Pro-angiogenic and osteogenic effects of adipose tissue-derived pericytes synergistically enhanced by Nel-like protein-1

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Research

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Abstract

Background

One of important objectives of regeneration of vascularized tissues is to develop agents for osteonecrosis. We aimed to determine pro-angiogenic and osteogenic efficacies of adipose tissue-derived (AD) pericytes combined with Nel-like protein-1 (NELL-1) to investigate their therapeutic effects for osteonecrosis.

Methods

The experimental groups were configured to evaluate additional effects of NELL-1 on pericytes (i.e., Control, the group treated with only NELL-1, the group treated with only pericytes, and the group treated with pericytes and NELL-1). Tube formation and cell migration were assessed to determine pro-angiogenic efficacy. In vivo vessel formation was evaluated using the chorioallantoic membrane assay. A mouse model with a 2.5-mm necrotic bone fragment at the femoral shaft was used as a substitute for osteonecrosis in humans. Bone formation was assessed radiographically (plain radiographs, three-dimensional images, and quantitative analyses). Histomorphometric analyses were also performed. To identify factors related to additional effects of NELL-1, microarray, qRT-PCR analysis, and western blot were performed.

Results

The results for pro-angiogenic efficacy indicated there were synergistic effects of pericytes and NELL-1 on tube formation, cell migration, and vessel formation. For the results for osteogenic efficacy, the mouse models of osteonecrosis treated with pericytes and NELL-1 revealed the greatest bone formation in radiographic images and quantitative analyses, compared with other settings. The corresponding models treated with pericytes and NELL-1 revealed robust bone and vessel formation in the histomorphometric analyses. Using additional arrays, we found an association of FGF2 between the effects of NELL-1.

Conclusion

Combinational therapy using AD pericytes and NELL-1 has potential as a novel treatment for osteonecrosis.

Introduction

Angiogenesis is a vital process for normal tissue development and supports skeletal growth. It is also associated with a variety of pathological conditions [1, 2]. Pathological angiogenesis is a hallmark of various ischemic conditions [3]. Therefore, restoring vascular homeostasis and therapeutic angiogenesis...
have great potential for treatment of ischemic conditions. Pursuit of a safe and efficacious approach to restore angiogenesis is a field of ongoing research [2].

Avascular necrosis is defined as cellular death of bone caused by interruption of normal blood flow. It most frequently affects the femoral head (i.e., osteonecrosis of the femoral head, ONFH). The affected limb becomes progressively more painful and debilitated. To overcome limitations of current treatments [4], it is highly desirable to develop agents to enhance angiogenesis and bone formation.

Pericytes surround endothelial cells of micro-vessels. They have critical roles in regulation of endothelial cell function and angiogenesis [5–8]. During angiogenesis, pericytes participate in recruitment, extracellular matrix modulation, paracrine signaling, and direct interactions with endothelial cells [9]. Study results indicate that pericytes participate through direct cell contact and communication. Their main functions include angiogenesis stimulation, regulation of blood vessel diameter [9], and maintenance of vascular permeability and integrity [10]. Because of their effects on endothelial cells, the study of pericytes is a research area of increasing interest that includes evaluation as potential targets of pro-angiogenic therapies [11]. While their presence in the microcirculation has long been documented, their functional roles and importance have been largely under-investigated. In addition to pericytes, Nel-like protein-1 (NELL-1) is a potential additive to enhance angiogenesis and bone formation [12]. NELL-1 has robust bone formation effects in the axial skeleton and the extremities in in vivo settings [13–18].

We focused on the synergistic effects of pericytes combined with NELL-1. Our examination of the potential for this combination as a novel therapeutic agent for osteonecrosis included use of a mouse model with a necrotic bone fragment.

**Methods**

*Human stromal vascular fraction (hSVF) isolation from human adipose tissue and purification of human pericytes from hSVF*

