Change of angiogenesis-related protein expression by polydeoxyribonucleotide (PDRN)treatment in chondrocyte-like cells

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ABSTRACT

Change of angiogenesis-related protein expression by polydeoxyribonucleotide (PDRN) treatment in chondrocyte-like cells

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(Directed by Professor Sang Chul Lee)

The purpose of this study was to investigate the effect of polydeoxynucleotide (PDRN) on factors associated with angiogenesis after administration of PDRN in chondrocyte-like cells.

Interleukin (IL)-1β or phosphate buffered saline (PBS) was used to treat human chondrocytic cell line in hypoxic condition for 24 h (IL-1β group or control group). PDRN was then used to treat IL-1β group cells for 24 h (PDRN group). Angiopoietin-2 (ANG-2), platelet-derived growth factor (PDGF) related to pro-angiogenesis and angiostatin and endostatin related to anti-angiogenesis were chosen by Label-based Human Antibody Array 1000 for further validation studies.

Quantitative real-time reverse transcription polymerase chain reaction and western blot analysis validated that levels of PDGF and ANG-2 were significantly increased in the PDRN group compared to those in the control group or the IL-1β group. However, levels of endostatin and angiostatin were significantly decreased in the PDRN group compared to those in the control group or the IL-1β group. In *in vitro* scratch assay, wound closure was significantly increased in the PDRN group compared to that in the control group or the IL-1β group. Moreover, PDRN decreased expression of MMP13 (a catabolic factor for OA) but increased expression of aggrecan (an anabolic factor for OA).

These data suggest that PDRN may promote angiogenesis and wound healing via down-regulation of catabolism and up-regulation of

anabolism in chondrocyte-like cells.

Key words : Polydeoxyribonucleotide (PDRN), chondrocyte, inflammation

Change of protein expression in a polydeoxyribonucleotide (PDRN)-treated inflammatory chondrocyte-like cells

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I. INTRODUCTION

Knee osteoarthritis (OA) involves degeneration of the articular cartilage and meniscus in the knee joint, synovial hyperplasia, subchondral bone sclerosis, and edema, leading to joint pain, swelling, stiffness, muscle atrophy, and functional disability[1]. To date, pharmacological armamentarium for OA is limited to symptomatic treatments with goal to diminish functional impairments and pain severity[2]. Currently, corticosteroids and hyaluronic acid (HA) are used for intra-articular injection therapy of OA. However, corticosteroid injections only provide short-term pain relief by modulating inflammation. They can lead to further joint destruction[3]. HA, a large non-sulfated glycosaminoglycan found in synovial fluid, has been injected into arthritic knees to aid and improve articular cartilage lubrication[4]. Nevertheless, recent clinical practice guidelines issued by the American Academy of Orthopedic Surgeons in 2013 stated that they were unable to recommend HA for treatment of symptomatic OA in the knee based on the lack of evidence of its effectiveness from high and moderate quality research studies[5]. Thus, ideal intra-articular treatment for OA should not only mechanically protect the damaged cartilage surface, but also restore chondrocytes' homeostasis by reestablishing the physiological articular micro-environment.

Recently published studies have reported that polydeoxyribonucleotide (PDRN) may represent a new and safe injection therapy for knee OA. Gennero et al.[6] have estimated the efficacy of PDRNs on cartilage degradation and found that PDRNs are suitable for long-term cultivation of *in vitro* cartilage with therapeutic effects on chondrocytes by protecting cartilage. A randomized and double-blind clinical trial published in 2014 on 75 patients has assessed the efficacy and safety profiles of intra-articular PDRN injection in treatment of knee OA associated with persistent pain, showing a reduction of pain and an increase of function in daily living and sport activity from baseline values comparable to those obtained with the use of HA[7]. Another study that followed up 95 patients with knee OA or chondropathy (grade III or IV) for 60 days after injecting PDRN has found that intra-articular administration of PDRN in subjects with both severe knee arthritis and chondropathy can reverse short and medium term symptoms and function with significant improvement in quality of life[8].

PDRN is extracted from the sperm of trout bred for human food purposes. It is regarded as a source of pyrimidines and purines, thereby stimulating nucleic acid synthesis through the salvage pathway[9]. Despite increasing interest in PDRN for treatment of OA, precise mechanisms underlying actions of PDRN on OA have not been well studied yet. By activating purinergic A2A receptor, PDRN enhances cell proliferation and promotes rapid healing process via stimulating angiogenesis in experimental models of autologous skin graft donor sites, diabetic pressure ulcer, and varicocele[9-12].

Angiogenesis defined as blood vessel outgrowth from pre-existing vasculature is essential for growth and development, the reproductive cycle, and tissue repair. However, unlike skin wound or diabetic pressure ulcer, angiogenesis is known to play a key role in the progression of cartilage degradation in OA[13]. Angiogenesis contributes to synovitis, osteochondral damage, osteophyte formation, and meniscal pathology in patients with OA. Nerve growth along new blood vessels into structures normally not innervated could also contribute to pain in OA[13]. Therefore, if PDRN has positive effect on OA, it is necessary to clarify whether

PDRN can stimulate angiogenesis in OA or whether angiogenesis has a positive or negative impact on OA as in other tissue injuries.

An *in vivo* study has investigated the effect of PDRNs on collagen-induced arthritis in mice[14]. Results of that study showed that PDRNs treatment improved clinical signs of arthritis and histological damage, reduced cartilage expression and inflammatory cytokine production from stimulated human chondrocytes, thus representing a valid alternative for treatment of arthritis[14]. If PDRN can stimulate angiogenesis in OA, angiogenesis may weaken the above positive effect of PDRN. Otherwise, angiogenesis in PDRN may have a positive effect on OA, unlike previous reports showing negative effects of angiogenesis on OA. However, the effect of PDRN on angiogenesis in OA has not been reported yet. Therefore, the aim of this study was to investigate the effect of PDRN on factors associated with angiogenesis and determine changes of pro-angiogenic and anti-angiogenic factors after administration of PDRN in chondrocyte-like cells.

