.15	0.31
Q1-5	0.00	0.09	0.32	0.03	−0.02
Q1-4	0.10	0.11	0.28	0.13	0.05
Q1-10	0.14	0.04	−0.04	**0.62**	0.03
Q2-4	**0.39**	0.05	−0.08	**0.61**	−0.13
Q1-12	−0.05	0.01	0.01	**0.46**	0.06
Q1-14	−0.07	0.06	0.20	0.34	0.13
Q2-2	0.30	−0.03	0.19	0.26	0.05
Q1-1	0.03	0.11	0.00	0.04	**0.46**
Q1-3	0.18	0.13	−0.05	0.13	**0.43**
Q1-7	0.07	0.06	0.20	0.01	**0.41**
Q1-11	0.10	0.04	0.16	0.25	0.28

Bold typeface indicates absolute value of the factor loading of more than 0.26

Table 6. Coefficient for each item of the formula for measurement scale

Item	1 Social function	2 Mental health	3 Lumbar function	4 Walking ability	5 Low back pain
Q1-1					20
Q1-2	2				
Q1-3					20
Q1-4			10		
Q1-5			10		
Q1-6			20		
Q1-7					20
Q1-8			10		
Q1-9			30		
Q1-10				30	
Q1-11					10
Q1-12				20	
Q1-13		3			
Q1-14				10	
Q2-1		−4			
Q2-2				10	
Q2-3			20		
Q2-4	4			30	
Q2-5	6				
Q2-6	10				
Q2-7		6			
Q2-8		6			
Q2-9		−3			
Q2-10		−3			
Q2-11		3			

the five factors' scores; rather, they should be treated by nonparametric analysis. The reliability of the questionnaire including 25 items for the JOABPEQ was confirmed in Part 2 of this project. The validity of the questionnaire was evaluated using factor analysis, and the measurement scale was established in Part 3 of this study. Further studies must be performed to confirm the responsiveness of the calculations of the severity score.

Conclusions

We confirmed the validity of the JOA Back Pain Evaluation Questionnaire (JOABPEQ) and established a measurement scale.

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Appendix 1. Items selected for the draft of a JOABPEQ document

With regard to your health condition during the last week, please choose the item number among the answers for the following questions that best applies as your condition varies depending on the day or time. Circle the item number when your condition is at its worst.

Q1-1 To alleviate low back pain, you often change your posture.
1) Yes
2) No

Q1-2 Because of low back pain, you do not do any routine housework these days.
1) No
2) Yes

Q1-3 Because of low back pain, you lie down more often than usual.
1) Yes
2) No

Q1-4 Because of low back pain, you sometimes ask someone to help you when you do something.
1) Yes
2) No

Q1-5 Because of low back pain, you refrain from bending forward or kneeling down.
1) Yes
2) No

Q1-6 Because of low back pain, you have difficulty standing up from a chair.
1) Yes
2) No

Q1-7 Your lower back aches most of the time.
1) Yes
2) No

Q1-8 Because of low back pain, turning over in bed is difficult.
1) Yes
2) No

Q1-9 Because of low back pain, you have difficulty putting on socks or stockings.
1) Yes
2) No

Q1-10 Because of low back pain, you walk only short distances.
1) Yes
2) No

Q1-11 Because of low back pain, you cannot sleep well. (If you take sleeping pills because of the pain, select "No.")
1) No
2) Yes

Q1-12 Because of low back pain, you stay seated most of the day.
1) Yes
2) No

Q1-13 Because of low back pain, you become irritated or angry at other persons more often than usual.
1) Yes
2) No

Q1-14 Because of low back pain, you go up stairs more slowly than usual.
1) Yes
2) No

Q2-1 How is your present health condition?
1) Excellent
2) Very good
3) Good
4) Fair
5) Poor

Q2-2 Do you have difficulty in climbing stairs?
1) I have great difficulty
2) I have some difficulty
3) I have no difficulty

Q2-3 Do you have difficulty with any one of the following motions: bending forward, kneeling, stooping?
1) I have great difficulty
2) I have some difficulty
3) I have no difficulty

Q2-4 Do you have difficulty walking more than 15 minutes?
1) I have great difficulty
2) I have some difficulty
3) I have no difficulty

Q2-5 Have you been unable to do your work or ordinary activities as well as you would like?
1) I have not been able to do them at all.
2) I have been unable to do them most of the time.
3) I have sometimes been unable to do them.
4) I have been able to do them most of the time.
5) I have always been able to do them.