Human adipose tissue was obtained from eight patients who underwent total knee arthroplasty due to degenerative osteoarthritis (Supplemental Table A). The adipose tissue samples were stored (4°C) until they were processed. All samples were processed within 48 h after collection. Human stromal vascular fraction (hSVF) was prepared by digesting the adipose tissue using collagenase digestion, as previously described [19]. The adipose tissue was briefly washed in an equal volume of phosphate buffered saline (PBS). Collagenase digestion was performed using Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 3.5% bovine serum albumin (Sigma-Aldrich) and 1 mg/ml collagenase type II for 70 min under agitation at 37°C. The filtered solution was centrifuged to separate and exclude adipocytes. The processed hSVF was suspended in red cell lysis buffer (155 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA) and incubated for 10 min at room temperature. The hSVF filtrate was immediately processed for human pericyte purification.
A fluorescence-activated cell sorter (FACS) was used to purify human pericytes from isolated hSVF, as previously described [20, 21]. The isolated hSVF was centrifuged and the resulting pellet was incubated (4°C for 15 min in the dark) with conjugated antibodies (anti-CD34-phycocerythrin (Dako), anti-CD45-allophycocyanin (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and anti-CD146-fluorescein isothiocyanate (AbD Serotec)). The hSVF pellet was then resuspended in PBS and 4’, 6-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA, USA) and was filtered through a 70 µm cell filter for removal of nonviable cells. The solution was processed on a FACS Aria cell sorter (BD Biosciences, San Jose, CA, USA) to isolate populations of cells that constituted human pluripotent stem cells, based on cell surface markers: pericytes (CD146+, CD34-, CD45-).

**Osteogenic and adipogenic differentiation of human pericytes**

The human pericytes were cultured separately in the PBS control and NELL-1 solutions to determine the differentiation potential of human pericytes and to test the biologic activity of NELL-1 protein during osteogenesis and adipogenesis. Osteogenic differentiation of the pericytes occurred over a period of 15 d. The cells were added to 24-well plates (3 x 10⁴ cells/well density) with DMEM + 10% fetal bovine serum (FBS). Within 24 h, osteogenic differentiation of the cells was induced in the PBS control and NELL-1 (800 ng/ml) treatments using osteogenic differentiation medium (DMEM + 10% FBS + 50 µg/ml ascorbic acid, and 3 mM β-glycerophosphate). The medium was changed every 3 d. Alizarin Red staining was used to assess osteogenic differentiation.

For adipogenic differentiation, human pericytes were added to 24-well plates (5 x 10⁴ cells/well density) with DMEM + 10% FBS. In 24 h, cells were induced to adipogenic differentiation in the PBS control and NELL-1 (800 ng/ml) treatments using adipogenic differentiation medium (Human MesenCult™ Adipogenic Differentiation Medium; STEMCELL TECHNOLOGIES, Catalog #05412). Adipogenic differentiation was performed over 12 d. The medium was changed every 3 d. Adipogenic differentiation was assessed at 12 d using Oil Red O staining.

**Endothelial cell culture**

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and cultured in endothelial growth medium (Lonza, Basel, Switzerland). The cells were used between passages 4 and 7 [22].

**Viability test**

To determine the dose of NELL-1 protein, we performed a viability test after adding various doses of NELL-1. Two thousand pericytes were added to 100 µl DMEM in each well of two 96-well plates. The cells were incubated in DMEM with 100 ng/ml or 800 ng/ml NELL-1 for 72 h. Control cells were incubated with only DMEM. A water-soluble tetrazolium salt (WST) assay (Cell Counting Kit-8; Dojindo, Kumamoto, Japan) was used to measure cell proliferation. Then, WST (10 µl) was added to each well and the cultures were incubated for an additional 2 h at 5% CO₂ and 37°C before evaluation using
spectrophotometry. Conversion of WST to formazan was measured at 450 nm. The results were normalized and were presented as percentage of the viable cells in the control group [23, 24].

**Tube formation assay**

A capillary tube formation assay (Matrigel, Corning, Corning, NY, USA) was used to assess the effects of human pericytes and NELL-1 on endothelial cell morphogenesis. Briefly, $4 \times 10^4$ cells per well were added to Matrigel-pre-coated 96-well plates and treated with pericytes ($0.2 \times 10^4$ cells/well) or NELL-1 (800 ng/ml), or both. Suramin (40 mM; Sigma-Aldrich) was included as a negative control. After 12 h, cell changes were recorded using a microscope (Nikon ECLIPSE Ti2) and analyzed using the Image J software program. This program provided automated quantitative measurements of tube characteristics (e.g., number of connected tubes, tube area, and angiogenic index).