II. MATERIALS AND METHODS

1. Cell culture and IL-1β stimulation

SW1353 cells, which have been reported as human chondrocytic cell line[15-17]. They were obtained from were obtained from American Type Culture Collection (ATCC HTB-94, Manassas, VA, USA) and maintained in Dulbecco's modified Eagle medium-High Glucose (DMEM-HG, Gibco, Carlsbad, CA) containing 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA) and 1% 60 U/ml penicillin at 37℃. After reaching 80% confluence, cells were harvested using 0.05% trypsin-EDTA (Gibco, Carlsbad, CA). Cells were washed, centrifuged, resuspended and seeded into new plates. Culture medium was replaced every 2 to 3 days. In each experiment, cells were rendered quiescent for 12 hours by adding DMEM-HG without serum under hypoxic condition. Cells were stimulated with phosphate buffered saline (PBS) or 10 ng/ml of interleukin (IL)-1β (R&D Systems, Minneapolis, MN, USA) for 24 hours to establish an *in vitro* OA model16,[18-20]. After stimulation with IL-1β, cells were treated with PDRN (Placentex Integro, Mastelli Srl, Italy) for 24 hours and harvested using

0.05% trypsin-EDTA as described above. Three experimental groups were used in this study and as follows: PBS treated group (Control group); IL-1β treated group (IL-1β group); IL-1β followed by PDRN treatment group (PDRN group) (Fig. 1A).

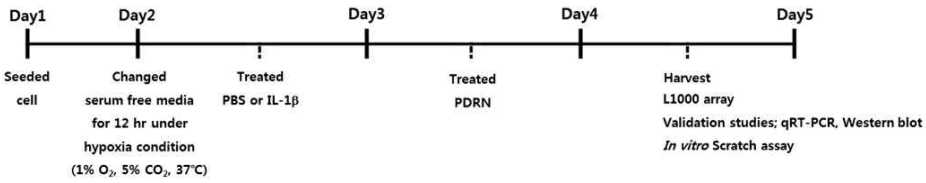

Figure 1. Experimental scheme and L1000 array analysis. (A) Seeded SW1353 cells were serum starved under hypoxic condition for 12 h and then stimulated with 10 ng/mL of IL-1β or PBS for 24 h followed by treatment with 100 mg/ml of PDRN. After 24 h, cells were harvested for L1000 array. To validate L1000 array study and OA pathogenesis, qRT-PCR and western blot were conducted. To examine migration efficiency *in vitro* scratch assay was performed.

2. Label-based Human Antibody Array 1000

To detect relative expression levels of 1000 human proteins, whole cell lysates from experimental groups were homogenized and dissolved in RIPA buffer (Thermo Scientific, Rockford, IL, USA) with protease inhibitors (Abcam, Cambridge, MA, USA) to prepare protein for human L1000 array. Human L1000 glass slide array (RayBiotech, Norcross, GA, USA) was performed by eBiogen Inc. (Seoul, Republic of Korea).

3. Bioinformatics analysis

Enriched proteins from the IL-1β compared to the PDRN group were categorized according to biological process of gene ontology term analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) 6.8 annotation tool to identify protein function.

To identify protein function, enriched protein-coding genes were used with

biological process in DAVID 6.8 annotation tool. Within several biological processes, proteins coding genes, involved in angiogenesis, were focused for further validation studies.

4. Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was reverse-transcribed into cDNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Expression levels of genes of interest were determined using qPCRBIO SyGreen Mix Hi-ROX (PCR BIOSYSTEMS, London, UK) in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Data analysis was performed using the $2^{-\Delta\Delta \text{ cycle threshold (CT)}}$ method[21]. Primers used for qRT-PCR are listed in Table 1.

Table 1. Primers used for qRT-PCR.

Gene symbol	Forward primer (5′→3′)	Reverse primer (5′→3′)
PDGF	GCA CCG GCT CAT CTT TGT CTA	TTC GGT ACA AGT CTG TGA GGT G
ANG-2	ATA CGA TGA CTC GGT GCA GA	TGC TCC GCT GTT TGG TTC AA
Angiostatin	TAA TCC CAG CTT GTC TGC CA	TTC GGT GGA TTG GAC TCT TCC
Endostatin	CCC AGC CGT GGC ATT CCT A	TGA TGC GCT CTG AAG ATG GTG G
GAPDH	AAG GGT CAT CAT CTC TGC CC	GTG AGT GCA TGG ACT GTG GT

PDGF, Platelet-derived growth factor; ANG-2, Angiopoietin-2; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase

5. Western blot analysis

To assess protein levels of platelet-derived growth factor (PDGF), angiopoietin-2 (ANG-2), endostatin, angiostatin, matrix metalloproteinase 13 (MMP13), and aggrecan, proteins from cell pellets were harvested as described

above. Total proteins were quantified using BCA™ Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Samples were denatured and separated by 4–12% Bis-Tris gels in 1× NuPage MOPS SDS running buffer (Invitrogen, Eugene, OR, USA). Proteins were transferred onto PVDF membranes (Invitrogen, Eugene, OR, USA) in 20% (vol/vol) methanol in NuPage Transfer Buffer (Invitrogen, Eugene, OR, USA) at 4°C. Membranes were blocked and then incubated at 4°C overnight with the following antibodies: anti-PDGF, anti-ANG-2, anti-endostatin, anti-angiostatin, anti-vascular endonthelial growth factor (VEGF), anti-MMP13, anti-aggrecan (1:1000, Abcam, Cambridge, MA, USA), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:3000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The next day, blots were washed three times with TBS plus Tween 20 and incubated with horse-radish peroxidase–conjugated secondary antibodies (1:4000; Santa Cruz, CA, USA) at room temperature for 1 hour. After washing three times with TBS plus Tween 20, proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Little Chalfont, UK).

6. In vitro scratch assay

To determine the effect of PDRN treatment on cell spreading and migration capabilities, cells were seeded into 6-well tissue culture dishes. A linear wound was then generated in the monolayer of cells with a 200 μl of plastic pipette tip. Any cellular debris was removed by washing with PBS, DMEM medium with PBS (control group), or DMEM medium with IL-1β (IL-1β group). After 24 h, PDRN was added (IL-1β+PDRN group). After 24 h of incubation, images of migrated cells were taken using a digital camera connected to an inverted microscope to observe the closure of wound area. Wound was quantified with Image J software (NIH, Baltimore, MD, USA). All scratch assays were performed in quadruplicates.

7. Statistical Analysis

All results are expressed as means ± standard error of the mean (SEM) from at least three independent experiments. Statistical analyses were conducted using the Statistical Package for Social Sciences (SPSS) version 23.0. One-way analysis of variance followed by post hoc Bonferroni comparison was performed to confirm statistical results. P-value < 0.05 was considered statistically significant.

