Osteoinduction of Human Ligamentum Flavum by Osteoinductive Gene Therapy

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저자 씀

Table of Contents

LIST OF FIGURES

<Abstract>

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Bone morphogenetic protein-2 (BMP-2) was widely known as a pivotal agent for osteoinduction. Clinically and experimentally, BMP-2 proved to be effective in spinal fusion. Since degenerated LF has only been implicated as a pathophysiological significance in spinal stenosis, LF tissue engineering for osteogenesis to achieve spinal fusion has not attempted before. In vitro experiment was performed using human ligamentum flavum (LF), adenovirus lacZ construct, adenovirus BMP-2 construct, and recombinant human BMP-2. The objectives of this study were to demonstrate the feasibility of marker gene to human LF cells and the effect of BMP-2 and Ad/BMP-2 on osteogenic differentiation of human LF cells. Human LF and cancellous bone from ilium were harvested from patients with lumbar spinal stenosis. LF cells and osteoblasts were isolated and cultured. Adenovirus lacZ construct (Ad/lacZ), adenovirus luciferase construct (Ad/luciferase), and adenovirus BMP-2 construct (Ad/BMP-2) were produced. Also human recombinant BMP-2 was synthesized by transfecting human BMP-2a cDNA to Chinese hamster ovary cells. Then LF cell cultures were exposed to various concentrations of BMP-2, Ad/lacZ,

and Ad/BMP-2. Transgene expression of lacZ was assessed by X-gal stain, β-galactosidase assay, Western blot analysis, and Northern blot analysis. Alkaline phosphatase, von Kossa, and Alizarin red-S stains were utilized to confirm osteogenic differentiation and bone nodule formation. Immunocytochemical staining was also performed to detect expression of osteocalcin. Genetically modified human LF tissue with Ad/BMP-2 was implanted into nude mouse to test in vivo osteogenic effect. The LF cell cultures transduced with Ad/lacZ showed extensive expression of X-gal and increased β-galactosidase activity compared to viral (Ad/luciferase) and saline controls. In the LF cultures treated with BMP-2 or Ad/BMP-2, there were robust expression of alkaline phosphatase, bone nodule formations as evidenced by positive von Kossa and Alizarin red-S stains, and a strong expression of osteocalcin. The osteogenic response of LF cells to BMP-2 or Ad/BMP-2 was dose dependent. Human LF cells were susceptible to adenovirus–mediated marker gene transfer, which opens new research arena for genetic modification of LF. In human LF, BMP-2 clearly upregulates expression of osteogenic phenotypes and induces bone nodule formation. Furthermore, genetic modification using osteoinductive gene (BMP-2 cDNA) rendered upregulation of osteogenic phenotype in human LF and de novo osteogenesis in experimental animal. The results of this study support the notion that biologically modified LF i.e., LF treated with BMP-2, LF with adenovirus-mediated BMP-2 cDNA gene transfer can be a substitute for autogenous bone graft in spinal fusion.

Key words: adenovirus, gene therapy, bone morphogenetic protein-2, ligamentum flavum, de novo osteogenesis

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I. INTRODUCTION

Osteogenesis of ligamentum flavum (LF) has been widely known as a pathophysiologic factor in spinal stenosis and other pathological ossification of spinal ligament [1,2]. In histologic studies of human specimen, LF from spinal stenosis showed marked hypertrophy, degeneration of collagen and elastic fiber, calcium crystal deposition, chondroid metaplasia of fibroblast, and ossification [3-5]. In bone morphogenetic pathway, cartilage differentiation, hypertrophy, and cell death are followed by bone formation [6]. Therefore, differentiation of ligament fibroblast toward chondrocyte-like cell in the process of chondroid metaplasia indicates the susceptibility of LF to proper osteogenic stimuli i.e., parathyroid hormone and bone morphogenetic proteins. Moreover, bone morphogenetic protein-2 (BMP-2) and BMP-2 receptor were reported to be present in the ossification of LF [7], suggesting a mechanism of osteogenic differentiation of degenerated LF. In this regard, several

studies successfully demonstrated spontaneous expression of osteogenic phenotype from degenerated LF [8] and also ossification of spinal ligament in response to in vivo and vitro application of BMP-2 [2, 9, 10].

Bone morphogenetic proteins are pleiotropic morphogens and also regulate hematopoiesis, stimulate extracellular matrix synthesis, and influence cell survival maintenance and death [11]. In addition, BMPs demonstrated a powerful osteoinductive property in various conditions in clinical and experimental studies [12 - 14]. Although autogenous bone graft is firmly known as a gold standard to achieve solid spinal fusion, morbidity of bone harvesting procedure alerts spine surgeons to search for alternatives. Hence, it can be hypothesized that degenerated LF and powerful osteoinduction by BMP-2 may provide a mechanism of facilitated osteogenesis. In other words, autogenous LF tissue removed from decompressive spinal surgery, could be modified by exogenous biologic agents and reimplanted into spinal fusion bed as a substitute for autogenous bone graft. This notion also opens new research field regarding tissue engineering or recycling of degenerated LF in various spinal conditions, which need an accelerated bone formation.