Q2-6 Has your work routine been hindered because of the pain?
1) Greatly
2) Moderately
3) Slightly (somewhat)
4) Little (minimally)
5) Not at all

Q2-7 Have you been discouraged or depressed?
1) Always
2) Frequently
3) Sometimes
4) Rarely
5) Never

Q2-8 Do you feel exhausted?
1) Always
2) Frequently
3) Sometimes
4) Rarely
5) Never

Q2-9 Do you feel happy?
1) Always
2) Almost always
3) Sometimes
4) Rarely
5) Never

Q2-10 Do you think you are in reasonable health?
1) Yes (I am healthy)
2) Fairly (my health is better than average)
3) Not very much (my health is average)
4) Barely (my health is poor)
5) Not at all (my health is very poor)

Q2-11 Do you feel your health will get worse?
1) Very m uch so
2) A little bit at a time
3) Sometimes yes and sometimes no
4) Not very much
5) Not at all

Appendix 2. Measurement scale for JOABEPQ

Social life function
('Q1-2' × 2 + 'Q2-4' × 4 + 'Q2-5' × 6 + 'Q2-6' × 10 − 22) × 100 ÷ 74

Mental health
('Q1-13' × 3 + 'Q2-1' × 4 + 'Q2-7' × 6 + 'Q2-8' × 6 + 'Q2-9' × 3 + 'Q2-10' × 3 + 'Q2-11' × 3 − 28) × 100 ÷ 103

Lumbar function
('Q1-4' × 10 + 'Q1-5' × 10 + 'Q1-6' × 20 + 'Q1-8' × 10 + 'Q1-9' × 30 + 'Q2-3' × 20 − 100) × 100 ÷ 120

Walking ability
('Q1-10' × 30 + 'Q1-12' × 20 + 'Q1-14' × 10 + 'Q2-2' × 10 + 'Q2-4' × 30 − 100) × 100 ÷ 140

Low back pain
('Q1-1' × 20 + 'Q1-3' × 20 + 'Q1-7' × 20 + 'Q1-11' × 10 − 70) × 100 ÷ 70

ARTICLE

A Functional Polymorphism in *THBS2* that Affects Alternative Splicing and MMP Binding Is Associated with Lumbar-Disc Herniation

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Lumbar-disc herniation (LDH), one of the most common musculoskeletal diseases, has strong genetic determinants. Recently, several genes that encode extracellular matrix (ECM) proteins in the intervertebral disc have been reported to associate with LDH. Thrombospondins (THBSs) 1 and 2 are good candidates for the LDH susceptibility gene: They are intervertebral disc ECM proteins that regulate the effective levels of matrix metalloproteinases (MMPs) 2 and 9, which are key effectors of ECM remodeling. Here, we report that *THBS2* is associated with LDH in Japanese populations. An intronic SNP in *THBS2* (IVS10-8C → T; rs9406328) showed significant association ($p = 0.0000028$) with LDH in two independent Japanese populations. This SNP, located in a polypyrimidine tract upstream of the 3′ splice site of intron 10, exerts allelic differences on exon 11 skipping rates in vivo, with the susceptibility allele showing increased skipping. Skipping of exon 11 results in decreased THBS2 interaction with MMP2 and MMP9. Further, a missense SNP in *MMP9* (Q279R; rs17576) is also strongly associated with LDH in the Japanese population ($p = 0.00049$) and shows a combinatorial effect with *THBS2* (odds ratio 3.03, 95% confidence interval 1.58–5.77). Thus, a splicing-affecting SNP in *THBS2* and a missense SNP in *MMP9* are associated with susceptibility to LDH. Our data indicate that regulation of intervertebral disc ECM metabolism by the THBS2-MMP system plays an essential role in the etiology and pathogenesis of LDH.

