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Human Hypertrophic Nonunion Tissue Contains Mesenchymal Progenitor Cells with Multi-lineage Capacity *in vitro*

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Summary

Hypertrophic nonunion usually results from insufficient fracture stabilization. Therefore, most hypertrophic nonunions simply require the stabilization of the nonunion site. However, the reasons why union occurs without treating the nonunion site directly is not well understood biologically. In this study, we hypothesized that the intervening tissue at the hypertrophic nonunion site (nonunion tissue) could serve as a reservoir of mesenchymal progenitor cells and investigated whether the cells derived from nonunion tissue had the capacity for multilineage mesenchymal differentiation. After nonunion tissue was obtained, it was cut into strips and cultured. Homogenous fibroblastic adherent cells were obtained. Flow cytometry revealed that the adherent cells were consistently positive for mesenchymal stem cell related markers CD13, CD29, CD44, CD90, CD105, CD166, and negative for the hematopoietic markers CD14, CD34, CD45, and CD133, similar to control bone marrow stromal cells. In the presence of lineage-specific induction factors, the adherent cells differentiated *in vitro* into osteogenic, chondrogenic and adipogenic cells. These results demonstrated for the first time that hypertrophic nonunion tissue contains multilineage mesenchymal progenitor cells. This suggests that hypertrophic nonunion tissue plays an important role during the healing process of hypertrophic nonunion by serving as a reservoir of mesenchymal progenitor cells which are capable of transforming into cartilage and bone forming cells.

Abstract

Hypertrophic nonunion usually results from insufficient fracture stabilization. Therefore, most hypertrophic nonunions simply require the stabilization of the nonunion site. However, the reasons why union occurs without treating the nonunion site directly is not well understood biologically. In this study, we hypothesized that the intervening tissue at the hypertrophic nonunion site (nonunion tissue) could serve as a reservoir of mesenchymal progenitor cells and investigated whether the cells derived from nonunion tissue had the capacity for multilineage mesenchymal differentiation. After nonunion tissue was obtained, it was cut into strips and cultured. Homogenous fibroblastic adherent cells were obtained. Flow cytometry revealed that the adherent cells were consistently positive for mesenchymal stem cell related markers CD13, CD29, CD44, CD90, CD105, CD166, and negative for the hematopoietic markers CD14, CD34, CD45, and CD133, similar to control bone marrow stromal cells. In the presence of lineage-specific induction factors, the adherent cells differentiated *in vitro* into osteogenic, chondrogenic and adipogenic cells. These results demonstrated for the first time that hypertrophic nonunion tissue contains multilineage mesenchymal progenitor cells. This suggests that hypertrophic nonunion tissue plays an important role during the healing process of hypertrophic nonunion by serving as a reservoir of mesenchymal cells which are capable of transforming into cartilage and bone forming cells.

Key words : hypertrophic nonunion; fracture healing; mesenchymal progenitor cells

Introduction

Long bone fractures may be treated with nonoperative or operative methods depending on a number of factors including individual circumstances, patients characteristics or surgeon or patient preferences. Despite recent advances in biological fixation techniques and implant developments, cases of failed union after long bone fractures still occasionally occur. Of the approximately 5.6 million bone fractures in the United States each year, up to 10% do not heal and require further treatment.¹

Traditionally, aseptic nonunions are classified as atrophic or hypertrophic radiographically.²⁻⁶ Atrophic nonunion usually reflects poor vascularity at the fracture site and shows little callus formation. In general, the appropriate approach for dealing with atrophic nonunion is decortication and bone grafting together with stabilization. Resection of the nonviable bone and the intervening tissue at the nonunion site (nonunion tissue) is often necessary. On the other hand, hypertrophic nonunion largely depends on an impaired mechanical stability at the fracture site and shows hypertrophic callus formation on X-ray indicating it is biologically active. Although the nonunion site is well vascularized and the fracture is ready to unite, the biologic process to union is inhibited by the lack of mechanical stability.⁴⁻⁶ In such situations, most hypertrophic nonunions simply require the stabilization of the nonunion site, while resection of

nonunion tissue, bone grafting or decortication is optional for healing. In fact, a successful outcome can often be achieved with rigid fixation without resection of nonunion tissue in the treatment of hypertrophic nonunion with plating and exchange nailing.^{7,8} However, the reason why union occurs in most hypertrophic nonunions simply by the stabilization of the nonunion site without treating the nonunion site directly is not well understood biologically.

Recently, we demonstrated that haematoma at the fresh fracture site contains multilineage mesenchymal progenitor cells and indicated that it plays an important role in bone healing.⁹ When a fracture occurs, haematoma forms at the fracture site, and is organized into granulation tissue. It is transformed to callus and continuously union occurs.¹⁰⁻¹² However, in hypertrophic nonunion, the healing process is inhibited mainly by a lack of mechanical stability and the osseous transformation of nonunion tissue fails to occur adequately with the resultant failure of the osseous bridge at the nonunion site.⁶ Previously Boyan et al reported that the cells derived from hypertrophic nonunion tissue (nonunion cells; NCs) retain their ability to respond to bone morphogenetic protein *in vitro* in the same manner as mesenchymal cells,¹³ but there have been no reports of detailed cellular analysis. Therefore, we hypothesized that one reason why union occurs in most hypertrophic nonunion simply by the stabilization of the nonunion site without treating the nonunion site directly is that hypertrophic nonunion tissue can serve as a reservoir of mesenchymal progenitor cells which are capable of transforming into cartilage and bone forming cells.

In this *in vitro* study, we targeted NCs and investigated whether NCs have the capacity for multilineage mesenchymal differentiation.