Recently the popularity of gene therapy has grown in various musculoskeletal conditions [15] including spinal fusion [16] and intervertebral disc regeneration [17, 18]. Gene therapy is believed to provide an excellent mechanism to upregulate the expression of certain gene and render subsequent biologic events. In that regard, genetically modified LF with osteoinductive gene can be a therapeutic agent and/or biological carrier to facilitate spinal fusion in more efficient and

sustained manner. As far as LF concerns, gene transfer to human LF for any reason was not attempted before.

Accordingly, the objectives of this study are threefold, first, to test the feasibility of gene therapy to LF, second, to demonstrate the effect of BMP-2 on fibroblasts from degenerated LF in terms of osteogenesis, and third, to prove the effect of genetically modified human LF in vivo osteogenesis, and finally to suggest biologically modified LF as a bone graft substitute or biological carrier for facilitating spinal fusion.

II. MATERIALS AND METHODS

All of the experimental protocols were approved by the human subjects Institutional Review Board of the institution.

1. Patient data and tissue acquisition procedures

The LF tissue was obtained from twelve patients (age range: 56 to 68 years) during surgical spinal procedures. All patients had severe spinal stenosis of the lumbar spine. Thickened LF was noted from magnetic resonance imaging preoperatively and also from direct visualization intraoperatively. An attempt was made by the operating surgeons (SHM, HML) to carefully obtain tissue as an en-bloc from the central portion of the LF to minimize tissue damage and optimize harvest of

only ligament proper neither insertion nor origin site of the ligament. Patients' cancellous bone from ilium was also harvested during spinal fusion procedure and only a small portion of cancellous bone was utilized for experiment.

The LF tissue and cancellous bone specimens were washed with Hank's balanced salt solution (HBSS, Gibco-BRL, Grand Island, NY, USA) to remove blood and bodily fluid contaminants, and were then transported in sterile HBSS to the laboratory, less than 20 minutes following surgical removal.

2. Ligamentum flavum cell culture

The LF cells were then isolated from the ligament as described before [8]. Briefly the dissected specimens were minced with a scalpel into pieces of approximately two cubic millimeters in volume. Then LF tissues were digested for 60 minutes at 37° C in 5% CO_2 under gentle agitation in a medium composed of serumless Dulbecco's Modified Eagle's medium (DMEM, Gibco-BRL) containing 250U/ml type 1A collagenase (Sigma, St. Louis, MO, USA) . Collagenase-treated ligament chips were removed after 80 min and washed with serum-containing medium and placed in 25-mm culture flask in DMEM containing 10% FBS, 1% v/v penicillin, streptomycin and nystatin (all antibiotics from Gibco-BRL) in a 5% CO_2 incubator with humidity. Culture medium was changed twice a week.

3. Osteoblast culture

Bone chip was rinsed with PBS and minced to pieces of 1-2mm^3 and digested with medium with 0.1% collagenase type IV (Sigma) and 0.05% trypsin, 136mM NaCl, 2.6mM KCl, 0.36mM NaH2PO4, and 5mM EDTA for 20 minutes at 37° C. Bone chip and the supernatant fraction from third and fourth digestion was collected and washed with DMEM twice and plated in 25-mm culture flask. Primary cultures were sustained for 2 weeks in DMEM/F12 containing 10% FBS, 1% v/v penicillin, streptomycin and nystatin in a 5% CO_2 incubator with humidity.

4. Adenoviral vectors

Adenovirus lacZ construct (Ad/lacZ) and adenovirus luciferase construct (Ad/luciferase) were prepared for this study. Recombinant adenoviral vector originated from replication-deficient type 5 adenovirus lacking the E1 and E3 regions of the genome [19, 20]. The lacZ gene or luciferase gene was cloned into the E1 region under the control of the human cytomegalovirus early promoter. Recombinant virus was grown in transformed human embryonic kidney 293 cells and purified by CsC1 density gradient purification. Titers were determined by optical density at 260nm (OD260) and standard plaque assay [21, 22]. The five doses utilized in this study were: $1.25x10^7$ plaque-forming unit (PFU), giving a multiplicity of infection (MOI) = 25; $2.5x10^7$ PFU, MOI = 50; $3.75x10^7$ PFU, MOI = 75; $5x10^7$ PFU, MOI = 100; $7.5x10^7$

PFU, MOI = 150.

5. Preparation of recombinant human BMP-2

The recombinant human BMP-2 was prepared as described elsewhere [12]. Briefly cDNA for BMP-2A was inserted into a mammalian expression vector pcDNA3.1/hygro (6.7kb). Chinese hamster ovary cells were transduced by pcDNA3.1/hygro/BMP-2A by Lipofectamine PLUS(Gibco-BRL). Transformants were selected with Hygromycin-containing medium(Gibco-BRL). Then supernatant was harvested every 24 hours for 4 days and applied to Heparin-Sepharose (Amersham Pharmacia, Uppsala, Sweden) column. Finally, recombinant protein was concentrated by ultrafiltration with YM10 (Amicon, Bedford, MA, USA) membrane. Purity assay was performed on silver stained gel.