**Wound migration assay**

HUVECs were added at a density of $3 \times 10^3$ cells per well in the ibidi Culture-Insert 2 Well in a µ-Plate 24 Well plate and allowed to grow into a confluent monolayer overnight. Fresh medium containing indicated concentrations of human pericytes and NELL-1 was added. The Culture-Insert 2 Well was then removed with sterile tweezers. After 24 h, the cells were photographed using a JuLI™ Stage Cell History Recorder (NanoEntek, Guro-gu, Seoul, Korea). Migration rate was calculated using the JuLI™ Stage. Three measurements of three independent wounds were taken for each monolayer sample.

**Chick chorioallantoic membrane assay**

The chick chorioallantoic membrane (CAM) assay was used to assess the effect of NELL-1 on ex vivo angiogenesis. Briefly, fertile chicken eggs were candled on embryonic day 3. A small opening was made at the top of the live eggs and a sterilized ThermaNox™ Coverslip (Nunc™) saturated with either PBS or NELL-1 was placed on the CAM. The holes were then sealed with cellophane tape. The eggs were photographed after a 72-h incubation. Image J software was used to quantify blood vessel density; the results were presented using bar diagrams [24].

**Animals**

Twenty-four 8-week-old male NOD SCID mice (CHA Institute Animal Experimentation, Pangyo, Seongnam, Korea) were used for the study to prevent immune reactions to implants containing human cells. Each mouse was housed alone in a pathogen-free ventilated cage, fed a standard rodent chow diet, provided tap water *ad libitum*, and experienced 12-h light and dark cycles. The mice were cared for following the Chancellor's Animal Research Committee for Protection of Research Subjects guidelines at the CHA medical university (IACUC190082).

**Implant preparation and grouping**

Recombinant human NELL-1 was purchased from Bone Biologics Inc. (UCLA, CA, USA). An absorbable collagen sponge (ACS) of defined dimensions ($0.5 \text{ cm} \times 0.5 \text{ cm} \times 1.0 \text{ cm}$, Lyoplant, 1066102; Aesculap AG, Tuttlingen, Germany) was used. Because intramuscular implantation of ACS alone has no known
bone-forming effects, this carrier was chosen for its nonosteoinductive characteristics [25]. Defined concentrations of viable cells and NELL-1 in PBS suspension (20 ul) from the hSVF were applied and allowed to saturate the ACS. The cell and protein scaffold suspensions were kept on ice until implantation. The four treatment groups used were (a) control with PBS (b) high concentration (800 ng/ml) NELL-1, (c) Pericytes, and (d) Pericytes loaded with a high concentration (800 ng/ml) of NELL-1. 

**Surgical procedure for mouse models of osteonecrosis**

We used a mouse model with a necrotic bone fragment to replace osteonecrosis in humans. A total of 30 mice (6 for control, 8 for NELL-group, 8 for Pericyte group, and 8 for Pericytes + NELL-1 group) were used for the experiment. All mice were prepared at the age of 3 months. Anesthesia was initiated in a 5% gaseous isoflurane-filled holding chamber and maintained with 3–4% gaseous isoflurane through a nose cone. Before making an incision, the thigh of the mouse was shaved, and the skin prepared using an alcohol and betadine solution. Sterile ophthalmic lubricant ointment was applied to each eye and a buprenorphine injection (0.05 mg/kg) was given via the subcutaneous route.

All surgical procedures were performed by the senior author, an experienced orthopedic surgeon. The femoral bone was exposed using an incision made on the anterolateral aspect of the thigh. A PEEK plate was located on the anterior femur. The most proximal hole of the plate was gently drilled using a 0.3 mm drill bit and the first screw was inserted. Additional distal screws were inserted in a similar fashion. The 0.22 mm Gigli saw wire was closely placed around the bone in a medio-lateral orientation and inserted in the slots of the customized jig to create a 2.5-mm bone defect. The jig was inserted on the stem of the two last screws and applied above the plate. Next, a 2.5-mm long mid-diaphyseal femoral ostectomy was performed using the Gigli saw while applying a constant steady tension. Care was taken to avoid excess movement to obtain a straight bone cut. After the ostectomy, the Gigli saw was removed and the saw wire was cut close to the bone on one side. The jig and remaining stems of the screw were removed. The bone fragment from the ostectomy was removed and immersed in liquid nitrogen for 5 min to induce necrosis. The ACS treated with the assigned material based on treatment group (± Pericytes ± NELL-1) was put below the femoral shaft and the necrotic bone fragment was taken back to the original site. It was then wrapped using the ACS. Finally, the ACS was sutured to prevent displacement of the necrotic bone fragment and the wound was closed (Fig. 1).