III. RESULTS

1. L1000 array analysis

Enriched proteins were categorized according to fold change ratio ≥ |1.5|. Up- and down-regulated proteins at ≥ 1.5-fold were counted and summarized in Fig. 1B. Enriched protein-coding genes from the IL-1β group compared to the PDRN group were categorized according to biological process of gene ontology term analysis using the DAVID 6.8 annotation tool and listed in Table 2 (FDR< 0.001). Within several biological processes, proteins coding genes, involved in angiogenesis such as angiopoietin-2 (ANG-2)[28,29] and platelet-derived growth factor (PDGF)[30,31] related to pro-angiogenesis and angiostatin and endostatin related to anti-angiogenesis[32], were chosen for further validation studies. These proteins are presented in Table 3.

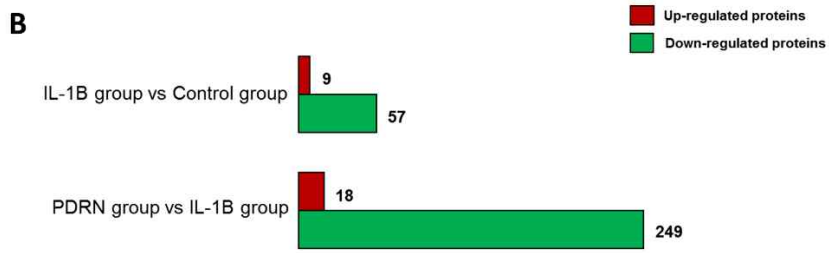

Figure 1. Experimental scheme and L1000 array analysis. (B) The number of enriched proteins is indicated by bar graphs. The number of up-regulated proteins is indicated by red bar while, the number of down-regulated proteins is indicated by green bar.

Table 2. Biological process of gene ontology analysis from the IL-1β group compared to the PDRN group.

Term	Count	*p*-value	FDR
Immune response	55	6.13E-37	1.06E-33
Inflammatory response	48	2.11E-31	3.64E-28
Positive regulation of cell proliferation	47	2.24E-26	3.86E-23
Signal transduction	70	2.43E-26	4.19E-23
Cell-cell signaling	36	5.14E-25	8.87E-22
Cytokine-mediated signaling pathway	28	2.73E-24	4.71E-21
Response to lipopolysaccharide	25	4.72E-18	8.14E-15
Peptidyl-tyrosine phosphorylation	22	2.54E-15	4.41E-12
MAPK cascade	27	3.24E-15	5.55E-12
Tumor necrosis factor-mediated signaling pathway	19	4.01E-14	6.91E-11
Chemotaxis	19	7.28E-14	1.26E-10
Positive regulation of ERK1 and ERK2 cascade	21	4.11E-13	7.09E-10
Positive regulation of inflammatory response	15	1.12E-12	1.93E-09
Regulation of phosphatidylinositol 3-kinase signaling	15	2.92E-12	5.03E-09
Positive regulation of phosphatidylinositol 3-kinase signaling	14	4.15E-12	7.16E-09
Neutrophil chemotaxis	14	5.11E-12	8.81E-09
Positive regulation of MAP kinase activity	13	2.30E-11	3.96E-08
Angiogenesis	**21**	**3.75E-11**	**6.47E-08**
Cell chemotaxis	13	7.68E-11	1.33E-07
Chemokine-mediated signaling pathway	13	2.27E-10	3.92E-07
Phosphatidylinositol phosphorylation	14	5.35E-10	9.23E-07
Positive regulation of cell migration	18	7.32E-10	1.26E-06
Positive regulation of pathway-restricted SMAD protein phosphorylation	11	8.62E-10	1.49E-06
Positive regulation of peptidyl-tyrosine phosphorylation	13	1.29E-09	2.22E-06
Phosphatidylinositol-mediated signaling	14	2.44E-09	4.21E-06
Positive regulation of interferon-gamma production	10	1.11E-08	1.91E-05
Activation of cysteine-type endopeptidase activity involved in apoptotic process	12	1.86E-08	3.21E-05
Positive regulation of tyrosine phosphorylation of Stat3 protein	9	4.03E-08	6.94E-05
Cell surface receptor signaling pathway	19	5.10E-08	8.78E-05
Cellular response to lipopolysaccharide	13	5.26E-08	9.07E-05
Transforming growth factor beta receptor signaling pathway	12	5.56E-08	9.58E-05
Positive regulation of GTPase activity	27	8.06E-08	1.39E-04
Extracellular matrix disassembly	11	8.98E-08	1.55E-04
Positive regulation of DNA replication	9	9.27E-08	1.60E-04
Extrinsic apoptotic signaling pathway	9	9.27E-08	1.60E-04

Positive regulation of MAPK cascade	11	1.66E-07	2.87E-04
Platelet degranulation	12	1.80E-07	3.11E-04
Negative regulation of interleukin-17 production	6	2.08E-07	3.59E-04
Positive regulation of cell division	9	2.33E-07	4.01E-04
Regulation of apoptotic process	16	2.81E-07	4.84E-04
Regulation of cell proliferation	15	2.95E-07	5.08E-04
Positive regulation of JAK-STAT cascade	7	4.04E-07	6.97E-04

Angiogenesis is relevant to PDRN treatment and is shown in a bold font.

These biological processes are statistically significant (FDR < 0.001).

Table 3. L1000 array analysis of protein related to angiogenesis

Antibody Name	IL-1β/Control	IL-1β+PDRN/IL-1β
ANG-2	0.818	1.717
PDGF-D	1.166	1.504
Endostatin	1.219	0.640
Angiostatin	0.834	0.607

ANG-2, Angiopoietin-2; PDGF, Platelet-derived growth factor

2. Validation for L1000 array

qRT-PCR and western blot analysis were conducted to validate L1000 array results for proteins involved in angiogenesis. By qRT-PCR, there were no significant changes in protein expression in the IL-1β group compared to the control group (Fig. 2A). Expression change results for the four selected proteins were as follows: PDGF, 0.887-fold; ANG-2, 1.089-fold; endostatin, 0.842-fold; and angiostatin, 0.937-fold. In the PDRN group, PDGF and ANG-2 were significantly increased whereas endostatin and angiostatin were significantly decreased compared to those in the control group. Their expression changes were as follows: PDGF, 1.259-fold ($p < 0.05$); ANG-2, 1.454-fold ($p < 0.05$); endostatin, 0.654-fold ($p < 0.01$); and angiostatin, 0.746-fold ($p < 0.05$). PDGF level was significantly ($p < 0.01$) whereas angiostatin level was significantly decreased ($p < 0.05$) in the PDRN group compared to those in the IL-1β group.