6. In vitro transduction of human LF cells

At confluence, the LF cell cultures were rinsed with PBS three times and exposed to 50 ul of HBSS with various doses of Ad/lacZ. Cultures with Ad/luciferase were utilized as viral control in X-gal stain and ß-galactosidase assay. All cultures were incubated in 5% CO_2 at 37° C with humidity for one hour. Caution must be given to prevent dry up during transduction. Then culture medium (950 ul) was added to each well, and the cells were further incubated in 5% CO_2 at 37° C with humidity.

7. Measurement of lacZ gene expression (X-gal stain and ß-galactosidase assay)

ß-galactosidase gene expression was assessed two days after transduction using the 5-bromo-4-chloro-3-indolyl-ß-galactosidase (X-Gal, Sigma, St. Louis, MO) staining technique. In detail, cells were washed with phosphate buffered saline (PBS, Gibco-BRL) and were fixed with 0.5% glutaraldehyde (Sigma) for 10 minutes followed by two rinses in PBS containing 1mM MgCl2. The cells were finally incubated with X-Gal substrate (1 mg/ml X-Gal, 1 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6/K_3Fe(CN)_6$ in PBS) for four hours at 37° C. LacZ gene expression are presented as percent X-Gal positive cell per total cell number (mean % ± the standard deviation%) in microscopic field (original magnification x100). Ten fields of view are randomly selected for counting X-Gal positive cells % of cell with positive X-Gal stain.

ß-galactosidase assay was performed using ONPG (o-nitrophenyl-b-D-galactopyranoside, Sigma) assay as described. The cells were washed once in PBS with calcium and magnesium and lysed with enough volume of lysis buffer (0.1% Triton X-100, 0.1M Tris (pH8.0). 30ul of the lysate was mixed with an equal volume of 2 x ß-galactosidase substrate (Promega, Madison, WI, USA) and incubated at 37° C for 15-30 min. The ß-galactosidase activity was determined by plate reader at 405nm wavelength.

8. Alkaline phosphatase, von Kossa, and Alizarin red-S staining

For alkaline phosphatase staining, LF cells and osteoblasts were fixed for 10 minutes with 3.7% formaldehyde at room temperature. After washing with PBS, the cells were incubated for 30 minutes with a mixture of 0.1mg/ml of naphthol AS-MX phosphate (Sigma), 0.5% N, N-dimethylformamide, 2mM $MgCl_2$, and 0.6mg/ml of fast blue BB salt (Sigma) in 0.1M Tris-HCl, pH 8.5, at room temperature in dark condition. For von Kossa stain, the LF cells and osteoblasts were fixed with 0.1M sodium cacodylate and then washed. 3% Sliver nitrate was added to the cell cultures in dark room and the cells were incubated for 30 minutes at room temperature in dark condition followed by exposure to light for 1hour. For Alizarin red-S staining, LF cells and osteoblasts were fixed for 2 hours with ice-cold 70% ethanol. After washing with distilled water, the cells stained with 40mM Alizarin red-S, pH4.2, for 10 minutes at room temperature. Stained cell layers were further processed by five rinses with distilled water followed by a 15 minutes washed with PBS. Histochemical stainings were compared qualitatively.

9. Western blot for osteocalcin

Cell lysates obtained in lysis buffer consisted of 0.5% Triton X-100 in 10Mm HEPES, pH 7.4, containing 150Mm NaCl, 0.02% sodium azide, and protease inhibitor mixture(Sigma) were separated on 13% tricine-SDS gel and transblotted

onto membrane. For the osteocalcin detection, the blotted membrane was incubated with 1: 10,000 dilution of rabbit anti-osteocalcin (Chemicon international, Temecula, CA, USA) for 1hour at room temperature after 1hour blocking in 1x TBST with 5% skim milk. The membrane was washed three times with 1x TBST, and incubated with secondary antibody(1:10,000 dilution of goat anti-rabbit IgG, horseradish peroxidase conjugated, Santa Cruz, CA, USA) at room temperature for 45 min. The immuno-reactive bands were visualized using ECL kit(Amersham Pharmacia, Piscataway, NJ, USA) after three times washes with 1xTBST.

10. Reverse transcription-polymerase chain reaction of human osteocalcin and BMP-2

Total cellular RNA was isolated using RNasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. cDNA was synthesized from 1μg total RNA using RT-premix system (Bioneer, South Korea) with the oligo(dT) priming method in a 50μl reaction mixture. 1μl aliquots were amplified in a 20μl reaction mixture that contained 1U Taq DNA polymerase (Bioneer), 250Mm of each dNTP, 10mM Tris-HCl(pH9.0), 40mM KCl, and 1.5mM $MgCl_2$. The same reaction profile was used for all primer sets: an initial denaturation at 94° C for 1 min, followed by 30 cycles of: 94° C for 5 sec; 60-65° C for 5 sec; and 72° C for 30 sec; and an additional 2 min extension step at 72° C after the last cycle. The primers used for human osteocalcin were: cactcctcgccctattggcc(forward) and gccaactcgtcacagtccgg(reverse). The primer

used for human BMP-2 were: aacggacattcggtccttgc(forward) and cgcaactcgaactcgctcag (reverse). PCR products (5 μl) were analysed by electrophoresis in 2 % agarose gels, and detected by staining with ethidium bromide.

