Original Article

Isolation of mesenchymal stem cells from bone marrow wastes of spinal fusion procedure (TLIF) for low back pain patients and preparation of bone dusts for transplantable autologous bone graft with a serum glue

Tomoko Ichiyanagi[1], Kouki Anabuki[2], Yoji Nishijima[3], Hirohisa Ono[3,*]

[1] *National Institute of Genetics, Mishima, Shizuoka, Japan;*
[2] *Department of Orthopedic Surgery, Nishijima Hospital, Numazu, Shizuoka, Japan;*
[3] *Department of Neurosurgery, Nishijima Hospital, Numazu, Shizuoka, Japan.*

Summary **Low back pain and subsequent disabilities are common. A lumbar spinal fusion procedure is an effective treatment with autologous bone grafts, but harvesting the bone from the iliac crest is associated with risks of complications. New treatments using stem cells together with osteoconductive and otesoinductive materials have made the procedure safer, but the inconsistency of the amount of stem cells harvested from bone marrow aspirate still remains to be solved. This study reports that the bone dusts, usually discarded as surgical wastes during transforaminal lumbar interbody fusion procedure (TLIF procedure), yielded cells which had the characteristics of mesenchymal stem cells (MSCs) *in vitro*. The cells were positive for the MSC markers and were able to differentiate in osteogenic and adipogenic directions. The cells grew robustly in an osteoconductive material, Bolheal (serum glue), and also proliferated well in culture medium supplemented with autologous serum. Therefore, the bone dust is a good candidate for the alternative source of stem cells other than bone marrow aspirate to increase the safety of the TLIF procedure.**

Keywords: Mesenchymal stem cells, bone dusts, TLIF procedure, serum glue, xenogenous material-free preparation

1. Introduction

More than 80 percent of Japanese men and women complained of low back pain in 2004 and the prevalence further increased, according to a Japanese national survey (*1*). Approximately 15 to 20% of these people suffered and experienced interference with daily activities (*2*) and it has been a major social burden with enormous cost and labor for taking care of these patients. In the U.S., the cost has been estimated to be more than $100 billion a year and is still increasing (*3-6*). It became clear that one of the major sources of the increasing cost was due to spinal fusion procedure, which is more prevalent in older populations (*4*). The spinal fusion surgery is a particular surgical procedure aimed to stabilize painful spine by connecting abnormally unstable parts of the spine, which is caused by degeneration or aging, with new bone growth by inserting bone fragments (autologous bone grafts or allografts). Recently, synthetic materials have been used although these materials themselves cannot grow the new bone but rather, attract bone forming cells, osteoblasts, on them and participate in bone growth in the surgical area as scaffolding materials (*7*,*8*). The number of fusion procedures in the lumbar region, such as transforaminal lumbar interbody fusion procedure (TLIF procedure) rose more rapidly than other fusion procedures (*4*).

The bone tissue takes much longer time to heal or to complete fusion than the soft tissue does. During the early era of the bone fusion procedures, it was a common practice to keep a patient postoperatively in an uncomfortable body cast immobilized in the bed until the bone fusion is completed in about 12 weeks after surgery, which was the estimated period required for the fusion. Recent technical and instrumental advancements

**Address correspondence to:*
Dr. Hirohisa Ono, Department Neurosurgery, Nishijima Hospital, 2835-7 O-oka, Numazu, Shizuoka 410-0022, Japan.
e-mail: hirohisa.ono@hotmail.co.jp

made the immobilization and use of the cast obsolete. The postoperative patients are much more comfortable and can be up and about the next day after the surgery as the results of intraoperative use of stabilizing metal screws (pedicle screws) and cages. However, these significant advancements in techniques and materials which provided an instant stabilization of the surgical area had not shorten the time required for the bone healing, or improved the fusion rate, and therefore the old fashioned autologous bone graft still remains as the best fusion material. The bone healing requires three fundamental factors, osteoconduction, osteoinduction, and osteogenic cells (*9*) and the autologous bone graft is the only material that processes these factors in one peace.

However, harvesting the autologous bone grafts, usually from patient's own hipbone, or the iliac crest, is well known to be associated with very high risks and surgical complications. Approximately 30% of patients developed donor site morbidity (*10*).

Despite of a complete relief of preoperative clinical symptoms from the fusion surgery, these patients suffered new symptoms after surgery such as pain and numbness in the iliac crest area, which can be severe enough to cause problems in ambulation (*11*). Other complications from harvesting autograft bone from iliac crest include wound dehiscence, infection, blood clot formation, and fracture of iliac crest with prolonged debilitating pain (*12,13*).