**In vivo plain radiograph**

In vivo bone regeneration was assessed using plain radiography images (GIX-I, Genoray, Seongnam, Korea; Ultra Light Portable X-ray, Nanoray, Deagu, Korea) of the femur under the conditions of 70 kV/20 mA, 0.06 sec; 20 lines per mm spatial resolution. Standard lateral digital radiographs of the femur were taken immediately after surgery and 4 weeks later under volatile anesthesia [26].

**Ex vivo micro computed tomography**

The mice were euthanized using CO₂ asphyxiation 4 weeks after surgery. High-resolution micro computed tomography (CT) scanning of each sample was performed (Bruker microCT Skyscan 1173). Each piece
of femur bone was in a polyethylene tube filled with alcohol (75 volume percent) during scanning. The radiographic projections were acquired at 130 kV and 60 uA with a fixed exposure time of 500 ms, an A1 1.0 mm filter, and a 6.04 um pixel size. Four frames were averaged for each rotation increment of 0.9. Three-dimensional images with a voxel average size of 13 mm were reconstructed using a Hamming-filtered back-projection, and the manufacturer’s reconstruction software (NRecon; Skyscan, Aartselaar, Belgium). Bone mineral density (BMD) of the femur was measured using the Bruker microCT with a phantom. The analyses were performed in the same manner for each mouse, with a volume of interest corresponding to the respective defect. The number of united cortices in two orthogonal reconstructed views was recorded for the qualitative analysis. Bone union was defined as union of four out of four cortices. Resident software (CTAn; Skyscan, Aartselaar, Belgium) was used to obtain the BV/TV and BMD for quantitative analysis of bone formation within a region of interest. A lower gray threshold of 45 grayscale indices (attenuation coefficient of 0.035) and an upper gray threshold of 240 grayscale indices (attenuation coefficient of 0.186) were used for each mouse [27].

**Histology and histomorphometric analysis**

The animals were euthanized 4 weeks after surgery. Histologic specimens were fixed in 4% paraformaldehyde at 4°C for 1 d, followed by decalcification (Calci-Clear™ Rapid Decalcifying Solution, HS-105; National Diagnostics) for 3 h at room temperature with gentle mechanical stirring. The specimens were then dehydrated and embedded in paraffin. The tissue blocks were sectioned to 3-mm thicknesses along longitudinal planes (Leica RM2235 microtome; Leica Microsystems GmbH, Wetzlar, Germany). All sections were stained with hematoxylin and eosin and alcian blue stain.

**Microarray**

Microarrays were used to measure expression levels of genes related to NELL-1 activity. NELL-1 treated Pericyte samples were used. Briefly, total RNA was isolated using RNeasy columns (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol. The RNA samples were quantified after processing with DNase digestion and clean-up procedures. An Ambion Illumina RNA amplification kit (Ambion, Austin, TX, USA) was used to amplify and purify the total RNA. Total RNA (550 ng) from each sample was converted to double-strand cDNA. Using a T7 oligo (dT) primer, amplified RNA (cRNA) was generated from the double-stranded cDNA template using an *in vitro* transcription reaction and purified using the Affymetrix sample clean-up module. An ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA) was used to quantify cDNA after purification. Uracil-DNA glycosylase and apurinic/apyrimidinic endonuclease and restriction endonucleases were used to fragment the cDNA. It was end-labeled using a terminal transferase reaction incorporating a biotinylated dideoxynucleotide. Fragmented end-labeled cDNA was hybridized to the GeneChip Human Gene 2.0 ST arrays manual (Affymetrix, Santa Clara, CA, USA). After hybridization, the chips were stained and washed (GeneChip Fluidics Station 450; Affymetrix) and scanned (GeneChip Array scanner 3000 G7; Affymetrix) by Macrogen Ltd. (Seoul, South Korea). Affymetrix® GeneChip™ Command Console software was used to compute signal values.