11. Immunocytochemical analysis of osteocalcin

Fixed cells were processed by the standard avidin-biotin peroxidase complex procedure (Dako, Carpinteria, CA, USA). Non-specific binding was blocked with 3% BSA in PBS for 30 minutes at room temperature, then cells were incubated with polyclonal anti-osteocalcin antibody (Chemicon, Temecula, CA, USA) (1:3000) for overnight at 4℃. Rabbit immunoglobulins at the same dilutions as the primary antibodies were used as controls. Immunocytochemical stains were compared qualitatively.

12. Experimental design

As a marker gene study, the LF cell cultures were organized into three groups: (1) lacZ gene group, (2) luciferase gene group, (3) saline control group. In Group 1, 50 ul of HBSS with various doses of Ad/lacZ (25, 50, 75, 100, 150 MOI) were added to the culture wells. In Group 2, 50 ul of HBSS containing Ad/luciferase (100 MOI) was added to the culture wells. In Group 3, 50 ul of HBSS without adenovirus was added to the culture wells. Group 2 was used as the viral control for

X-Gal stain and ß-galactosidase assay. The LF cell cultures were maintained for 2 days, then each culture was subjected to X-gal stain and ß-galactosidase assay.

To analyze biologic responses of human LF cells to BMP-2, cultures were exposed to various concentration of BMP-2 (50, 100, 500, 1000, and 2000 ng/ml) for 4 weeks with daily medium change. As a positive control, osteoblast cultures were subjected to same experimental protocol. The LF cells cultures without BMP-2 served as negative control. Each culture underwent alkaline phosphatase, von Kossa, and Alizarin red-S stain to demonstrate expression of osteogenic marker and formation of bone nodule, and immunocytochemistry for osteocalcin expression.

To analyze biologic responses of human LF cells to Ad/BMP-2, cultures were exposed to various concentration of Ad/BMP-2 (50, 100, 150 MOI) and cultured for 4 weeks with daily medium change. The LF cells cultures without Ad/luciferase and saline served as negative control. Each culture underwent alkaline phosphatase, von Kossa, and Alizarin red-S stain to demonstrate expression of osteogenic marker and formation of bone nodule, and immunocytochemistry for osteocalcin expression.

III. RESULTS

1. LacZ gene expression

The control cultures for X-Gal staining assay (Groups 2 and 3) exhibited negative X-Gal staining (Figure 1A). Ad/lacZ at an MOI of 150 rendered

approximately 65% of cultured cells in Group 1 (lacZ gene group) positive X-gal stain without evidence of cytotoxicity (Figure 1E). In semiquantitative analysis using microscope, Ad/lacZ at MOI of 25, 50, 75, 100, and 150 transduced approximately 7 ± 4 %, 21 ± 9 %, 40 ± 8 %, 52 ± 10 %, and 65 ± 8 % of the LF cells, respectively (Figure 1B, C, D, E).

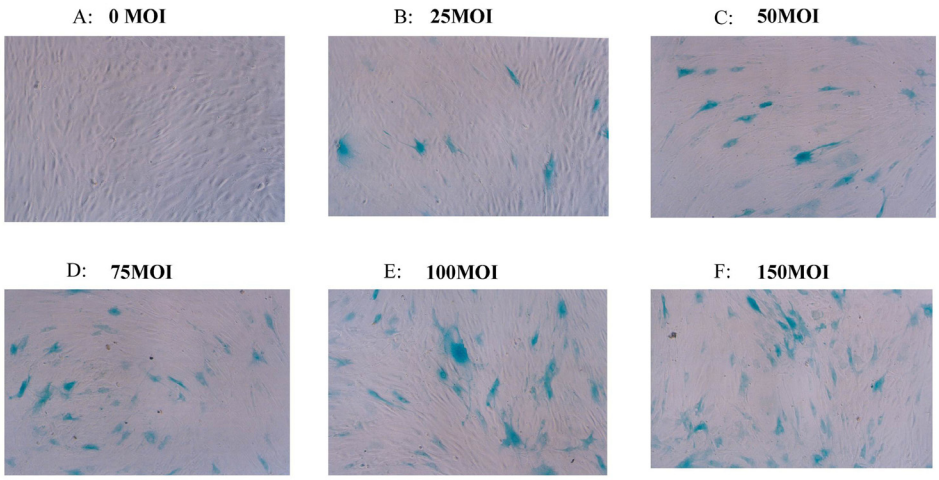

Figure 1. Human ligamentum flavum cell cultures at 2 days after transduction with Ad/lacZ in vitro at different MOIs (0, 25, 50, 75, 100, and 150). The culture with 0 MOI denotes control culture without adenovirus transduction. Blue stains indicate presence of ß-galactosidase, the enzyme encoded for by the adenovirus-lacZ construct. Percentage transduction of human ligamentum flavum cell increased with higher dose of adenovirus. A concentration of 150 MOI resulted in transduction of 65% of cells (original magnification: X 100).

The control cultures exhibited negative expression in ß-galactosidase assay while cultures with Ad/lacZ at an MOI of 25, 50, 75, 100, and 150 demonstrated increased production of ß-galactosidase i.e., 2.23, 6.34, 12.60, 17.03, and 19.92 ug/ml respectively (Figure 2). ß-galactosidase production was dose dependent to given concentration of Ad/lacZ.