Growing concerns on these risks and complications for autograft harvesting have prompted investigations on the other sources for the bone fusion and the mesenchymal stem cells have recently attracted researchers as the source of osteogenic cells. A major source of the stem cells, including the mesenchymal stem cells has been bone marrow blood cells obtained by aspiration of the iliac crest or the sternum (*14*). The aspiration is simpler than surgical harvesting of the bone tissue. However, the bone marrow aspiration can be associated with serious complications such as nerve, arterial and ureteral injury, herniation of abdominal contents, sacroiliac joint instability, pelvic fracture, hematoma and infection (*15*). The aspirant has to be less than 2 cc. at a site in order to avoid contamination by peripheral blood, yet a bone marrow aspirant processing device, such as Bone Marrow Aspirate Concentrate (BMAC) system (Harvest Technologies Corporation), requires a large quantity of the aspirant to collect enough amount of cells for a graft. The system collects mononuclear cells from the bone marrow aspirant where the stem cells reside, but the number of the cells obtained remains inconsistent due to the technical difficulties.

In order to substitute the role of autologous bone graft by the stem cells, additional factor(s) have to be considered, because stem cells alone at the fusion site failed to produce meaningful bone fusion (16-*18*). Some factors for osteoconduction and osteoinduction other than the stem cells are thought to be required. For the osteoconductive material, many biological and synthetic carriers for the stem cells have been examined (*19*), and the endeavor for finding osteoinductive element(s) resulted in finding of bone morphogenetic protein (BMP)-2 (*20,21*) which is currently commercially available. However, since the source and the harvesting method are inconsistent, the use of stem cells has not exceeded the fusion rate or the surgical results of the traditional autologous bone graft operation.

Our current study is aimed to find a more consistent and safe source for the autologous bone graft and refinement of the harvesting technique during lumbar spine fusion surgery, TLIF. First, *in vitro* isolation and expansion of the stem cells from the new source was attempted, and then the behavior of the isolated stem cells in an osteoconductive material, Bolheal (a serum glue) was tested. An attempt to remove all xenogenic materials from the processing of the graft for transplantable grade was also performed by culturing the stem cells by autologous serum supplement. This study is a first step towards our final goal to establish a fusion surgery with more affordable cost and to improve safety and efficiency of the spine fusion procedure.

2. Materials and Methods

2.1. *Harvesting bone marrow wastes*

The bone marrow wastes were harvested from 10 patients who underwent spinal surgery (TLIF procedure) with pedicle screws and a cage insertion in the disc space in the lumbar area. The bone marrow wastes, consisted of blood clot and bone dusts, were usually discarded as a waste immediately to clean the surgical field. However, the wastes were collected for this study according to the method approved by the Nishijima Hospital (Numazu City, Japan) Ethics Committee after informed consent process. In brief, a regular 5 or 6 mm drill (depending upon the size of the pedicles) for the TLIF procedure was inserted into the center of the bone marrow of designated lumbar spine vertebral bodies through the pedicles of the vertebral body at the planned surgical site. This step is an essential part of the regular technique for the TLIF procedure to prepare for the stabilizer screw insertion. When the drill was removed, the attached blood clot and bone dusts were immediately washed off in heparinized saline solution. If a first attempt with 5 or 6 mm size drill yielded less bone dusts, then a 4.5 mm drill was re-inserted into the same hole for removing the blood clots in the hole (Figure 1, *right*). The remaining blood clots were also collected from the drilled hole by suction since these usually contained bone dusts fallen off the drill during withdrawal of the drill. The free flowing blood, which is thought to be contaminated by the peripheral blood,