Materials and Methods

Patient characteristics

We enrolled seven consecutive patients suffering from hypertrophic nonunion in this study. Nonunion was diagnosed because a minimum of 9 months had elapsed since injury and the fracture showed no visible progressive signs of healing for 3 months, in line with the definition of the FDA panel.² Nonunions were classified according to the guidelines set out by the AO-ASIF Group, stating that hypertrophic nonunions are those of a florid bone reaction and flaring of bone ends.¹⁴ A small amount of nonunion tissue was obtained from patients undergoing surgical treatment for their nonunions. Sample data are shown in Table 1. The Institutional Review Board of Kobe University Hospital approved this study and informed consent was obtained from all patients involved. Reasons to exclude patients from this study were infections, tumors, autoimmune or other systemic bone-related diseases, or treatment with hormones, steroids, vitamin D, or calcium. Non-steroidal anti-inflammatory drugs were allowed.

Patients characteristics were as follows: age, 37-74 years old; mean age, 53.0; sex, 6 males and 1 female; fracture site,

3 femoral diaphysis fractures, 2 tibial diaphysis fractures, 1 humeral diaphysis fracture, and 1 ulnar diaphysis fracture. All seven patients underwent surgical operation in the initial treatment: intramedullary locking nail was applied in five patients, plate-and-screw fixation in one patient, and external fixation in one patient. There was no evidence of infection in any patients. All seven patients were receiving their first nonunion operation. The duration from first operation to the operation for nonunion was 9 to 14 months (mean, 11 months). On radiographs, all seven nonunions showed callus formation and the bones were thickened at the fracture ends. In all seven patients, the fixation devices were loosened, and in four patients the fixation device screw had broken. These findings suggest mechanical instability at the nonunion site.

Histological analysis

Following exposure of the nonunion site, nonunion tissue intervening at the nonunion site was obtained and placed in a sterile polypropylene container with attention paid to avoiding contamination of the periosteum or bone. The mean wet weight of the obtained tissue was 0.661g (0.167 to 1.322). The central portion of nonunion tissue excised by sharp dissection was fixed in 4% paraformaldehyde. After fixation, the tissue was embedded in paraffin, and 5 μ m sections stained with hematoxylin and eosin were analyzed using light microscopy to assess general morphology.

Isolation and culture of nonunion cells

After dissecting the central portion of nonunion tissue for histological analysis, nonunion tissue was treated as previously described for fracture haematoma.⁹ In brief, nonunion tissue was washed with phosphate-buffer saline (PBS)(Wako, Osaka, Japan) and divided with a scalpel into small pieces with the addition of the original medium, α -Modified Minimum Essential Medium (Sigma, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (FBS) (Sigma), 2mM L-glutamine (Gibco BRL, Grand Island, NY, USA) and antibiotics on 100mm diameter culture dish. The cultures were incubated at 37°C with 5% humidified CO₂.

Seven days after initial incubation, the culture dish was washed with PBS to remove nonviable cells and debris, and thereafter the culture medium was changed twice weekly. Approximately 3-4 weeks later, the adherent cells were harvested with 0.05% trypsin-0.02% EDTA (Wako) and passaged into non-coated 75 cm² culture flasks at a density of approximately 3 $\times$ 10⁵ cells per flask for further expansion.

Population-doubling (PD) is the method of calculating the proliferative capacity of NCs. The PD level was calculated for each subcultivation using the following equation: PDs = [Log₁₀ (N_H)–Log₁₀(N₁)]/log₁₀(2), where N₁ is the inoculum number, N_H is the cell harvest number and log, the logarithm.¹⁵ The calculated PD increase was added to the PD levels of the previous passages to yield the cumulative PD level.

In order to compare the PDs and the following differentiation capacities of NCs with those of fracture haematoma cells (HCs), fracture haematoma samples were obtained with informed consent from seven patients (all tibial fractures; age range, 19-57) during osteosynthesis, and cultured under the same conditions as the NCs.⁹

Cells from passage 1 to passage 3 were used in the following differentiation assays on each sample. All experiences were performed with all seven NCs.

Immunophenotyping of nonunion cells by flow cytometry

The adherent cells from nonunion tissue were harvested after the first passage and evaluated for cell-surface protein expression using flow cytometry. After several washes with PBS-3% FBS, cells were incubated for 30 minutes at 4°C in the dark with the following phycoerythrin(PE)-conjugated mouse anti-human antibodies: CD13, CD14, CD29, CD34, CD44, CD45, CD90, CD133, and CD166 (BD Biosciences, San Jose, California), CD105 (Ancell, Bayport, Minnesota). The nonspecific mouse PE-conjugated IgG (BD Sciences) was substituted as an isotype control. After incubation, cells were then washed twice with PBS-3% FBS and analyzed using FACSaria flow cytometry system (BD Sciences). In order to compare the data with those of bone marrow stromal cells (BMSCs), BM samples were obtained with informed consent from seven patients (age range, 46-74) undergoing hip surgery, and BMSCs were obtained by isolating with a density gradient (Ficoll-Paque, PLUS, Amersham Bioscience, Uppsala, Sweden) and culturing under the same conditions as NCs. Positive staining was defined as the emission of a fluorescence signal that exceeded the level obtained by >99% of cells from the control population stained with a matched isotype antibody. For each sample, at least 10,000 list mode events were collected.

Human Dermal Fibroblasts as Controls

Normal human dermal fibroblasts (DFs) (kindly provided by Dr. H. Terashi, Department of Plastic Surgery, Kobe University Graduate School of Medicine) served as negative controls in the differentiation studies. The cells were cultured under the same conditions as NCs.