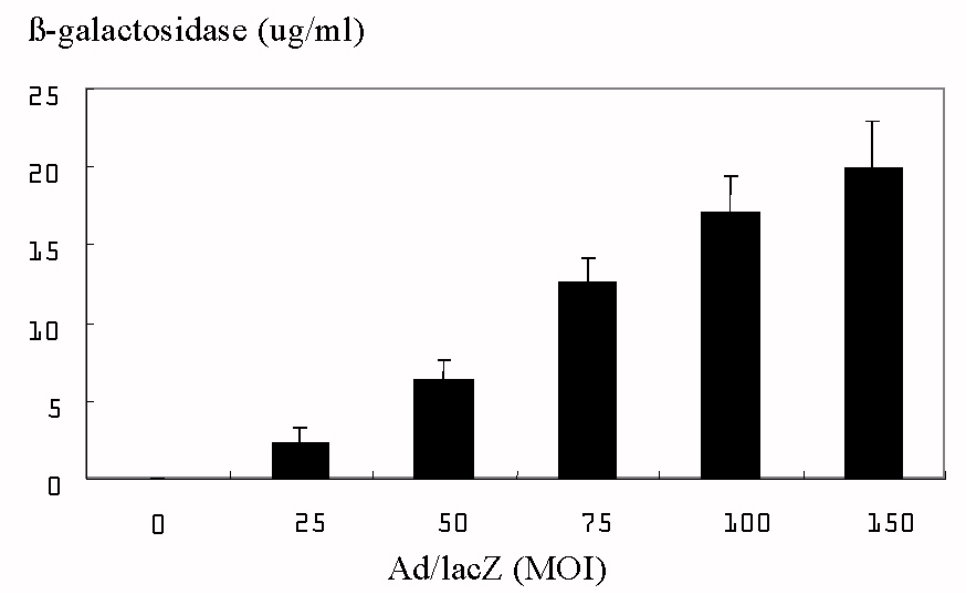

Figure 2. Production of ß-galactosidase in response to Ad/lacZ with an MOI of 25, 50, 75, 100, and 150 analyzed by ß-galactosidase assay. Control culture exhibited negative for ß-galactosidase while cultures with Ad/lacZ showed increased production of ß-galactosidase in dose dependent manner.

2. Validation of biologic activity of BMP-2 in osteoblast culture

To prove biologic activity of synthesized BMP-2, osteoblast cultures treated with BMP-2 were subjected to immunocytochemistry for osteocalcin expression. Osteoblast cultures without BMP-2 showed positive stain for osteocalcin due to inherent osteogenic potential (Figure 3A). Osteoblast cultures with BMP-2 (2000ng/ml) demonstrated an extensive expression of osteocalcin (Figure 3B) indicating BMP-2 was biologically active for osteoinduction.

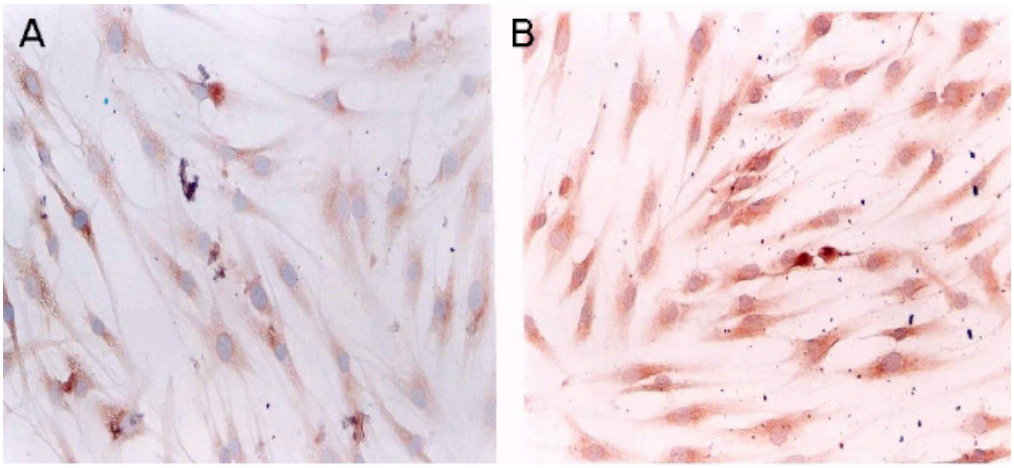

Figure 3. A, Osteoblast culture without BMP-2 showed moderate expression of osteocalcin. **B**, Osteoblast culture with BMP-2 (1500ng/ml) as a positive control demonstrated an extensive expression of osteocalcin in immunocytochemical stain (original magnification X100).

3. Alkaline phosphatase, Alizarin red-S, and von Kossa stain

The LF cell cultures treated with given dose of BMP-2 (50, 100 , 500, 1000, and 1500ng/ml) and extended culture period over 4 weeks demonstrated positive in alkaline phosphatase, Alizarin red-S stain, and von Kossa (Figure 4 A, B, C). Although there was an individual variation in the expression of alkaline phosphatase and intensity of Alizarin red-S stain and von Kossa stain (data not demonstrated), the effect of BMP-2 was uniformly positive and also dose dependent. As a positive control, osteoblast showed strong positivity in alkaline phosphatase stain, Alizarin red-S stain, and von Kossa stain. As a negative control, LF cells without BMP-2 showed negative in all stains.