Expression levels of these proteins in the IL-1β group compared to those in the control group by western blot were as follows: PDGF, 4.444-fold ($p < 0.01$); ANG-2, 0.965-fold; endostatin, 3.526-fold ($p < 0.001$); and angiostatin, 1.754-fold ($p < 0.05$) (Fig. 2B and 2C). In the PDRN group, PDGF and ANG-2 levels were significantly increased whereas endostatin and angiostatin levels were significantly decreased compared to those in the control group. Their expression values were as follows: PDGF, 8.659-fold ($p < 0.01$); ANG-2, 1.320-fold ($p < 0.05$); endostatin, 0.041-fold ($p < 0.001$); and angiostatin, 0.165-fold ($p < 0.05$).

In the PDRN group, PDGF and ANG-2 levels were significantly increased whereas endostatin and angiostatin levels were significantly decreased compared to those in the IL-1β group. Their expression values were as follows: PDGF ($p< 0.05$), ANG-2 ($p< 0.05$), endostatin ($p< 0.001$), and angiostatin ($p< 0.001$).

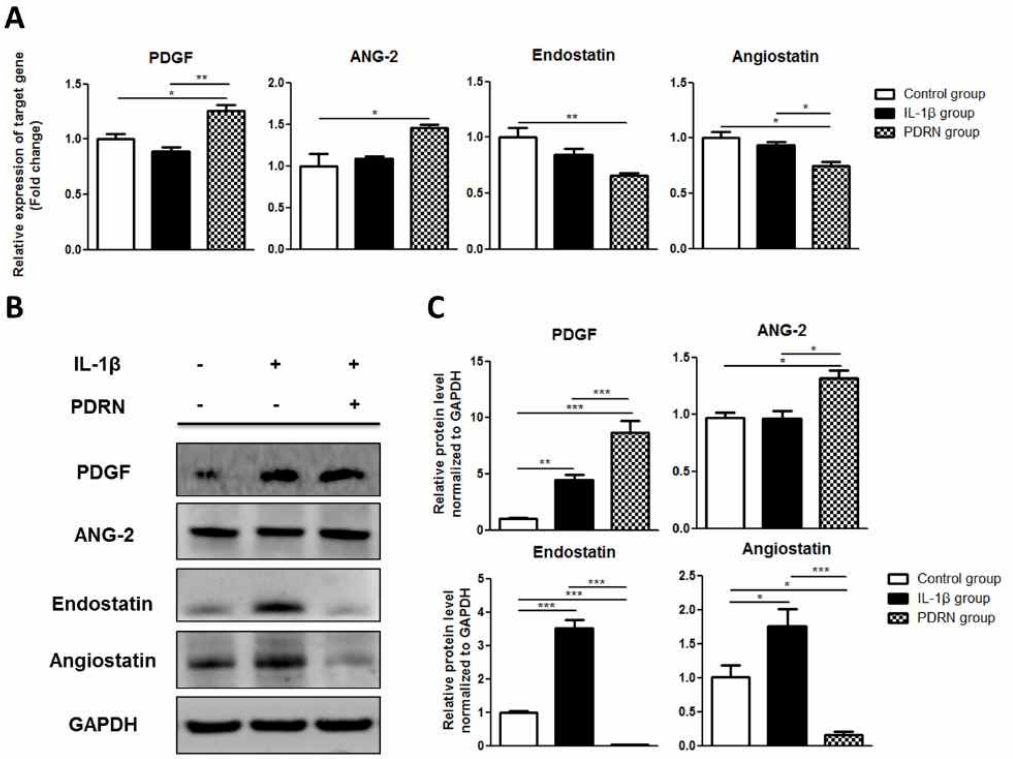

Figure 2. Effects of PDRN on mRNA and protein levels of angiogenesis.

(A) PDGF, ANG-2, endostatin, and angiostatin expression levels were validated by qRT-PCR. Relative expression levels of target genes were calculated with the $2^{-\Delta\Delta Ct}$ method. (B) Western blot analysis was performed with anti-PDGF, anti-ANG-2, anti-endostatin, anti-angiostatin, and anti-GAPDH antibody (as a control). (C) Relative protein expression levels in the IL-1β group and PDRN group compared those in the control group by western blot. All results are expressed as mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3. Effects of PDRN on wound healing

It has been reported that PDRN, an adenosine receptor A_{2A} agonist, can facilitate wound healing[22]. Thus, *in vitro* scratch assay was conducted in this study to determine the effect of PDRN on wound healing. *In vitro* cell migration ability was determined by the number of cells that migrated across scratch parts of cells. As shown in Fig. 3A, a significant increase in cell migration was observed in the PDRN group compared to the control group or the IL-1β group (Fig. 3B). The percentage of wound closure was as follows: Control group, 4.68%; IL-1β group, 5.51%; and PDRN group, 17.33%.

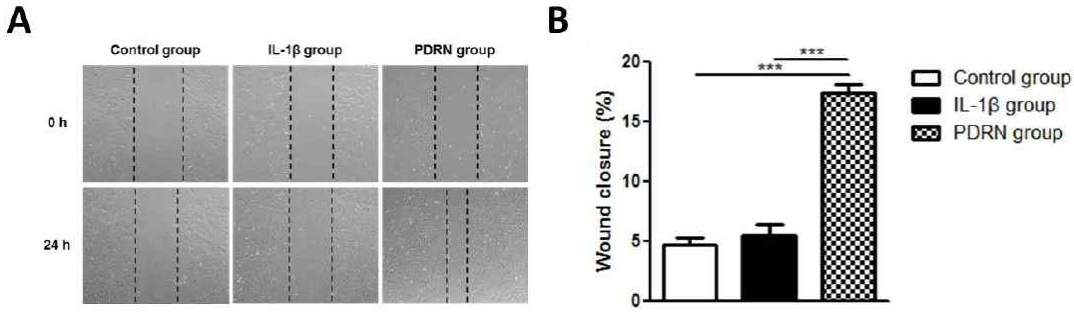

Figure 3. Effects of PDRN on cell migration. (A) Representative data of wound healing experiment. The beginning of the experiment is before treatment with PDRN and indicated as 0 h. After treatment with PDRN for 24 h is indicated as 24 h. (B) The area of the wound closure was quantified, and the ratio of wound closure was expressed as a percentage of recovered wound compared to the area at 0 h of each groups. All results are expressed as mean ± SEM. *$p < 0.05$; ***$p < 0.001$.