Differentiation studies

Osteogenic induction. NCs and DFs were cultured for 21 days in an osteogenic medium consisting of the original medium plus 10 nM dexamethasone (Dex) (Sigma), 10 mM β -glycerophosphate (Sigma), and 50 μ g/ml ascorbic acid (Wako).^{9,16-20} The control cells were maintained in the original medium. After 21days, osteogenic differentiation was evaluated by calcium deposition, which was stained by 1% Alizarin Red S (Hartman Leddon, Philadelphia, PA, USA). In order to quantitatively compare the mineralization of NCs with that of HCs, cells stained with Alizarin Red were

destained with ethylpyridinium chloride (Wako Industries, Ltd.) and then the extracted stain was transferred to a 96-well plate, and the absorbance at 562 nm was measured using a microplate reader, as previously described.²¹ Expression of osteoblast-related genes, alkaline phosphatase (ALP), osteocalcin and bone sialoprotein (BSP) was also measured by reverse transcription polymerase chain reaction (RT-PCR). Furthermore, ALP activities were also assayed and compared between NCs and HCs. The cell layer from each well was washed twice with phosphate buffered saline, sonicated with a Microson Ultrasonic Cell Disruptor XL2000 (Misonix, Farmingdale, New York) and stored at -20°C until assayed for ALP activity. ALP activity was assayed as the release of p-nitrophenol from p-nitrophenylphosphate, pH 9.8, and the p-nitrophenol release was monitored by optical density at 405 nm using LabAssay ALP (Wako Pure Chemical Industries Ltd, Osaka, Japan). Protein concentration in the sonicate was measured by BCA Protein Assay Kit (Pierce Chemical Co, Rockford, IL). The results are expressed as p-nitrophenol produced in nmol/min/mg of protein.

Chondrogenic induction. For chondrogenic differentiation, a pellet culture was performed for three-dimensional culture.^{9,16,19,20} About 2.5×10^5 cells in the 15 ml polypropylene tube were centrifuged at 2000 rpm for 4 minutes to form a pellet. The cells were treated with chondrogenic medium consisting of high glucose Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) with 10^{-7} M Dex, 50 µg/ml L-ascorbic acid-2-phosphate (Sigma), 0.4 mM proline (Sigma), 1% ITS⁺1 (Sigma), 10 ng/ml recombinant human TGF-β3 (R&D Systems, Minneapolis, MN, USA), and 500 ng/ml recombinant human BMP-6 (Sigma).^{9,16,19,20} The cells were recentrifuged to form a pellet. After 21 days, chondrogenic differentiation was assessed by staining with Toluidine Blue (Muto Pure Chemicals). For microscopy, the pellets were embedded in paraffin and sectioned. Expression of chondrocyte-specific genes, type II collagen (Col II) and type X collagen (Col X), Sry-type high-mobility group box 9 (SOX9), aggrecan were also measured by RT-PCR.

Adipogenic induction. To induce adipogenic differentiation, NCs and DFs were cultured for 21 days in an adipogenic medium consisting of low growth DMEM (Sigma) with 1 µM Dex, 0.5mM 3-isobutyl-1-methylxanthine (Sigma), 10 µg/ml insulin (Sigma), 0.2 mM indomethacin (Sigma), and 10% FBS.^{9,16,19,20} The control cells were maintained in low growth DMEM containing 10% heat-inactivated FBS and antibiotics. After 21 days, adipogenic differentiation was evaluated by the cellular accumulation of neutral lipid vacuoles that were stained with Oil-red O (Muto Pure Chemicals, Tokyo, Japan). Expression of adipocyte-specific genes, lipoprotein lipase (LPL) and peroxisome proliferator-activated receptor (PPAR)-γ2 were also measured by RT-PCR.

Total RNA extraction and RT-PCR.

To detect mRNA levels of specific genes related to each differentiation event, differentiated and undifferentiated cells were harvested. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. From each sample, approximately 1 µg of total RNA was reverse-transcribed using oligo

(dT) primer, dNTP, 10×PCR buffer, MgCl₂, RNase inhibitor, and Mulv Reverse Transcriptase (all from Applied Biosystems, Branchburg, New Jersey, USA). The converted cDNA samples were amplified by PCR using Taq Gold DNA polymerase (Applied Biosystems). In all RT-PCR assays, the house-keeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was analyzed to monitor RNA loading. The primers used for amplification are as following: ALP (357bp), 5'-CCCAAAGGCTTCTTCTTG-3' and 5'-CTGGTAGTTGTTGTGAGC-3'; OC (371bp), 5'-TCACACTCCTCGCCCTATTGG-3' and 5'-GGGCAAGGGGAAGAGGAAAGA-3'; BSP (447bp), 5'-ATTTCCAGTTCAGGGCAGTAG-3' and 5'-ACACTTTCTTCTTCCCCTTCT-3'; Col II (517bp), 5'-TCTGCAACATGCAGACTGGC-3' and 5'-GAAGCAGACAGGCCCTATGT-3'; Col X (703bp), 5'-GCCCAAGAGGTGCCCCTGGAATAC-3' and 5'-CCTGAGAAAGAGGAGTGGACATAC-3'; SOX9 (143bp), 5'-AACATGACCTATCCAAGCGC-3' and 5'-ACGATTCTCCATCATCCTCC-3'; Aggrecan (350bp), 5'-TGAGGAGGGCTGGAACAAGTACC-3' and 5'-GGAGGTGGTAATTGCAGGGAACA-3'; LPL (276bp), 5'-GAGATTTCTCTGTATGGCACC-3' and 5'-CTGCAAATGAGACACTTTCTC-3'; PPAR-γ2 (380bp), 5'-TGGGTGAACTCTGGGAGATTC-3' and 5'-CATGAGGCTTATTGTAGAGCTG-3'; GAPDH (593bp), 5'-CCACCCATGGCAAATTCATGGCA-3' and 5'-TCTAGACGGCAGGTCAGGTCCACC-3'.^{6,14-16}

Statistical analysis

Stat View-J 4.5 software (HULINKS Inc., Tokyo, Japan) was used for statistical analysis. Data were presented as mean $\pm$ standard deviation (SD). To assess differences of ALP activity and mineralization assay between treated and control cells, or between HCs and NCs, and to compare the mean cumulative population doublings of NCs with those of HCs at each passage, Student's two-tailed and unpaired *t*-test was performed. A value of $p < 0.05$ was considered statistically significant.

Results

Histology

Histological examination revealed mainly fibrous tissue and no ossicles were seen in any sections examined. Nonunion tissue contained various amounts of fibroblast-like cells. There were no findings of infection or malignancy (Fig. 1).

Morphological characteristics and immunophenotypes of adherent Nonunion cells.