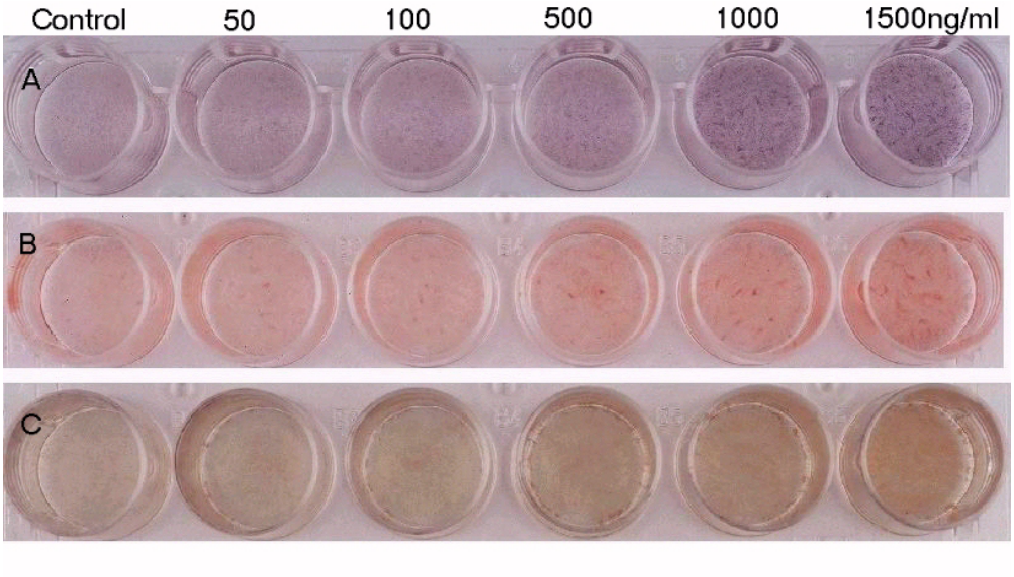

Figure 4. The LF cell cultures in 24 well culture plate, treated with a given dose of BMP-2 (50, 100, 500, 1000, and 1500ng/ml) and extended culture period over 4 weeks demonstrated positive in **A,** alkaline phosphatase stain, **B,** Alizarin red-S stain, and **C,** von Kossa stain in dose dependent manner to BMP-2.

4. Western blot for osteocalcin

The LF culture with Ad/BMP-2 revealed osteocalcin protein expression in dose dependent manner (Figure 5).

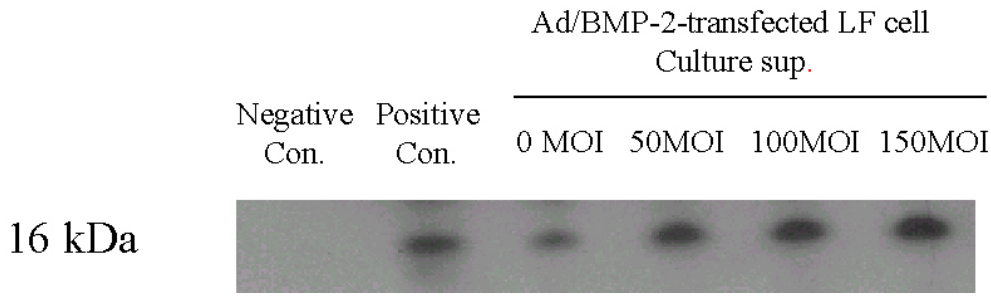

Figure 5. Western blot analysis of secreted osteocalcin protein from in human LF transfected with Ad/BMP-2 (MOI of 50, 100, 150)

5. Reverse transcription-polymerase chain reaction of human osteocalcin and BMP-2

The LF culture with Ad/BMP-2 revealed transgene expression (BMP-2 mRNA) and consequent osteocalcin mRNA expression in dose dependent manner (Figure 6).

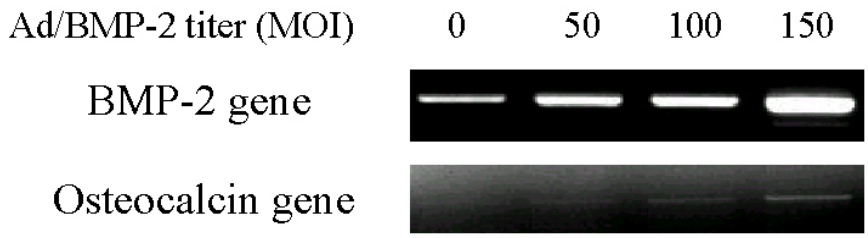

Figure 6. mRNA expression of BMP-2 and osteocalcin in human LF transfected with Ad/BMP-2 (MOI of 50, 100, 150)

6. Immunocytochemistry for osteocalcin expression

The LF cell cultures treated with BMP-2 exhibited positive staining in immunocytochemistry of osteocalcin while LF cells culture without BMP-2 showed negative staining (Figure 7).