4. Effects of PDRN on expression of MMP13 and aggrecan.

It has been reported that imbalance of anabolic and catabolic activity is crucial for OA pathogenesis[33-36]. Thus, we examined the effect of PDRN on expression of anabolic and catabolic factors.

MMP13 and aggrecan are components of cartilage. They can regulate cartilage destruction. It has been reported that the expression of MMP13, one of catabolic

genes for OA, increased the expression of aggrecan, one of anabolic genes for OA, is down-regulated in IL-1β treated chondrocytes[37]. Thus, we examined expression levels of MMP13 and aggrecan in this study.

In the IL-1β group, MMP13 expression was statistically increased compared to that in the control group (Fig. 4A). On the other hand, aggrecan expression was significantly decreased in the IL-1β group compared to that in the control group (Fig. 4A). Their expression values were as follows: MMP13, 1.359-fold ($p < 0.05$); and aggrecan, 0.765-fold ($p < 0.05$) (Fig. 4B). MMP13 expression was significantly decreased while aggrecan expression was significantly increased in the PDRN group compared to those in the control group (Fig. 4A). Their expression values were as follows: MMP13, 0.589-fold ($p < 0.05$); and aggrecan, 2.211-fold ($p < 0.001$) (Fig. 4B). MMP13 expression was significantly ($p < 0.001$) decreased while aggrecan expression was significantly ($p < 0.001$) increased in the PDRN group compared to those in the control group (Fig. 4A and B).

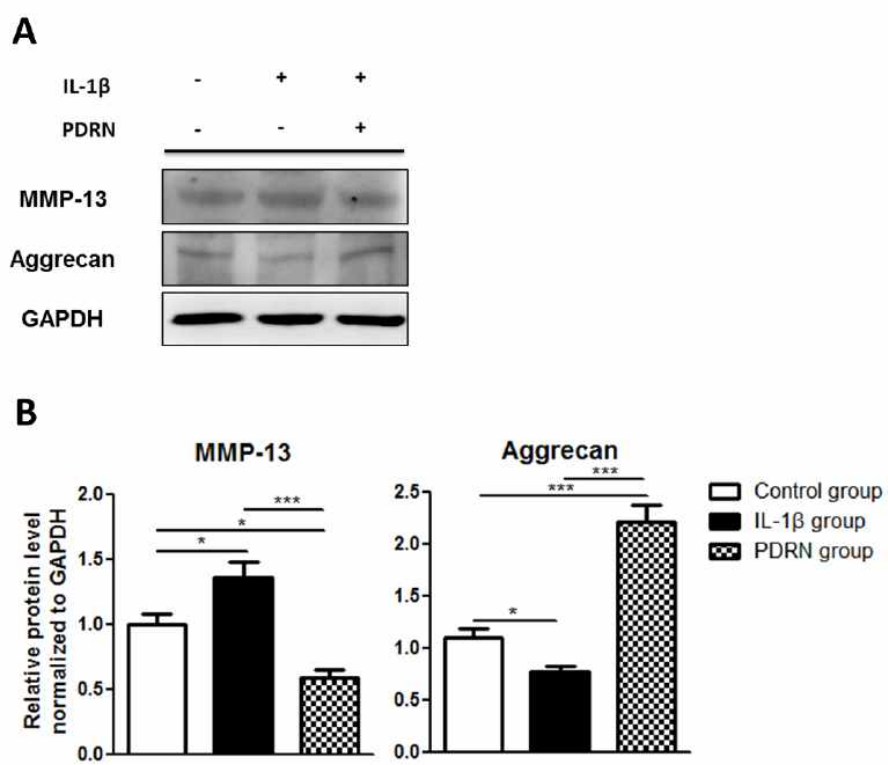

Figure 4. Effects of PDRN on expression of MMP13 and Aggrecan. (A) Western blot analysis using anti-MMP13, anti-Aggrecan, and anti-GAPDH (as a control). (B) Relative protein expression in the IL-1β group and the PDRN group compared to the control group by western blot. All results are expressed as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.

IV. DISCUSSION

Findings from this study indicated that PDRN could promote the expression of pro-angiogenic factors and inhibit the expression of anti-angiogenic factors in chondrocyte-like cells. This is the first study that compares changes in factors influencing angiogenesis after PDRN treatment in this model.

To detect relative expression levels of 1000 human proteins including angiogenic factors, label-based Human Antibody Array 1000 was used. Among angiogenic factors evaluated, PDGF and ANG-2 levels were significantly increased whereas endostatin and angiostatin levels were significantly decreased in the PDRN group compared to those in the IL-1ß group based on western blot analysis. These factors play important roles in angiogenesis. Activation of angiogenesis is basically the result of an imbalance between pro- and anti-angiogenic factors[38]. PDGF[39,40] and ANG-2[41] are known as pro-angiogenic factors whereas endostatin[42,43] and angiostatin[44,45] are known as anti-angiogenic factors.

The mechanism underlying preservation of avascularity in articular cartilage remains unclear. However, it has been suggested that hyaline cartilage contains high concentrations of endogenous inhibitors of angiogenesis[46]. Angiogenic and antiangiogenic factors might be upregulated in the osteoarthritic joint. However, when vascular growth predominates, articular cartilage will lose its resistance to vascularization[13]. Blood vessel and nerve growth are linked by common pathways involving the release of proangiogenic factors. As sensory nerves grow along new blood vessels in osteoarthritic joints, they eventually penetrate non-calcified articular cartilage, osteophytes, and inner regions of menisci. Therefore, angiogenesis can contribute to structural damage and pain in OA, thus providing potential targets for new OA treatments[47].

Considering these points, it is very difficult to interpret whether the angiogenic effect of PDRN has a positive effect on OA. Angiogenic effect of PDRN on recovery of chondrocyte in OA cannot be determined by results of this study. At the present time, there are two reasonable hypotheses. First, as is known, increased angiogenesis by PDRN may adversely affect chondrocytes in OA.

Nevertheless, positive effects of PDRN on OA patients such as anti-inflammatory and cell repair effects are greater than those of angiogenesis. Consequently, PDRN may help OA patients. Second, it can be assumed that the angiogenesis effect of PDRN itself does not have a bad influence on OA, unlike what is known in the past. If the first hypothesis is correct, PDRN will be considered as a treatment for OA without violating the existing theory of "angiogenesis plays a key role in the progression of cartilage degradation in OA."[13] For the second hypothesis to be persuasive, it is necessary to redefine the concept of angiogenesis in OA (especially in the early stage of OA).