In the primary culture, colonies of adherent cells exhibiting a fibroblast-like spindle shape began to appear in the culture dish about 5 days after plating. These colonies resemble colony-forming unit-fibroblasts (CFU-F) observed in BMSCs.¹⁹ The colony size increased rapidly, and after three to four weeks the cells merged and formed a subconfluent monolayer of fibroblastoid cells.

From the calculation of PD, NCs could be cultured for up to eight passages and approximately 10 PDs, but showed a gradual decline of PD compared with HCs which could be cultured for at least eight passages with a minimal decline in their proliferation capacity and approximately 20 PDs. Moreover, the proliferation capacity of NCs is significantly low compared to that of HCs (Fig. 2). Fig. 2B shows the morphology of HCs and NCs at passage 0 and 8. Both HCs and NCs have similar fibroblastic morphology, but NCs show lower cell density than HCs at passage 8.

The cell-surface antigen profile of adherent NCs was analyzed and compared with that of BMSCs. Both NCs and BMSCs were positive for mesenchymal stem cell (MSC)-related markers CD13, CD29, CD44, CD90, CD105 and CD166, but negative for hematopoietic markers CD14, CD34, CD45 and CD133 (data not shown).

Adherent Nonunion cells exhibited in vitro osteogenic, chondrogenic and adipogenic potential.

Adherent NCs were cultured under conditions favorable for osteogenic, chondrogenic or adipogenic differentiation, respectively. After a 21-day incubation under osteogenic conditions, induced NCs and HCs formed a mineralized matrix as evidenced by Alizarin Red S staining (Hartman-Leddon Company, Philadelphia, Pennsylvania), contrasting with an absence of mineralized matrix under undifferentiated conditions after the same duration (Fig. 3). In addition, the mineralization of NCs was significantly higher ($p < 0.05$) than that of HCs under differentiated conditions (Fig. 4A). This osteogenic potential was further confirmed by RT-PCR analysis, showing the expressions of ALP, osteocalcin, BSP under osteogenic conditions after a three-week culture period (Fig. 5). The expression of ALP, osteocalcin, BSP under osteogenic conditions was higher than under undifferentiated conditions in the control group. Neither mineralization (Fig. 3) nor up-regulation of the mRNA expression of osteoblast-related genes (data not shown) was observed in DFs by osteogenic induction for 3 weeks.

The level of ALP activity under osteogenic conditions was significantly higher ($p < 0.05$) than under control conditions on day 21 (Fig. 4B), and ALP activity of NCs was significantly higher ($p < 0.05$) than that of HCs under differentiated conditions (Fig. 4B).

After a 21-day incubation under chondrogenic conditions, cell pellets had a spherical and glistening transparent appearance. The development of a cartilage matrix from cell pellets was shown by staining the proteoglycans with toluidine blue (Fig. 3). The RT-PCR analysis showed the expression of mRNA of Col II, Col X, SOX9, and aggrecan chondrogenic conditions after a 21-day induction (Fig. 5). In contrast, there was no proteoglycan present in sections from

the DF cell pellets (Fig. 3) and no expression of those chondrocyte-related genes was observed (data not shown) in the DF cell pellets by chondrogenic induction for 3 weeks.

After a 21-day incubation period under adipogenic conditions, adherent NCs showed the formation of neutral lipid vacuoles, visualized by staining with Oil-red O, but not DFs (Fig. 3). Under in undifferentiated conditions, no Oil-red O-positive lipid vacuole was observed in adherent NCs (Fig. 4). The RT-PCR analysis showed the expression of LPL and PPAR- γ 2 under adipogenic conditions after a 21-day culture period (Fig. 5). The expression of LPL and PPAR- γ 2 under adipogenic conditions was higher than under undifferentiated conditions in the control group. In contrast, no expression of these genes was observed in DFs by adipogenic induction (data not shown).

All 7 samples analyzed for differentiation assay showed osteogenic, chondrogenic, and adipogenic differentiation capacity.

Discussion

The current study has shown for the first time that NCs of hypertrophic nonunion have the multilineage differentiation potential *in vitro*, differentiating toward the osteogenic, chondrogenic, and adipogenic lineages when cultured in the presence of established lineage-specific differentiation factors, which is consistent with that reported for BMSCs. Although it goes without saying that the most important factor in the treatment of hypertrophic nonunion is stabilization of the fracture site, our result suggests that NCs as well as the cells derived from the periosteum and bone marrow also play important role in the consolidation of the intervening tissue at the nonunion site under abundant vascularity after stabilization of the fracture site.

The primary culture of NCs showed the formation of colonies of fibroblast-like spindle shape cells, resembling that of BMSCs. Cell-surface markers analyzed using FACS revealed that NCs expressed MSC-related markers, while lacking hematopoietic-lineage markers. The capacity of NCs to differentiate into osteoblast-lineage cells that produce mineralized matrices, chondrocyte-lineage cells that produce proteoglycans, and adipocyte-lineage cells that accumulate lipid vacuoles in the presence of established lineage-specific differentiation factors *in vitro* was consistent with that reported for BMSCs, and was confirmed by RT-PCR analysis and histochemical evaluation. There were no remarkable differences among donors in terms of cell-surface antigens at the end of passage 1, and osteogenic, adipogenic, and chondrogenic differentiation potentials. In addition, there was no significant correlation between population doublings (proliferation capacity) and age, gender, fracture site, or duration from fracture shown in our experiment (data not shown). Taken together, these findings indicate that nonunion tissue contains multilineage mesenchymal progenitor cells exhibiting characteristics similar to BMSCs.