**RNA isolation and qRT-PCR analysis**
Total RNA was extracted from cells using Trizol reagent (Invitrogen), according to the manufacturers' instruction. One micrograms of total RNA were used to determine the expression of mRNAs using AMPIGENE® qPCR Green Mix (Enzo Biochem, Inc.) and iCycler real-time PCR detection system (Bio-Rad, CA) according to the manufacturers' instruction. The sequences of the primers were as follows: FGF2, 5′-AGAAGAGCGACCCTCACATCA-3′ (forward) and 5′-CGGTTAGCACACACTCCTTTG-3′ (reverse); IL-6, 5′-ACTCACCTCTTCAGAAGCGATT-3′ (forward) and 5′-CCATTTTTGGGAGGTTCA-3′ (reverse); TGFβ2, 5′- CAGCACACTCGATGGAGCA-3′ (forward) and 5′-CCTCGGGCTCAGGATTTGCT-3′ (reverse); VEGFA, 5′-AGGGGACGGCTCAGGATTAAGT-3′ (forward) and 5′-AGGGTCTCGATTGGATGGA-3′ (reverse); β-actin, 5′-ACCGAGCGGCTACAG-3′ (forward) and 5′-CTTAATGTCACGCACGATTTCC-3′. β-actin was used for normalization of mRNA.

Western blot

Protein extraction buffer (Pro-Prep, iNtRON Biotechnology, Gyeonggi-do, Korea) was used to lyse cells for western blot. After centrifugation (4°C, 13,000 rpm for 15 min), protein content of lysed cells was assessed (Bradford assay). Equal total protein amounts were run on 12% SDS polyacrylamide gels and transferred to a nitrocellulose membrane. Membranes were blocked using 5% non-fat milk powder at room temperature and then incubated with primary antibodies in tris-buffered saline-tween 20 (TBS-T) overnight at 4°C. The primary antibodies FGF2 protein 1:1000 (Santa Cruz Biotechnology) were used in the assay. The membranes were then washed in TBS-T and incubated with 1:5000 goat anti-mouse IgG (Santa Cruz Biotechnology) secondary antibodies for 1 h at room temperature. An enhanced luminol-based chemiluminescence detection kit (Bio-Rad Laboratories, Hercules, CA, USA) was used to visualize the resulting bands. Protein quantification was performed using densitometric digital analysis of the protein bands (ChemiDoc™ XRS + with Image Lab™ Software ver. 6.0; Bio-Rad Laboratories). The membrane was re-probed with GAPDH (Santa Cruz Biotechnology) to confirm equal loading. After each sample was run at least three times on western blot analysis, densitometry analysis was performed for each of the three bands.

Statistical analysis

All numerical results were expressed as mean ± standard deviation (SD) values. Analysis of variance was used for comparisons between treatment groups; LSD was used as a post-hoc test or Student’s t-tests were performed. The statistical software IBM SPSS ver. 23.0 (IBM Corp., USA) was used for the analysis, and the significance level was set at P < 0.05.

Results

Pericytes and NELL-1 promote angiogenesis and migration in vitro and in vivo

To assess whether Pericytes and NELL-1 affected angiogenesis of HUVECs in vitro, the HUVECs were treated with only pericytes (Pericytes group, PG), or Pericytes and NELL-1 (Pericytes + NELL-1 group,
PNG). The results indicated that the PG had significantly greater numbers of tubes and branch points than the control group (HUVECs only) (Fig. 2). The presence of pericytes increased tube formation, and the increase intensified with addition of NELL-1. When NELL-1 was added, the numbers of tubes and branch points were greater than in the control group. In particular, the number of branch points in the PNG was greater than the PG (Fig. 2A). Addition of NELL-1 without pericytes to HUVECs did not increase tube formation, compared with the control group (Supplemental Fig. A). Because endothelial cell migration has an important role in angiogenesis [9], the effect of NELL-1 on wound closure was studied using a HUVEC scratch assay. Similarly, we found that compared with the PG, the PNG had markedly induced HUVEC migration (Fig. 2B).

We also performed a CAM assay to examine angiogenic effects of NELL-1 in vivo. The assay results indicated that during embryonic vascularization, the blood vessels in the NELL-1-treated group formed vessel densities, total numbers of branching points, and total numbers of segments that were significantly greater compared with the control group. These results suggested that NELL-1 induced angiogenesis in vivo (Fig. 3).

Pericytes and NELL-1 promote bone formation in vivo.

To investigate the effectiveness of NELL-1 in vivo, we established an osteonecrosis model using mouse and implanted femurs with only Pericytes (PG), or Pericytes and NELL-1 (PNG) (Fig. 4A). The plain radiographs and three-dimensional images revealed that the femurs treated with Pericytes and NELL-1 (PNG) had the greatest bone formation, compared with other treatments (Fig. 4B). The microCT results indicated that greater gains in BV/TV and BMD occurred in the PNG than in the PG (Fig. 4C). Next, we examined the extent of osteocytic death through quantification of empty lacunae in each group. As expected, we observed a synergistic decrease in the fraction of empty lacunae in PNG. Also, Immunohistochemical analysis revealed that CD31 and VEGF expression was higher in the PNG than in the PG. This result indicated that angiogenesis was induced in these groups (Fig. 5).