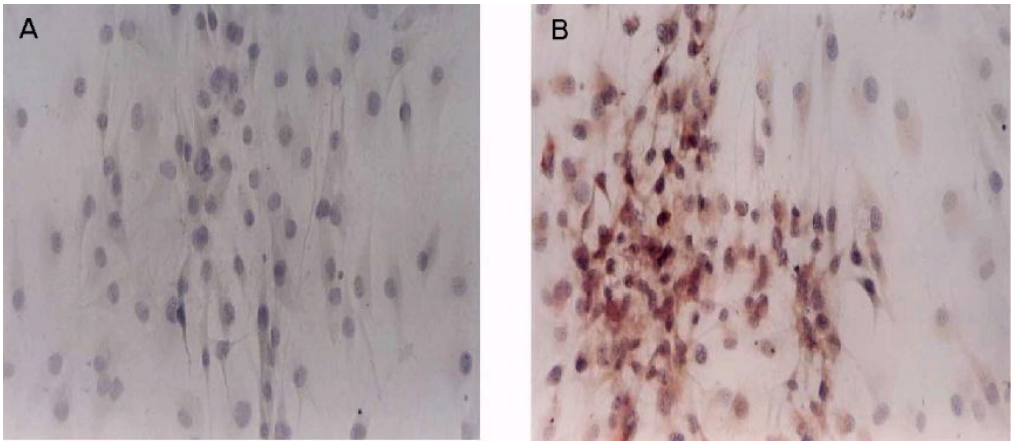

Figure 7. A, LF cell culture without BMP-2 showed negative in immunocytochemistry of osteocalcin, while B, LF cell culture with BMP-2 (1500ng/ml) exhibited extensive staining for osteocalcin (original magnification x100)

7. In vivo ossification of genetically modified human LF in nude mouse

The LF tissue transfected with Ad/BMP-2 exhibited de novo bone formation in nude mouse 4 weeks after implantation (Figure 8).

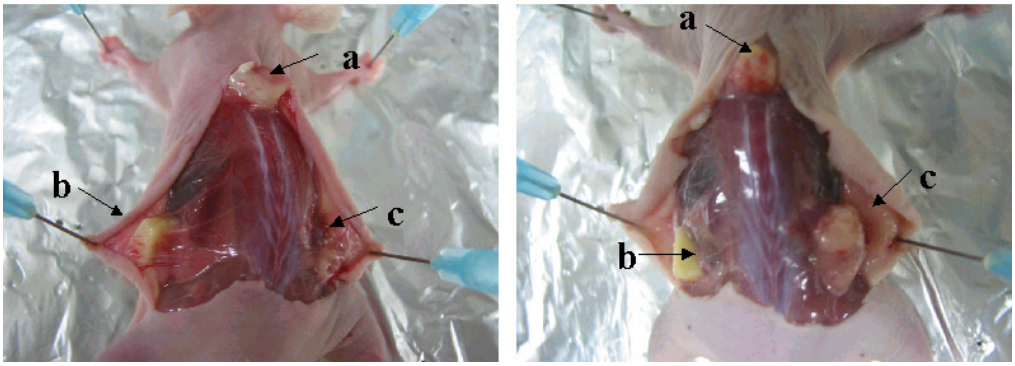

Figure 8. Nude mouse with implantation of genetically modified human LF (BMP-2 cDNA) exhibited de novo ossification 4 weeks after procedure. a: LF only, b: LF with BMP-2, c: LF with Ad/BMP-2.

IV. DISCUSSION

In the surgery of spinal stenosis, stenotic spinal canal can be decompressed by laminectomy, facectectomy, and extensive removal of thickened LF. Destabilized spinal motion segment caused by surgical decompression should be restabilized with spinal fusion with autogenous bone graft. It is well known that the role of autogenous bone graft in spinal fusion surgery is crucial to obtain solid bony fusion. Nevertheless,

morbidity and operation time mandate search for alternatives for autogenous bone graft. In this regard, numerous trials i.e., allogenous bone graft, osteoconductive and/or osteoinductive agent, have been attempted to substitute autogenous bone graft. Several successful results using BMP-2 and osteogenic protein-1 proved to be promising in spinal fusion [13, 14]. Meanwhile, extensive evidences revealed degenerated LF could differentiate into a bone under the influence of appropriate stimuli. Furthermore, degenerated and ossifying LF spontaneously expresses BMP and transforming growth factor (TGF) receptors [7] and TGF-β1[23], which support the notion that degenerated LF has high sensitivity to exogenous and endogenous osteogenic stimuli. Among those candidates for osteoinduction, BMPs seem to be most powerful agent inducing de novo osteogenesis from ligament fibroblasts [2, 9, 10]. Accordingly, the authors were interested in recycling of removed LF during spinal surgery as a substitute for autogenous bone graft avoiding morbidity related with bone harvesting procedure. Removed LF can be biologically modified with exogenous BMP-2 or osteoinductive gene transfer and then reimplanted into spinal fusion bed as a bone graft substitute or a biological carrier to induce spinal fusion. Hence, the authors attempted marker gene transfer to human LF cells to test the feasibility of gene therapy in LF tissue engineering and then apply BMP-2 to cultured human LF cells to prove osteogenic effect of BMP-2 in cytochemical and immunocytochemical stains.