The phenomenon of OA may be defined clinically, radiologically, and pathologically. However, its etiology remains poorly understood. The precise contribution of angiogenesis to symptoms and pathology of OA is currently unclear. Angiogenesis is a complex multistep process controlled by a wide range of positive and negative regulatory factors. Angiogenesis occurs during essential physiological processes such as embryogenesis and wound repair. However, angiogenesis can also contribute to a variety of pathological conditions, including unwanted vessel growth in chronic inflammatory diseases and growth or metastasis of tumors[48]. In OA, angiogenesis interacts closely with inflammation, compressive forces, and hypoxia. However, studies regarding the role of angiogenesis in early stages of OA, especially as a defense mechanism against the degenerative process, are insufficient. Further study is needed.

PDRN is expected to satisfy a double mechanism of action: mechanical protection for damaged cartilage and restoration of an ideal microenvironment for matrix production[49]. *In vitro* and *in vivo* studies have been carried out in order to verify the effects of PDRN on cartilage[6-10]. One of these studies has shown that intra-articular PDRNs are valid alternatives to traditional HA supplementation[7]. Chondrocytes have a pivotal role during OA. They are mainly responsible for the anabolic–catabolic balance required for matrix maintenance and tissue function[48]. On the other hand, OA chondrocytes are characterized by accelerated catabolic processes as well as suppression of anabolic processes. An imbalance in the expression of catabolic and anabolic factors can eventually lead

to osteoarthritic cartilage destruction[50]. In the *in vitro* scratch assay of our study, a significant increase in cell migration was observed in the PDRN group compared to either the control group or the IL-1β group. The percentage of wound closure values was as follows: control group, 4.68%; IL-1β group, 5.51%; and PDRN group, 17.33%. These results also support that PDRN might have therapeutic effect on OA chondrocyte regardless of whether the effect of angiogenesis on OA is positive or negative. Apart from the effect of PDRN on angiogenesis, the possibility that PDRN has therapeutic effect on OA cartilage is still high. Therefore, angiogenesis induced by PDRN on OA cartilage should be clarified.

This study has some limitations. Observations reported here should be interpreted with caution since factors involved in angiogenesis were not demonstrating real angiogenesis. However, the activation of angiogenesis is basically the result of an imbalance between pro- and anti-angiogenic factors. Increased expression of angiogenesis activators can lead to angiogenesis switch and act positively on neovascularization[51,52]. In addition, this study was based on cell line model instead of human subjects. Since this was the first study that examined the angiogenic effect of PDRN on chondrocyte-like cells, baseline study with cell line model was necessary. However, this model might differ from chronic OA condition. We conducted human chondrosarcoma cell line with addition of DMEM-HG under hypoxic condition for 12 hours and stimulation with 10 ng/mL concentration of IL-1 for 24 hours. This process might not be sufficient enough to reflect chronic status of OA. Additional study on chronic OA cell line or animal model is required.

V. CONCLUSION

In conclusion, PDRN may promote angiogenesis in OA chondrocytes. PDRN significantly increased levels of pro-angiogenic factors (PDGF, ANG-2) and decreased levels of anti-angiogenic factors (endostatin, angiostatin) PDRN in chondrocyte-like cells. PDRN also significantly facilitated cell migration

regardless of angiogenesis through down-regulation of catabolism and up-regulation of anabolism (Figure 5). Further studies about PDRN on angiogenesis are necessary to determine whether this effect is positive.

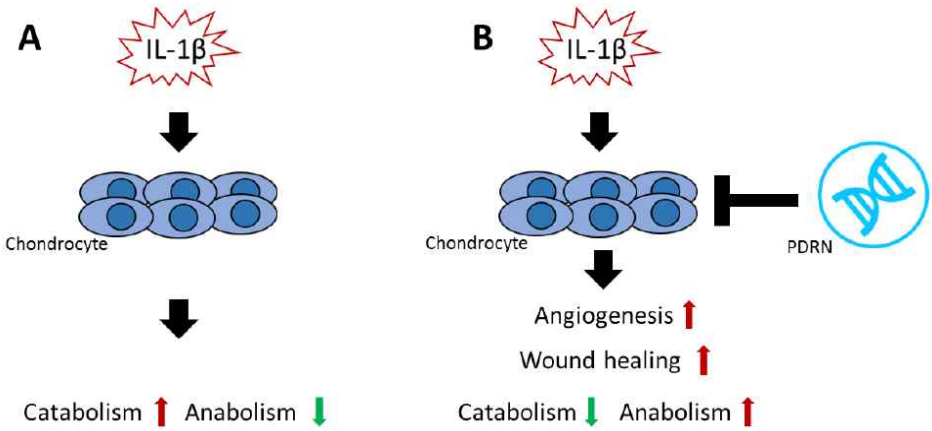

Figure 5. Effects of PDRN on *in vitro* OA model. (A) IL-1β induces the pathogenesis of OA in chondrocytes through up-regulation of catabolism and down-regulation of anabolism. (B) PDRN inhibit the pathogenesis of OA via up-regulation of angiogenesis and wound healing.

REFERENCES

1. Sakata R, Reddi AH. Platelet-rich plasma modulates actions on articular cartilage lubrication and regeneration. Tissue Eng Part B Rev 2016;22(5):408-19.
2. Daouti S, Latario B, Nagulapalli S, Buxton F, Uziel-Fusi S, Chirn GW, et al. Development of comprehensive functional genomic screens to identify novel mediators of osteoarthritis. Osteoarthritis Cartilage 2005;13(6):508-18.
3. Arroll B, Goodyear-Smith F. Corticosteroid injections for osteoarthritis of the knee: meta-analysis. BMJ 2004;328(7444):869.
4. Rutjes AW, Jüni P, da Costa BR, Trelle S, Nüesch E, Reichenbach S. Viscosupplementation for osteoarthritis of the knee: a systematic review and meta-analysis. Ann Intern Med 2012;157(3):180-91.
5. Brown GA. AAOS clinical practice guideline: treatment of osteoarthritis of the knee: evidence-based guideline, 2nd edition. J Am Acad Orthop Surg 2013;21(9):577-9.
6. Gennero L, Denysenko T, Calisti GF, Vercelli A, Vercelli CM, Amedeo S, et al. Protective effects of polydeoxyribonucleotides on cartilage degradation in experimental cultures. Cell Biochem Funct 2013;31(3):214-27.
7. Giarratana LS, Marelli BM, Crapanzano C, De Martinis SE, Gala L, Ferraro M, et al. A randomized double-blind clinical trial on the treatment of knee osteoarthritis: the efficacy of polynucleotides compared to standard hyaluronian viscosupplementation. Knee 2014;21(3):661-8.
8. Saggini R, Di Stefano A, Cavezza T, Saladino G, Bellomo RG. Intrarticular treatment of osteoartropaty knee with polynucleotides: a pilot study with medium-term follow-up. J Biol Regul Homeost Agents 2013;27(2):543-9.
9. Galeano M, Bitto A, Altavilla D, Minutoli L, Polito F, Calò M, et al. Polydeoxyribonucleotide stimulates angiogenesis and wound healing in the genetically diabetic mouse. Wound Repair Regen 2008;16(2):208-17.
10. Arena S, Minutoli L, Arena F, Nicotina PA, Romeo C, Squadrito F, et al. Polydeoxyribonucleotide administration improves the intra-testicular vascularization in rat experimental varicocele. Fertil Steril 2012;97(1):165-8.