Recently, we demonstrated that haematoma at the fracture site contains multilineage mesenchymal progenitor cells and indicated that haematoma plays an important role in bone healing⁹. Surprisingly, regardless of the extended period from fresh fracture to nonunion, NCs have similar characteristics to HCs in morphology, differentiation capacity and cell-surface markers. The localization of the haematoma and nonunion tissues intervening in the fracture gap is also similar. Therefore, we speculated that nonunion tissue could be derived from fracture haematoma. However, from the calculation of PD, the proliferation capacity of NCs was significantly inferior to that of HCs, and NCs showed lower cell density than HCs at passage 8. On the other hand, both ALP activity and mineralization of NCs under osteogenic conditions was significantly higher than that of HCs. As hypertrophic callus formation suggests that considerable proliferation had occurred in the tissue in vivo prior to isolation, the reduced ability of NC to proliferate in culture would result from prior excessive proliferation and the proliferative capacity would have been largely exhausted. On the other hand, the higher osteogenic capacity of NCs than of HCs may explain the fact that the callus is hypertrophic rather than atrophic. One reason why union occurs without treating the hypertrophic nonunion site may be that NCs retain enough differentiation capacity despite the proliferation capacity being reduced.

Hypertrophic nonunion usually results from a mechanical instability at the fracture site. In fact, in this study, all seven cases had loosening of the fixation device indicating the cause of nonunion as mechanical instability at the nonunion site. As the hypertrophic nonunion site is biologically active, in most hypertrophic nonunions, union occurs simply by the stabilization of the nonunion site without directly treating the nonunion site. In the past, several biological effects are reported. In hypertrophic nonunion of femoral and tibial diaphyseal fractures, exchange nailing which includes reaming of the medullary canal, and placement of a larger intramedullary nail is an excellent treatment.⁸ Exchange nailing improves mechanical instability, which is the main requirement for achieving osseous healing. Biologically, some reported that reaming of the medullary canal increases periosteal blood flow and stimulates periosteal new-bone formation,²²⁻²⁴ and others have suggested that reaming products which contains osteoblasts and multipotent stem cells, serve as local bone graft that stimulates medullary healing at the nonunion site.²⁵⁻²⁸ Each findings could provide one reason why union occurs simply by the stabilization of the nonunion site without directly treating the nonunion site. On the other hand, it is reported that union occurs by re-plating without treating the hypertrophic nonunion site directly.⁷ The local biological effect is inexplicable from the idea of periosteal blood flow or local bone graft by reaming of the medullary canal. However, in both cases of exchange nailing and re-plating, the stabilization procedure itself may have exerted systemic effects on healing. However, the reason why union occurs in most hypertrophic nonunions simply by the stabilization of the nonunion site without treating the nonunion site directly is not well understood biologically.

Meanwhile, Wu et al²⁵ compared the closed technique consisting of intramedullary reaming to a larger size and reinsertion of a stable intramedullary nail to the open technique consisting of debridement of nonunion tissue,

stabilization of the nonunion site, bone grafting after decortication. In both cases the nonunion rate was 100%, but the union period with the closed technique was significantly shorter than that of the open technique (4.0 +/- 0.6months vs. 5.1 +/- 0.8 months; $p<0.01$). They suggested that the resection of nonunion tissue would disturb the vascular supply. However, from our result, we speculate that the resection of nonunion tissue also leads to remove the mesenchymal cells which are capable of transforming into cartilage and bone forming cells.

Previously, Boyan et al reported that human NCs retain their ability to respond to bone morphogenetic proteins in vitro in the same way as mesenchymal cells.¹³ Guerkov HH et al reported that human NCs respond to pulsed electromagnetic fields in culture and produce transforming growth factor-beta 1 in the same way as mesenchymal cells.²⁹ From our results and previous reports, it is suggested that hypertrophic nonunion tissue could serve as a reservoir of mesenchymal progenitor cells, and we speculated that mechanical stability promotes the adequate proliferation and differentiation of NCs which have multilineage differentiation potential under the influence of several growth factors, finally allowing bony bridging and remodeling of the nonunion site.

We have shown for the first time that NCs can differentiate into osteogenic, chondrogenic and adipogenic cells in vitro, and thus hypertrophic nonunion tissue contains multilineage mesenchymal progenitor cells. This indicates that hypertrophic nonunion tissue could serve as a reservoir of mesenchymal cells which are capable of transforming into cartilage and bone forming cells, thus, in hypertrophic nonunion, union would occur without treating the nonunion site directly.

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Table 1. Cells Sample Data from 7 Patients

Patient	Gender	Age	Fracture site	Duration from fracture (months)	Population doublings at 8 passage
1	F	64	Tibial diaphysis	9	4.54
2	M	55	Femoral diaphysis	13	12.50
3	M	60	Femoral diaphysis	9	14.38
4	M	39	Femoral diaphysis	11	12.41
5	M	42	Tibial diaphysis	9	11.53
6	M	74	Humeral diaphysis	14	5.64
7	M	37	Ulnar diaphysis	9	12.98

Figure legends

Fig. 1 Histological examination of the intervening tissue at the hypertrophic nonunion site stained with hematoxylin and eosin using light microscopy. Mainly fibrous tissue was seen and no ossicles were seen in any sections examined. Nonunion tissue contains various amounts of fibroblast-like cells, and there were no findings of infection or malignancy. Scale bar = 200 μ m.

Fig. 2 (A) Mean values of the cumulating population doublings, determined at each subcultivation, with the cells in the intervening tissue at the nonunion site (NCs) shown (n=7) and fracture haematoma cells (HCs) shown (n=7). N.S. indicates no significance (p=0.14) and, * and ** indicate statistically significant difference between NCs and HCs (* p=0.041; ** p < 0.01). (B) Phase contrast images of adherent NCs and HCs displaying fibroblastoid morphology at passage 0 (primary culture) and passage 8. Scale bar = 200 μ m.

Fig. 3 Histochemical analysis of osteogenic, chondrogenic and adipogenic differentiation capacity of NCs and DFs (negative control). Alizalin Red S staining after 21-day incubation in osteogenic medium (NC Os+), undifferentiated medium (NC Os-) or negative control (DF Os+). Histological section stained with Toluidine Blue after 21-day incubation in chondrogenic medium (NC Ch+) or negative control (DF Ch+). Oil-red O staining after 21-day incubation in adipogenic medium (NC Ad+), undifferentiated medium (NC Ad-) or negative control (DF Ad+). Scale bar = 200 μ m.