NELL-1 promotes the FGF2 signaling pathway

To identify the pathway of pro-angiogenic and osteogenic effects enhanced by NELL-1, we performed heatmap analysis by using the microarray data to compare the control versus Nell-1 treated Pericyte. Among the genes upregulated by NELL-1, four candidate genes (FGF2, IL6, TGFB2, and VEGFA) were commonly regulated (Fig. 6A, B). To understand the role of NELL-1, we also examined whether NELL-1 modulated the FGF2 pathway. NELL-1 increased the m protein levels of FGF2 and the direct downstream genes of p-PLCγ, p-AKT, p-ERK and p-eNOS. These results indicated that NELL-1 induced the pro-angiogenic and osteogenic pathway (Fig. 6C).

Discussion

The principle finding of this study was that both angiogenesis and osteogenesis enhanced by pericytes were more intensified in the presence of NELL-1. We found a synergistic effect of pericytes combined with
NELL-1 in angiogenesis and osteogenesis [12], which are critically coupled processes for bone regeneration [28]. Vascular control is required for bone health. Blood vessels are key bone homeostasis regulators; they serve as structural templates and provide nutrients and minerals [29]. Changes in vascular growth can negatively affect physiological bone healing and result in osteonecrosis [30]. Development of animal models to test novel cell-based therapeutic options that prevent or delay osteonecrosis progression have been the focus of basic science and clinical research in this field. Cell-based therapies have been performed with surgical treatment and provide significant improvements in survivorship over time, compared with surgical treatment alone [4]. Given the growing evidence and non-invasiveness of regenerative therapies, more effort and related studies to support the efficacy of novel agents are necessary. To the best of our knowledge, this study is the first to use a mouse model with a necrotic bone fragment that mimics osteonecrosis in humans and reveal the potential of combined application of stem cells (pericytes) and an osteoinductive protein (NELL-1) in an in vivo setting. We hypothesized that pericytes with NELL-1 can reverse osteonecrosis lesions. We found potency after local delivery, which was also supported by radiographic images and histologic findings. These findings were the main achievements and strengths of this study.

Large animals, such as canine [31], porcine [32], and sheep [33] have been used to induce osteonecrosis. However, their use in studies that require a series of experiments can be limited due to high costs. On the other hand, rodents, such as a mouse, can be used with relatively lower costs and the feasibility of having a large sample size. For surgical procedures, the mouse models are somewhat small to underwent surgical procedures. An ideal animal model should not only be cost-effective, but also be large enough to investigate various procedures. Considering these points, we selected the femoral shaft instead of the proximal or distal end of the femur. We believe that our mouse model may provide the benefit of low cost and a large enough skeletal size for surgical procedures.

The regenerative potential of pericytes depends on tissue origin. AD pericytes have efficient osteogenic differentiation [34]. Therefore, we isolated and used AD pericytes with potency for osteogenic differentiation. To evaluate additional effects of NELL-1 on pericytes, determination of NELL-1 concentration was needed before further analyses. First, the concentration of NELL-1 was set based on Zhang et al.’s study [35], which verified the effect of NELL-1 on bone formation. In addition, effects of NELL-1 on proliferation of pericytes in live dead assays and WST-1 assays were assessed. Proliferation of pericytes without NELL-1 and of those treated with 100 ng/ml NELL-1 or 800 ng/ml NELL-1 was compared. We found they were not significantly different (Supplemental Fig. B.1). These results indicated that the direct effects of NELL-1 on proliferation of pericytes were not significant and that the proliferation of pericytes was comparable for NELL-1 concentrations between 100 ng/ml and 800 ng/ml. We assumed that concentrations between 100 and 800 ng/ml were feasible options for subsequent assays; we selected the highest concentration (800 ng/ml) to maximize the effects as we first set up. Using a NELL-1 concentration of 800 ng/ml, enhanced osteogenic differentiation was assessed (Supplemental Fig. B.2). On the other hand, NELL-1 concentrations between 300 and 600 ng/ml were used in Askarinam et al.’s study [12].
Effects of the pericyte-endothelial cell ratio on angiogenic potential have been examined [34]. Based on their results, Herrmann et al. used 10% pericytes and 90% HUVECs [34]. We used a 1:20 ratio of pericytes:HUVECs that was chosen based on an optimal vascular tube growth and stability protocol (VascuNet™ Pericyte Co-Culture Assay; ESI BIO, USA). Because we found synergistic effects of pericytes and NELL-1, the methods used in our study are feasible options for future studies.