Introduction

Lumbar-disc herniation (LDH) is a predominant cause of low-back pain and unilateral leg pain. Low-back pain affects 70%–85% of all people during their lifetime, and LDH is the most common cause of activity limitation in individuals younger than 45 years of age.[1] Twenty percent of individuals with LDH require surgical treatment to relieve prolonged or aggravated leg pain.[2] Although many risk factors have been reported for LDH, its etiology and pathogenesis are for the most part unknown. The strong familial predisposition for lumbar-disc degeneration has been established through a number of family and twin studies.[3–5] Recently, several genes have been reported to associate with lumbar disc disease. Most encode extracellular matrix (ECM) proteins in the intervertebral disc, suggesting the importance of ECM metabolism in LDH.[6,7]

The thrombospondins (THBSs) are a family of five secreted, modular glycoproteins whose functions in the ECM are diverse and poorly understood.[8,9] THBS1 (MIM 188060) and THBS2 (MIM 188061) are structurally more similar to each other than to THBS3–THBS5 and are, therefore, considered to constitute a subfamily.[10] Both THBS1 and THBS2 interact with matrix metalloproteinase 2 (MMP2 [MIM 120360]) and 9 (MMP9 [MIM 120361]) and regulate their effective levels in the pericellular ECM.[11,12] Mice with deficiencies in either *Thbs1* or *Thbs2* show abnormal spine curvature.[13,14] *Thbs2* knockout mice also exhibit increased levels of MMP2 after injury.[15] A more recent study has shown immunolocalization of THBSs in the human intervertebral disc.[16] These observations prompted us to examine *THBS1* and *THBS2* as candidate genes for LDH.

We have identified a significant association between *THBS2* and LDH in Japanese populations. We show that an associated SNP in *THBS2* affects the splicing and consequently alters the binding affinity to MMP2 and MMP9. We further show *MMP9* is also associated with LDH. Our findings indicate that THBS2 is involved in LDH through the regulation of MMP activity in intervertebral-disc ECM.

Material and Methods

Subjects

For the case-control association analysis of LDH, we recruited 525 cases (34.7% female; mean age ± standard deviation [SD] = 41.5 ± 14.9; mean body mass index (BMI) ± SD = 23.1 ± 3.2) and 564 controls (42.4% female; mean age ± SD = 62.4 ± 9.9; mean BMI ± SD = 23.6 ± 3.3) for the first screen (Japanese A

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population), and 322 cases (36.6% female; mean age ± SD = 35.7 ± 15.2; mean BMI ± SD = 22.6 ± 3.5) and 332 controls (36.4% female; mean age ± SD = 60.9 ± 14.1; mean BMI ± SD = 23.4 ± 2.9) for the replication study (Japanese B population). Affected individuals with LDH were recruited from 19 collaborating hospitals between November 2001 and February 2007. The diagnosis of LDH required the following three criteria: (1) diagnosis of LDH by magnetic resonance imaging (sagittal and axial images obtained with a 1.5-T imaging system), (2) treatment and monitoring for more than 1 year by orthopedic surgeons, and (3) a history of unilateral pain radiating from the back along the femoral or sciatic nerve to the corresponding dermatome of the nerve root for a longer than 3 months. Primary exclusion criteria included synovial cyst, spinal tumor, spondylolisthesis, spondylosis, trauma, and inflammatory disease. From a total of 847 individuals with LDH, 811 underwent surgery for LDH, including posterior discectomy, anterior lumbar interbody fusion, microendoscopic discectomy, and percutaneous nucleotomy. We extracted genomic DNA from peripheral blood leukocytes of affected individuals and controls by using standard protocols. We obtained normal intervertebral disc tissue from individuals with idiopathic scoliosis during surgery (seven samples). Written informed consent was obtained from each subject as approved by the ethical committees of the SNP Research Center at RIKEN and participating clinical institutes.

Genotyping

We genotyped SNPs by using the multiplex polymerase chain reaction (PCR)-based Invader assay (Third Wave Technologies),[17] TaqMan SNP genotyping assays (Applied Biosystems), or by direct sequencing of PCR products with ABI 3700 DNA analyzers (Applied Biosystems) according to the manufacturers' protocols.

Statistical Analysis

We assessed association and Hardy-Weinberg equilibrium by using the χ^2 test. We estimated haplotype frequencies with the expectation-maximization algorithm.[18] Linkage disequilibrium coefficients (D' and r^2) were calculated as described previously.[7] We used the permutation test to adjust significance in the analysis of association between the *THBS2* SNPs and LDH.[19] We performed 1,000,000 permutations of the cases and the controls. Detection of significance of stratification (p) and estimation of quantitative assessment of population stratification (λ) were done according to previous reports.[20,21] We assessed the relationship between the clinical profiles and the genotype information by the Kruskal-Wallis test and the χ^2 test. Combinatorial effects of *THBS2* and *MMP9* with regard to LDH susceptibility were also examined as described previously.[22] The odds ratio was defined against the genotype consisting of homozygotes of nonsusceptibility alleles from both loci.