Fig. 4 (A) The mineralization activity of HCs and NCs in the osteogenic medium (Os+) or control medium after 21 days in culture was measured as described in Materials and Methods. (B) Alkaline phosphatase (ALP) activity of HCs and NCs in the osteogenic medium (Os+) or control medium (Os-) after 21 days in culture. N.S. indicates no significance, while * and ** indicate statistically significant difference between each bar (* p < 0.05, ** p < 0.01).

Fig. 5 RT-PCR analysis of gene expression of alkaline phosphatase (ALP), osteocalcin (OC), bone sialoprotein (BSP), type II collagen (Col II), type X collagen (Col X), Sry-type high-mobility group box 9 (SOX9), aggrecan, lipoprotein lipase (LPL), and peroxisome proliferator-activated receptor (PPAR) γ 2 in NCs in differentiated medium (Os+, Ad+, Ch+) or in undifferentiated medium (Os-, Ad-) on day 21. Ch- = RT-PCR analysis of gene expression of Col II, Col X, SOX9 and aggrecan in the cells on day 0. GAPDH, glyceraldehyde-3-phosphate-dyhydrogenase.

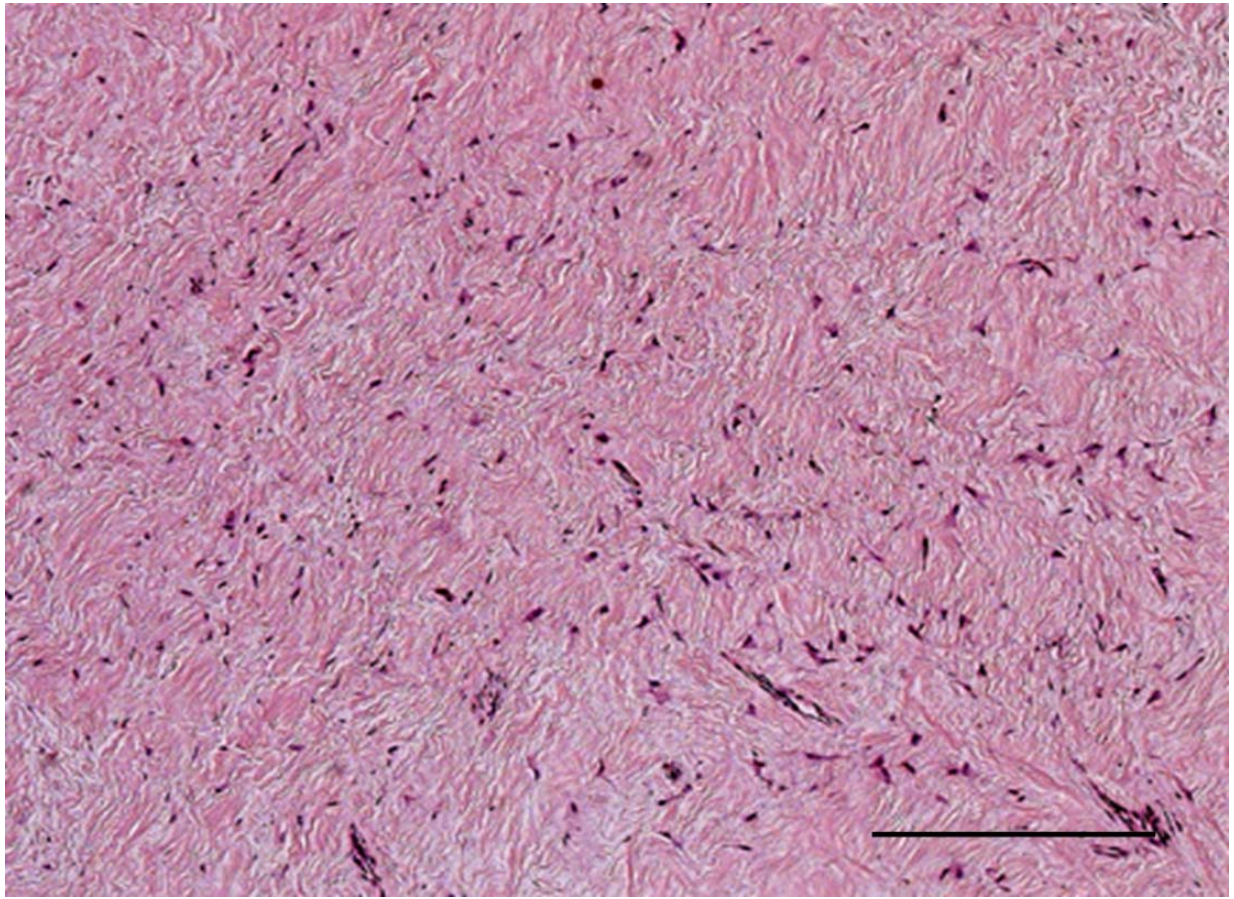


Figure 1.