Tube formation in HUVECs treated with NELL-1 without pericytes was not significantly different compared with the control (Supplemental Fig. A); NELL-1 did not have a direct effect on tube formation. Taken together, the results suggested that NELL-1 affected cell migration when combined with pericytes and that NELL-1 mainly affected the efficacy of pericytes through an increase in FGF2. The ability to regulate growth and function of vascular cells (e.g., endothelial and smooth muscle cells) is a well-characterized activity of FGF2. As a growth factor that increases vascularization, FGF2 not only induces angiogenesis [36]. It also stimulates mitogenesis of mesenchymal progenitors and osteoblasts [37]. Future studies should aim at further understanding the detailed mechanisms of NELL-1 combined with pericytes, especially as related to FGF2.

This study had some limitations. First, this study is just pre-clinical research. Therefore, a large number of future studies and convincing evidence must be needed for clinical use. Nevertheless, we believe that our mouse model is appropriate to assess the bone regeneration because our mouse models treated with pericytes and NELL-1 revealed robust bone and vessel formation, which were not observed with other settings. We believe that our mouse model could at least simplify osteonecrosis in humans. Second, reliability of mouse models of osteonecrosis is questionable. To be consistent, all surgical procedures were performed by the senior author, an experienced orthopedic surgeon. In addition, we believe that our mouse model is reliable because ostectomy using the customized jig and the Gigli saw is much simpler than the cauterization of the vessels to directly induce ischemic osteonecrosis [38]. Last, a sequence of vessel maturation in angiogenesis was not included. However, we assessed the bone regeneration that resulted from the critically coupled processes of angiogenesis and osteogenesis.

Conclusion

The results of this study indicated that AD pericytes combined with NELL-1 synergistically enhanced the sequence of angiogenesis. We used a mouse model of osteonecrosis and found significant bone formation in radiographic images and histologic findings. Combination therapy using AD pericytes with NELL-1 has potential as a novel treatment for osteonecrosis.

Abbreviations

AD adipose tissue-derived

NELL-1 Nel-like protein-1

ONFH osteonecrosis of the femoral head
hSVF human stromal vascular fraction
PBS phosphate buffered saline
DMEM dulbecco's modified eagle's medium
FACS fluorescence-activated cell sorter
FBS fetal bovine serum
HUVECs human umbilical vein endothelial cells
WST water-soluble tetrazolium salt
CAM chick chorioallantoic membrane
ACS absorbable collagen sponge
CT computed tomography
BMD bone mineral density
PG pericytes group,
PNG pericytes + NELL-1 group

Declarations

Ethics approval and consent to participate
The institutional review board at CHA University reviewed and approved the experimental protocol.

Consent for publication
Not applicable

Authors' contributions
Hyun-ju An and Kyung Rae Ko reviewed, edited, and revised the manuscript, provided administrative support, and analyzed data. They contributed equally and should be considered as co-first authors. Minjung Baek, Yoonhui Chung, Hyunhae Lee, Hyungkyung Kim, Do Kyung Kim and So-Young Lee collected and analyzed data. Soonchul Lee designed the study, wrote, reviewed, and edited the manuscript, and supervised the work.

Competing Interests
The authors declare that they have no competing interests.
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Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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Supplemental Table