Cell Culture and RNA Extraction

Fibroblast cells and the human chondrosarcoma cell lines OUMS-27[23] and CS-OKB[24] were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C under 5% CO_2. Total RNA was extracted from tissues and cells with Isogen (Nippongene) and purified with SV Total RNA Isolation System (Promega), according to the manufacturers' instructions.

RT-PCR and Real-Time PCR

We obtained complementary DNA (cDNA) from all tissues other than intervertebral disc from Multiple Tissue cDNA Panels (Clontech). For the intervertebral disc tissue and cell line, cDNA was synthesized from total RNA using Multiscribe reverse transcriptase and oligo-dT primer (Applied Biosystems). We performed quantitative real-time PCR with an ABI PRISM 7700 sequence detector with the Quantitect SYBR Green PCR Kit (QIAGEN) in accordance with the manufacturers' instructions. Primer sequences for reverse transcriptase (RT)-PCR and real-time PCR are available on request.

Expression of Recombinant Partial Human THBS2

We cloned a cDNA encoding wild-type (WT-THBS2) and exon-11-skipped partial human THBS2 (Skip-THBS2) into pET42b expression vectors that had both S tag and GST tag and expressed them in the *E. coli* Rosetta (DE3) pLys strain. Recombinant proteins were solubilized from inclusion bodies, renatured with the Protein Refolding Kit (Novagen), and used for pull-down and solid-phase binding assays.

Western Blotting

Membranes were blocked with 3% bovine serum albumin in Tris-buffered saline Tween 20 (TBST). We used polyclonal antibodies against MMP2 and MMP9 (R&D Systems) at a 1:1000 dilution in TBST with 1% bovine serum albumin. We used a horseradish peroxidase (HRP)-conjugated anti-goat immunoglobulin G (IgG) (Santa Cruz) as the secondary antibody at a 1:7000 dilution in TBST with 1% bovine serum albumin.

S Protein Pull-down Assay

We incubated S-tagged recombinant THBS2 (5 μM, 20 μl) with 1 μg of purified human MMP2 or MMP9 (CHEMICON) for 2 hr at 4°C in 0.3 ml of binding buffer (20 mM Tris-HCl [pH 7.5], 250 mM NaCl, 1% Triton X-100, and Complete protease inhibitor cocktail [Roche]). Twelve and a half microliters of S protein agarose (Novagen) was added to the reaction and incubated for 30 min at room temperature. Precipitates were washed three times with binding buffer and subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). We detected coprecipitated proteins by Western blotting with antibodies to MMP2 or MMP9 (R&D Systems) and S protein HRP conjugate (Novagen).

Solid-Phase Binding Assay

We coated Maxisorp enzyme-linked immunosorbent assay (ELISA) plate (Nunc) wells with 50 μl of 0.2 μM GST-tagged recombinant THBS2 in 100 mM Tris-HCl (pH 7.2) at 4°C overnight. We then blocked the wells with 300 μl of blocking buffer (100 mM Tris-HCl [pH 7.2], 5 mM $CaCl_2$, 1% bovine serum albumin) for 1 hr at room temperature, added 50 μl of 4 μg/ml purified human MMP2 or MMP9 (CHEMICON) to the wells, and incubated them for 2 hr at room temperature. Wells were washed twice with 100 mM Tris-HCl (pH 7.2) and incubated with antibodies to MMP2 or MMP9 (R&D Systems). Next, we washed the wells three times with 100 mM Tris-HCl (pH 7.2) and incubated them with alkaline-phosphatase-conjugated antibody to goat IgG (ZYMED). After washing the wells three times with 100 mM Tris-HCl (pH 7.2), we assayed bound phosphatase activity with the Alkaline Phosphatase Substrate Kit (Bio-Rad) and detection at 405 nm.

Results

To confirm the candidacy of *THBS1* and *THBS2* for LDH, we examined *THBS1* and *THBS2* expression in various