11. Rubegni P, De Aloe G, Mazzatenta C, Cattarini L, Fimiani M. Clinical evaluation of the trophic effect of polydeoxyribonucleotide (PDRN) in patients undergoing skin explants. A Pilot Study. Curr Med Res Opin 2001;17(2):128-31.
12. Valdatta L, Thione A, Mortarino C, Buoro M, Tuinder S. Evaluation of the efficacy of polydeoxyribonucleotides in the healing process of autologous skin graft donor sites: a pilot study. Curr Med Res Opin 2004;20(3):403-8.
13. Mapp PI, Walsh DA. Mechanisms and targets of angiogenesis and nerve growth in osteoarthritis. Nat Rev Rheumatol 2012;8(7):390-8.
14. Bitto A, Polito F, Irrera N, D'Ascola A, Avenoso A, Nastasi G, et al. Polydeoxyribonucleotide reduces cytokine production and the severity of collagen-induced arthritis by stimulation of adenosine A(2A) receptor. Arthritis Rheum 2011;63(11):3364-71.
15. Santoro A, Conde J, Scotece M, Abella V, Lopez V, Pino J, et al. Choosing the right chondrocyte cell line: Focus on nitric oxide. J Orthop Res 2015;33(12):1784-8.
16. Gebauer M, Saas J, Sohler F, Haag J, Soder S, Pieper M, et al. Comparison of the chondrosarcoma cell line SW1353 with primary human adult articular chondrocytes with regard to their gene expression profile and reactivity to IL-1 beta. Osteoarthritis Cartilage 2005;13(8):697-708.
17. Tew SR, Clegg PD, Brew CJ, Redmond CM, Hardingham TE. SOX9 transduction of a human chondrocytic cell line identifies novel genes regulated in primary human chondrocytes and in osteoarthritis. Arthritis Res Ther 2007;9(5):R107.
18. Zeng L, Wang W, Rong XF, Zhong Y, Jia P, Zhou GQ, et al. Chondroprotective effects and multi-target mechanisms of Icariin in IL-1 beta-induced human SW 1353 chondrosarcoma cells and a rat osteoarthritis model. Int Immunopharmacol 2014;18(1):175-81.
19. Jia P, Chen G, Zhou G, Zhong Y, Li R. Fuyuan Decoction inhibits nitric oxide production via inactivation of nuclear factor-kappaB in SW1353 chondrosarcoma cells. J Ethnopharmacol 2013;146(3):853-8.
20. Bao G, Xu L, Xu X, Zhai L, Duan C, Xu D, et al. SGTB Promotes the

Caspase-Dependent Apoptosis in Chondrocytes of Osteoarthritis. Inflammation 2016;39(2):601-10.

21. Livak, KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2[−Delta Delta C(T)] method. Methods 2001;25(4):402–8.
22. Bitto A, Galeano M, Squadrito F, Minutoli L, Polito F, Dye JF, et al. Polydeoxyribonucleotide improves angiogenesis and wound healing in experimental thermal injury. Crit Care Med 2008;36(5):1594-602.
23. Altavilla D, Bitto A, Polito F, Marini H, Minutoli L, Di Stefano V, et al. Polydeoxyribonucleotide (PDRN): a safe approach to induce therapeutic angiogenesis in peripheral artery occlusive disease and in diabetic foot ulcers. Cardiovasc Hematol Agents Med Chem 2009;7(4):313-21.
24. Kim S, Kim J, Choi J, Jeong W, Kwon S. Polydeoxyribonucleotide Improves Peripheral Tissue Oxygenation and Accelerates Angiogenesis in Diabetic Foot Ulcers. Arch Plast Surg 2017;44(6):482-9.
25. Noh TK, Chung BY, Kim SY, Lee MH, Kim MJ, Youn CS, et al. Novel Anti-Melanogenesis Properties of Polydeoxyribonucleotide, a Popular Wound Healing Booster. Int J Mol Sci 2016;17(9). pii: E1448.
26. Jeong W, Yang CE, Roh TS, Kim JH, Lee JH, Lee WJ. Scar Prevention and Enhanced Wound Healing Induced by Polydeoxyribonucleotide in a Rat Incisional Wound-Healing Model. Int J Mol Sci 2017;18(8). pii: E1698.
27. Yu M, Lee JY. Polydeoxyribonucleotide improves wound healing of fractional laser resurfacing in rat model. J Cosmet Laser Ther 2017;19(1):43-8.
28. Yoshiji H, Kuriyama S, Noguchi R, Yoshii J, Ikenaka Y, Yanase K, et al. Angiopoietin 2 displays a vascular endothelial growth factor dependent synergistic effect in hepatocellular carcinoma development in mice. Gut 2005;54(12):1768-75.
29. Coffelt SB, Chen YY, Muthana M, Welford AF, Tal AO, Scholz A, et al. Angiopoietin 2 stimulates TIE2-expressing monocytes to suppress T cell activation and to promote regulatory T cell expansion. J Immunol 2011;186(7):4183-90.