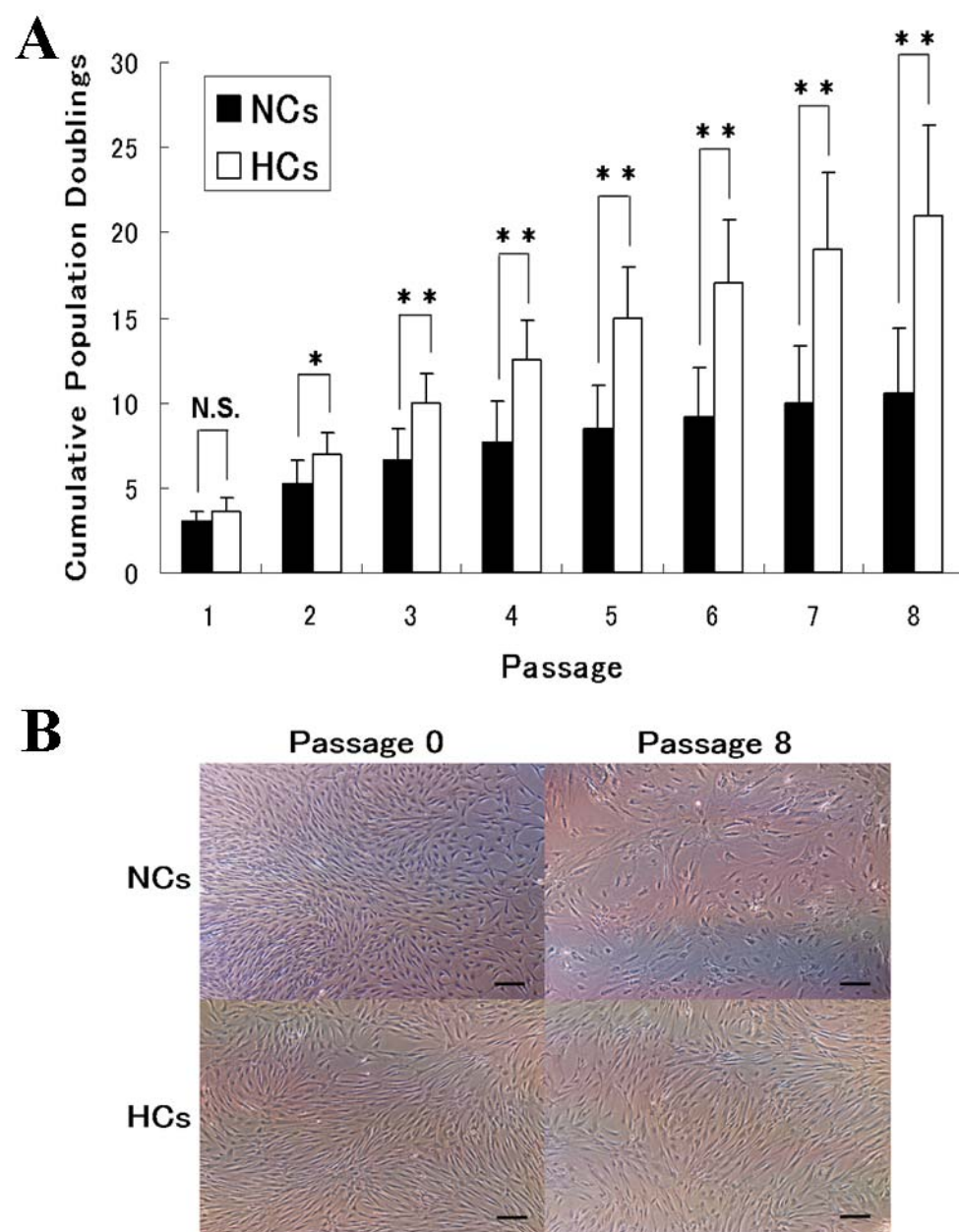


Figure 2.