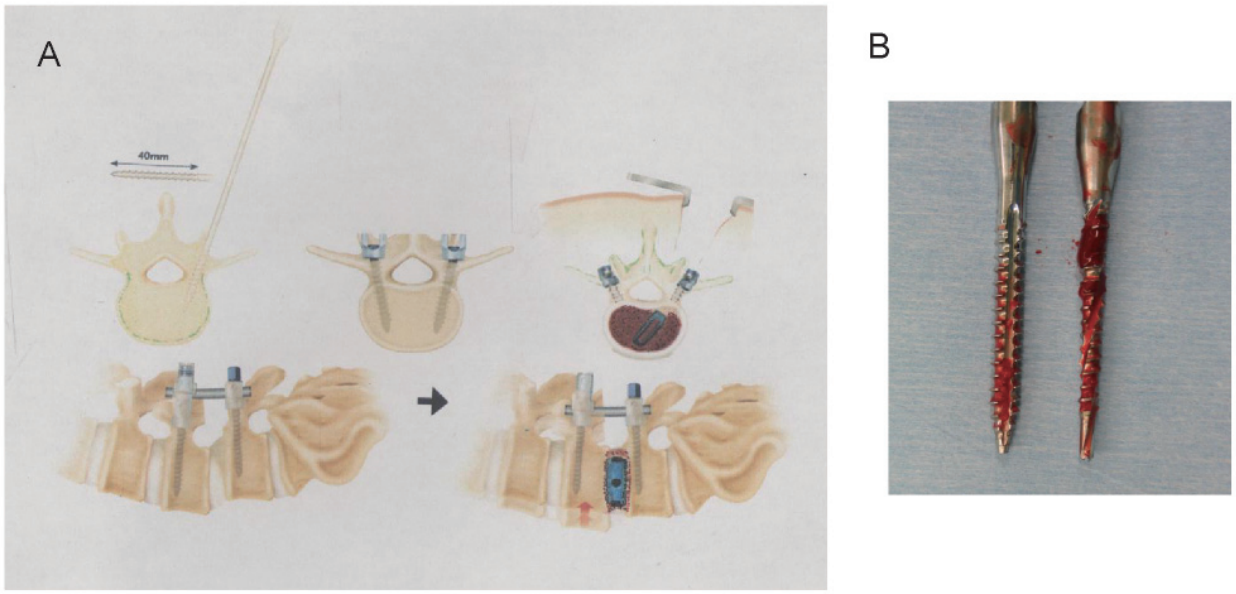

Figure 1. TLIF (transforminal lumbar interbody fusion) procedure and drills for the two-drill technique. Left: A regular TLIF drill is inserted into the center of the bone marrow and the bone dusts attached to it were harvested after making guide holes for pedicle screws. Then, a cage is inserted in the space between two vertebral bodies, and anterior sliding of one of the two unstable vertebral bodies is corrected with inserted screws and the cage (Modified from Medtronic Sofamor Danek Co., Ltd. brochure illustration). **Right**: 6 mm and 4.5 mm drills used in harvesting the bone dusts.

was not collected but rather, immediate hemostasis was obtained as in the regular surgery.

2.2. *Culture of the bone marrow wastes*

The bone marrow wastes, consisted of blood clot and bone dusts were washed 3 times in Hanks Balanced Salt Solution (Gibco) and then digested with 256 U/mL of Collagenase IX (Sigma) at 37°C for 3 h. After the treatment, samples were centrifuged to separate BD cells (bone derived cells) and CR cells (collagen released cells) according to Sakaguchi *et al*. (*22*). Obtained bone fragments or cells were cultured in DMEM with 2 mM L-Glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma), and 10% FBS (Gibco) or 10% autologous serum.

2.3. *Preparation of autologous serum*

Approximately 60 mL of the whole blood from the same patient who was subjected for collecting bone dusts were first centrifuged at 160 × g for 10 min at room temperature, and the upper layer were centrifuged again at 160 × g for 10 min at room temperature. The upper layer were taken as platelet-rich plasma (PRP) and centrifuged at 780 × g for 10 min, and the supernatant was pooled as platelet poor plasma (autologous serum) and the pellet (platelets) were resuspended with HBSS and stored frozen separately. Autologous serum was heat inactivated in 56°C waterbath for 30 min, and then centrifuged for 1,640 × g for 10 min at 4°C, and the supernatant was used in the culture where indicated.

2.4. *Flow cytometric analysis*

Surface molecule expression was examined by flow cytometric analyses by the following monoclonal antibodies (mAb): anti-CD14-fluorescein isothiocyanate (FITC), anti-CD19-FITC, anti-CD45, anti-CD105-PE, anti-HLA-DR-PE (eBioscience), anti-CD34-PE (Santa Cruz Biotechnology), anti-CD73-PE (BD Bioscience), anti-CD90 mAb (Serotec, Oxford, UK). FITC or PE labeled isotype control mAbs (eBioscience) were used for directly labeled mAbs, and Alexa488-labeled goat anti-mouse IgG secondary antibody (Molecular Probes) was used for anti-CD45 and anti-CD90 unlabeled mAbs. Cells were stained with mAbs on ice for 30 min, and washed twice by PBS. Secondary antibody was applied for unlabeled mAbs and incubated on ice for 30 min, then washed by PBS. Cells were resuspended in PBS, and analyzed by FACSCalibur (BD Bioscience).