30. Pietras K, Pahler J, Bergers G, Hanahan D. Functions of paracrine PDGF signaling in the proangiogenic tumor stroma revealed by pharmacological targeting. PLoS Med 2008;5(1):e19.
31. Ding W, Knox TR, Tschumper RC, Wu W, Schwager SM, Boysen JC, et al. Platelet-derived growth factor (PDGF)-PDGF receptor interaction activates bone marrow-derived mesenchymal stromal cells derived from chronic lymphocytic leukemia: implications for an angiogenic switch. Blood 2010;116(16):2984-93.
32. Kohn EC. Endostatin and angiostatin: the next anti-angiogenesis generation. Angiogenesis 1998;2(1):25-7.
33. Mueller MB, Tuan RS. Anabolic/Catabolic balance in pathogenesis of osteoarthritis: identifying molecular targets. PM R 2011;3(6 Suppl 1):S3-S11.
34. Kim JH, Jeon J, Shin M, Won Y, Lee M, Kwak JS, et al. Regulation of the catabolic cascade in osteoarthritis by the zinc-ZIP8-MTF1 axis. Cell 2014;156(4):730-43.
35. Caterson B, Hughes CE, Roughley P, Mort JS. Anabolic and catabolic markers of proteoglycan metabolism in osteoarthritis. Acta Orthop Scand Suppl 1995;266:121-4.
36. Wojdasiewicz P, Poniatowski LA, Szukiewicz D. The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis. Mediators Inflamm 2014;2014:561459.
37. Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier JP, Fahmi H. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. Nat Rev Rheumatol 2011;7(1):33-42.
38. Pesesse L, Sanchez C, Delcour JP, Bellahcène A, Baudouin C, Msika P, et al. Consequences of chondrocyte hypertrophy on osteoarthritic cartilage: potential effect on angiogenesis. Osteoarthritis Cartilage. 2013;21(12):1913-23.
39. Edelberg JM, Aird WC, Wu W, Rayburn H, Mamuya WS, Mercola M, et al. PDGF mediates cardiac microvascular communication. J Clin Invest 1998;102(4):837-43.
40. Lindahl P, Johansson BR, Levéen P, Betsholtz C. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. Science

1997;277(5323):242-5.
41. Distler JH, Hirth A, Kurowska-Stolarska M, Gay RE, Gay S, Distler O. Angiogenic and angiostatic factors in the molecular control of angiogenesis. Q J Nucl Med 2003;47(3):149-61.
42. Sasaki T, Larsson H, Kreuger J, Salmivirta M, Claesson-Welsh L, Lindahl U, et al. Structural basis and potential role of heparin/heparan sulfate binding to the angiogenesis inhibitor endostatin. EMBO J 1999;18(22):6240-8.
43. Yamaguchi N, Anand-Apte B, Lee M, Sasaki T, Fukai N, Shapiro R, et al. Endostatin inhibits VEGF-induced endothelial cell migration and tumor growth independently of zinc binding. EMBO J 1999;18(16):4414-23.
44. Claesson-Welsh L, Welsh M, Ito N, Anand-Apte B, Soker S, Zetter B, et al. Angiostatin induces endothelial cell apoptosis and activation of focal adhesion kinase independently of the integrin-binding motif RGD. Proc Natl Acad Sci U S A 1998;95(10):5579-83.
45. O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, et al. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell 1994;79(2):315-28.
46. Moses MA, Sudhalter J, Langer R. Identification of an inhibitor of neovascularization from cartilage. Science 1990;248(4961):1408-10.
47. Ashraf S, Mapp PI, Walsh DA. Contributions of angiogenesis to inflammation, joint damage, and pain in a rat model of osteoarthritis. Arthritis Rheum 2011;63(9):2700-10.
48. Bonnet CS, Walsh DA. Osteoarthritis, angiogenesis and inflammation. Rheumatology (Oxford) 2005;44(1):7-16.
49. Vanelli R, Costa P, Rossi SM, Benazzo F. Efficacy of intra-articular polynucleotides in the treatment of knee osteoarthritis: a randomized, double-blind clinical trial. Knee Surg Sports Traumatol Arthrosc 2010;18(7):901-7.
50. Kim H, Kang D, Cho Y, Kim JH. Epigenetic Regulation of Chondrocyte Catabolism and Anabolism in Osteoarthritis. Mol Cells 2015;38(8):677-84.
51. Ribatti D, Vacca A, Presta M. The discovery of angiogenic factors: a historical

review. Gen Pharmacol 2000;35(5):227-31.
52. Ribatti D, Nico B, Crivellato E, Roccaro AM, Vacca A. The history of the angiogenic switch concept. Leukemia 2007;21(1):44-52.

Polydeoxyribonucleotide (PDRN) 처리한 연골세포양 세포에서 혈관신생 관련 단백질 발현의 변화

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본 연구에서는 골관절염의 시험관내 모델에서 polydeoxynucleotide (PDRN)의 투여 후 혈관 신생과 관련된 인자에 대한 PDRN의 효과를 조사하고자 하였다.
골관절염의 시험관내 모델을 만들기 위해 인간 연골세포주에 Interleukin (IL)-1β와 phosphate buffered saline (PBS)을 첨가하여 24시간 동안 저산소 상태를 유지하였다. IL-1β군에 24시간 동안 PDRN 처리를 하여 PDRN군을 만들었다. Label-based Human Antibody Array 1000을 통해 IL-1β군과 PDRN군을 비교한 결과, 혈관생성인자인 angiopoietin-2 (ANG-2), platelet-derived growth factor (PDGF)와 혈관 생성 억제 인자인 angiostatin, endostatin에서 유의한 차이를 보였다. 상기 인자들에 대한 추가적 검증 연구를 진행하였다.
정량적 실시간 역전사 중합효소연쇄반응 및 western blot 분석을 통해 PDGF와 ANG-2가 대조군 또는 IL-1β 군에 비해 PDRN 군에서 유의하게 증가함을 확인하였다. 대조적으로, endostatin과 angiostatin은 대조군 또는 IL-1β 군에 비해 PDRN 군에서 유의하게 감소하였다. 다른 군에 비해 PDRN 군에서 시험관내 스크래치의 회복이 유의하게 증가하였다. 더불어, PDRN은 골관절염에 대한 대표적인 이화작용 인자인 MMP13의 발현을 감소시켰으나, 골관절염에 대한 동화작용 인자인 aggrecan의

발현을 증가시켰다.
본 연구의 결과는 PDRN이 골관절염의 시험관 내 모델에서 이화작용의 하향 조절 및 동화 작용의 상향 조절을 통해 혈관 신생 및 상처 치유를 촉진 할 수 있음을 시사한다.

핵심되는 말 : polydeoxyribonucleotide (PDRN), 연골세포양 세포, 염증