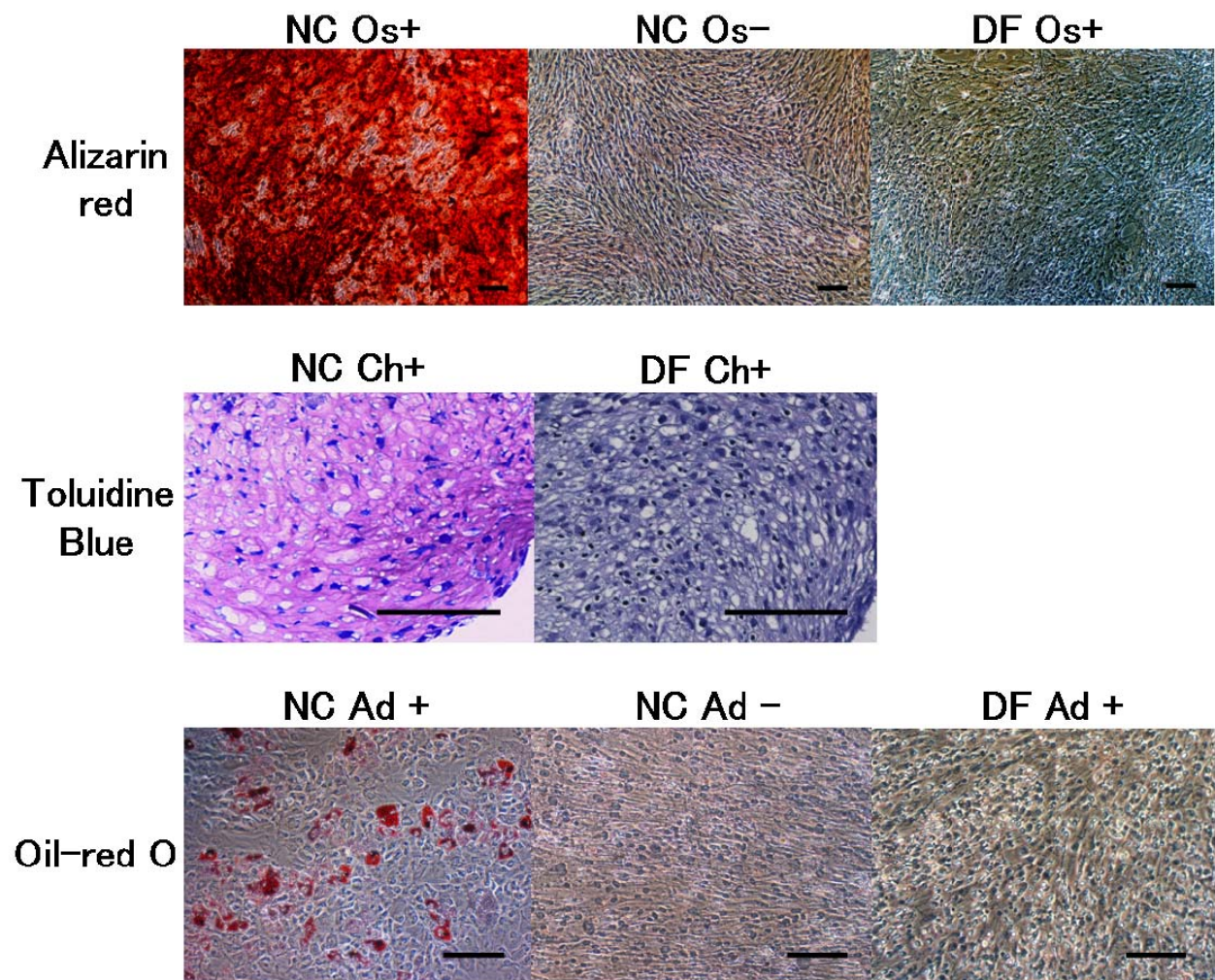


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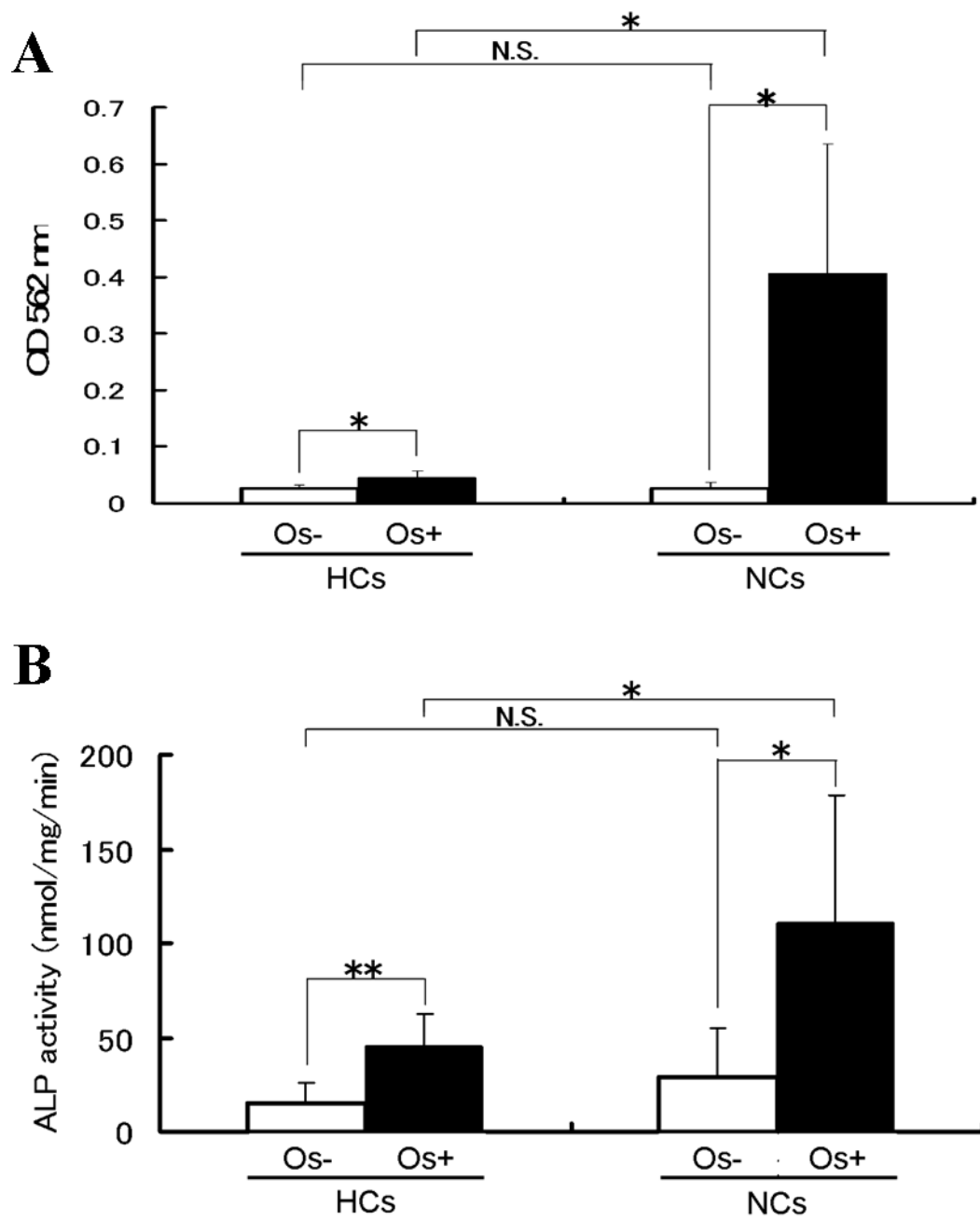


Figure 4.

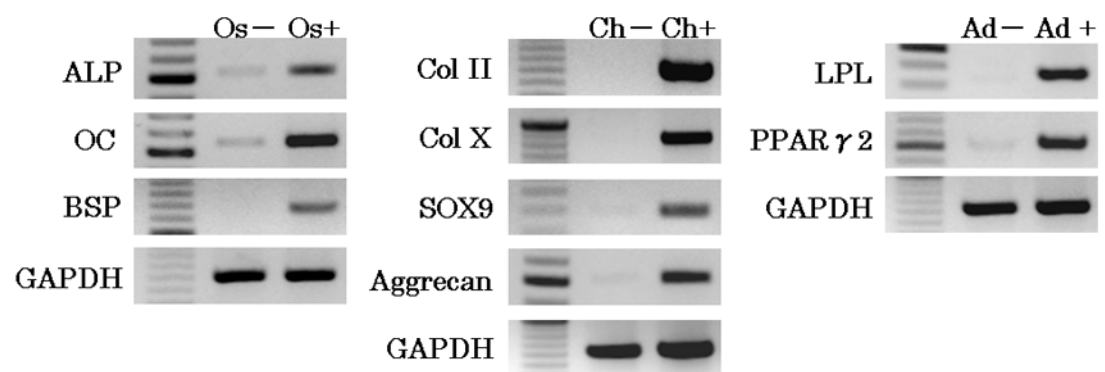


Figure 5.