Supplemental Table A. Human adipose tissue samples used study.
**Table:**

| Samples | Sex/Age | Past medical history | SVF yield | SVF viability | Ratio of pericytes in SVF | Pericytes yield |
|---------|---------|----------------------|-----------|--------------|--------------------------|-----------------|
| 1       | M/75    | Hypertension         | 22.4 x 10^6 | 90.3%        | 0.27                     | 5.46 x 10^6     |
| 2       | M/68    | Diabetes mellitus    | 25.1 x 10^6 | 85.2%        | 0.22                     | 4.70 x 10^6     |
| 3       | F/73    | None                 | 19.0 x 10^6  | 88.2%        | 0.24                     | 4.02 x 10^6     |
| 4       | F/82    | Hypercholesterolemia, Diabetes mellitus | 31.4 x 10^6 | 78.3%        | 0.31                     | 7.62 x 10^6     |
| 5       | F/66    | None                 | 29.2 x 10^6  | 79.2%        | 0.22                     | 5.09 x 10^6     |
| 6       | F/72    | None                 | 22.3 x 10^6  | 92.4%        | 0.19                     | 3.91 x 10^6     |
| 7       | F/65    | Hypertension, HBV carrier | 15.1 x 10^6 | 83.7%        | 0.29                     | 3.67 x 10^6     |
| 8       | F/70    | Hypertension         | 17.6 x 10^6  | 87.9%        | 0.23                     | 3.56 x 10^6     |

^Infrapatellar fat pads harvested during total knee arthroplasty were used for pericyte sorting.

**Figures**
Figure 1

Procedures for mouse models of osteonecrosis (A) After ostectomy, bone fragment was removed. (B) The bone fragment was immersed in liquid nitrogen for 5 min. (C) An absorbable collagen sponge (ACS), with or without pericytes and NELL-1, was put below femoral shaft. Necrotic bone fragment was transferred to defect site. (D) Necrotic bone fragment was wrapped by suturing the ACS to prevent displacement.
Pericytes and NELL-1 promote angiogenesis and migration of HUVECs. (A) Pericytes and NELL-1 promote tube formation of HUVECs. Matrigel-coated 96-well plates were seeded with HUVECs (1.5×10⁴) treated with pericytes and NELL-1 (800 ng/ml). After a 12-h incubation, an EVOS FL Cell Imaging System microscope was used to examine and photograph capillary networks. Tube number was quantified using Image-Pro Plus 6.0 software. (B) Pericytes and NELL-1 promote migration of HUVECs in wound-healing assay. HUVECs were treated with or without NELL-1 (800 ng/ml) for 24 h. Images were captured using an EVOS FL Cell Imaging System at 0 and 24 h. Migrated cells were quantified using Image-Pro Plus 6.0. *P < 0.05; **P < 0.01; ***P < 0.001.
Figure 3

NELL-1 protein enhances angiogenesis in CAM. Representative images of chick chorioallantoic membrane in CAM assay. Fertilized eggs were treated with or without NELL-1 (800 ng/ml) for 72 h. Microvessel images were captured using an EVOS FL Cell Imaging System (magnification, ×10). *P < 0.05.
Figure 4

Pericyte and NELL-1 promote bone formation in osteonecrosis animal model. (A) Upper four images taken immediately after surgical procedures. Lower four images taken at 4 weeks after surgical procedures. Bone union of necrotic bone treated with pericytes and NELL-1. (B) Representative images of femurs. Isolated from pericytes treated with or without NELL-1 mice were generated by μCT. (C) BV/TV and BMD of femur (n = 6) were analyzed using a μCT scanner and CTAn software. *Greatest compared with other treatments; P < 0.05.
Immunohistochemical analysis of femur sections for the expression of H&E, masson trichrome (MT) and relevant proteins. Immunohistochemical staining of femur sections for H&E, MT, angiogenesis marker CD31, and VEGF. At end of in vivo study, small pieces of femur sections taken from all groups were decalcified in EDTA, fixed in formalin, paraffin-embedded, sectioned, and immunostained for H&E (upper panel), MT (second panel), CD31 (third panel), and VEGF (lower panel). The right panel shows the quantification data of immunohistochemical staining and the percentage of empty lacunae per total lacunae. Images taken at each indicated magnification.
Figure 6

NELL-1 increases FGF2-AKT-eNOS pathway. (A) Heatmap indicating temporal expression patterns of angiogenesis-related mRNAs identified from mRNA sequencing and quantification Pericytes treated with NELL-1 (100 ng/ml and 800 ng/ml). Z-scores of normalized read counts indicated by colored bars. Red, high expression; green, low expression. (B) Validation of selected differentially expressed mRNAs in Pericytes treated with NELL-1 (800 ng/ml) by qRT-PCR. β-actin was used as a normalizer. (C) Western blot analysis of FGF2 pathway-related proteins by NELL-1 (800 ng/ml) after 72 h of stimulation. *P < 0.05; **P < 0.01; ***P < 0.001.

Supplementary Files

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- SupFigure1.jpg
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