With the results of this study, we clearly demonstrated that human LF cells were susceptible to adenovirus–mediated marker gene transfer, enabling genetic modification of LF with gene(s) for osteoinduction. Also our data confirmed that

BMP-2 upregulated osteogenic phenotypes of human LF cells, as evidenced by positive stains in alkaline phosphatase, von Kossa, Alizarin red-S, and immunocytochemistry for osteocalcin. Furthermore genetic modification of human LF with osteoinductive gene (BMP-2 cDNA) rendered powerful transgene expression and the upregulation of osteogeneic phenotype in vitro and in vivo. Taken together all these findings support the notion that feasibility of biologically modified LF i.e. LF treated with BMP-2, LF with adenovirus-mediated BMP-2 cDNA transfer as an alternative approach for autogenous bone graft.

The current study has unique differences from other previous studies on BMPs and spinal ligaments [2, 7, 9, 10]. First, we attempted marker gene transfer to human LF cells. Efficient marker gene transduction and therapeutic gene as shown on this study enables us to conduct further experiments of in vivo therapeutic gene transfer to LF to facilitate osteogenesis in spinal fusion. Second, this study dealt degenerated LF cells and proved osteogenic effect of BMP-2 in cellular level and histochemical stain, which rendered clinical implication of biologically modified LF cell therapy in spinal fusion. Third, this study has specific aim to utilize osteogenic potential of degenerated LF to achieve spinal fusion without emphasizing an etiological significance in ossification of spinal ligaments, suggesting degenerated LF as a material for tissue engineering and recycling.

V. CONCLUSION

In conclusion, degenerated LF cells are susceptible to adenovirus-mediated marker gene transfer and also LF with BMP-2 or Ad/BMP-2 has an osteogenic potential, which has important clinical implication in spinal fusion as a substitute for autogenous bone graft.

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국문요약

골형성 유전자 전달에 의한 척추 황색 인대에 골화 유도

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Bone morphogenetic protein-2 (BMP-2)는 골형성에서 중요한 역할을 하고 있다. 임상적으로 혹은 실험적으로 BMP-2 는 척추 골유합에서 그 효용성이 증명되었다. 퇴행, 비후된 척추 황색인대는 주로 척추관 협착증의 병리기전으로만 알려졌으므로 골형성을 유도하는 조직공학적 접근은 시도된 적이 없다. 그리하여 본 실험적 연구는 퇴행, 비후된 인간의 척추황색인대와 아데노바이러스 표식 유전자전달체 및 아데노바이러스 BMP-2 유전자 전달체 그리고 유전공학적으로 제조된 BMP-2 를 이용하였다. 본 연구의 목적은 인간 척추황색인대의 표식유전자 전달 가능성과, BMP-2 와 BMP-2 유전자 전달에 의한 척추황색인대의 골화 현상을 규명하기위함이다. 퇴행, 비후된 인간의 척추황색인대와 장골능에서 채취된 자가골을 이용하였으며 각각에서 척추황색인대세포와 조골세포가 분리배양되었다. 아데노바이러스 표식 유전자전달체 (Ad/luciferase, Ad/lacZ) 및 아데노바이러스 BMP-2 유전자 전달체 (Ad/BMP-2)가 제작되었으며 유전공학적으로 인간의 BMP-2 cDNA 를

Chinese hamster ovary 세포에 전이시킴으로서 재조합된 BMP-2 를 생산하였다. 인간의 척추황색인대 세포를 다양한 농도의 Ad/lacZ, Ad/luciferase, Ad/BMP-2, BMP-2 에 노출시켰고 전달유전자 발현검사로 X-gal 염색, b-galactosidae 염색, Western blot, Northern Blot 을 시행하였고 골형성능의 증명을 위해 alkaline phosphatase 염색, Von Kossa 염색, Alizarin Red-S 염색, 면역조직학염색을 시행하였다. 생체내 효과를 알기위해 유전적으로 조작된 인간 척추황색인대 조직을 nude mouse 에 이식하였다. 표식유전자가 전달된 황색인대 세포에서는 농도의존적으로 표식유전자가 발현되었으며 Ad/BMP-2 와 BMP-2 를 투여한 군에서도 역시 농도의존적으로 BMP-2 mRNA 가 발현하였고 이에 따라 Osteocalcin mRNA 도 발현증가하였다. 더구나 골형성 염색에서도 농도의존적으로 양성반응을 보였다. 이상의 연구 결과로 인간의 척추황색인대는 유전적 조작이 용이 하였으며 이는 새로운 연구 영역을 개척했다고 볼 수 있다. 그리고 BMP-2, Ad/BMP-2 는 명백히 척추황색인대의 골형성을 촉진시켰다. 결론적으로 BMP-2 혹은 BMP-2 유전자 치료는 척추 황색인대 골화를 촉진하며 이는 척추 유합술에서 자가골이식을 대체할 수 있는 가능성을 제시한다.

핵심되는 말: 아데노바이러스, 유전자 치료, bone morphogenetic protein-2, 황색인대, 신생골 형성.