2.5. *Mesenchymal lineage differentiation*

Abilities to differentiate into multiple mesenchymal lineages of expanded human mesenchymal stem cells were examined by Human Mesenchymal Stem Cell Functional Identification Kit (R&D Systems). Briefly, MSC were grown on cover glasses in 24 well plate to 50-70% confluent for osteogenic differentiation, and 100% confluent for adipogenic differentiation. Then, osteogenic differentiation was induced by adding Osteogenic Supplement (dexamthasone, ascorbate-phosphate, and β-glycerolphosphate), and adipogenic differentiation was induced by adding Adipogenic

Supplement (hydrocortisone, isobutylmethylxanthine, and indomethacin) in αMEM Basal Medium (αMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin). Fresh induction medium or αMEM Basal Medium for negative control was replaced every 3-4 days. After 3 weeks, osteogenic differentiated cells were washed by PBS and fixed by 70% ethanol on ice for 60 min and subsequently incubated with 1% Alzarin Red S (Sigma), pH 4.2 for 10 min (*23*). Adipogenic differentiated cells were fixed by 4% paraformaldehyde in PBS for 10 min and then subjected for Oil-Red O (Sigma) staining to visualize the lipid droplets in the cells. For chondrogenic differentiation, 2.5×10^5 cells were transferred in a 15 mL conical tube and cultured as a pellet after centrifugation (200 × g, 5 min) in 0.5 mL of DMEM/F-12 Basal Medium (DMEM/F-12 supplemented with ITS Supplement, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin) with or without Chondrogenic Supplement (dexamethasone, ascorbate-phosphate, proline, pyruvate, and TGF-β3). Medium was replaced with fresh medium every 2-3 days for 3 weeks. The chondrocyte pellet was then fixed with 4% paraformaldehyde in PBS for 20 min at room temperature and washed with PBS for 5 min. The pellets were frozen in O.C.T. compound (Tissue-Tek) and the sections were cut at thickness of 5-10 μm.

2.6. *hMSC proliferation in fibrin clot*

The bone dusts or grown MSC culture were put into fibrin glue, Bolheal (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) to see the growth in scaffold material. Bolheal is prepared by mixing Solution A and Solution B. Solution A contains 80 mg/mL fibrinogen, fibrin-stabilizing factor XIII (75 units/mL), and aprotinin (1,000 KIE/mL) and Solution B contains thrombin (250 units) and 40 μM $CaCl_2$ as effective ingredients. In clinical practices, Solution A and B are mixed in the ratio of 1:1 in volume, but here we diluted both Solution A and B by PBS to 1/8 of original solution before mixing. The bone dusts or expanded MSC culture were first put into diluted Solution A, and then equal volume of Solution B was added to form semi rigid gel. Culture medium was added over the gel for culture. The culture was observed by phase contrast microscope (Zeiss) or confocal microscope (Zeiss, LSM700). Cells were stained with Calcein AM (Invitrogen) 5 μg/mL for 20 min at room temperature.

3. Results

3.1. *Bone dust harvesting*

Use of the routine TLIF drills consistently yielded bone dusts. Although, the amount of bone dust differed and some bone marrow tissues were mixed with a large quantity of fat tissue, the two drill techniques provided sufficient bone dusts. The amount of the bone dusts thus obtained was enough to fill an interdiscal cage, if this is to be used in actual fusion procedure.

Postoperative course of these TLIF patients who donated the bone dusts was uneventful. No patient complained of any symptoms related to the drilling and the harvesting of the bone dusts.

3.2. *MSC-like cells from the bone dusts expanded adherent to plastic plates*

We attempted to culture bone dusts collected during the TLIF procedure from 10 patients (Ages 50-73, MSC1-MSC10). First, we cultured the bone dusts after collagenase digestion, separating BD cells and CR cells as described in "Material and Methods". Since both cells expanded adherently on plastic plates and both had MSC-like phenotype, we did not separate BD and CR cells in the later cultures because the objective of our study was to obtain as many MSCs as possible for the use in spinal fusion surgeries. Some of the bone dusts were immediately cultured without collagenase treatment and they still grew in 10% FBS supplemented DMEM (data not shown). In all cases, cells adherent to plastic plates proliferated and were able to expand to make frozen stock vials.

The bone dusts were also attempted to culture by autologous serum, so that the culture will not contain materials from other species. MSC4 was first plated in 4 groups with different conditioned medium. One was DMEM medium supplemented by 10% FBS, and the other three were supplemented with autologous serum (AS) by 5, 10, and 20%. Although the attached cells grew in 5% AS, the proliferation rate was a little lower than that of 10% FBS medium while 10% AS and 20% AS culture showed almost equal to or even better proliferation (data not shown). Hence, serum concentration of the culture medium supplemented with AS was decided at 10% for the later cell culture.

The culture grown in autologous serum was morphologically quite uniform with smooth outlines while the culture in FBS seemed heterogeneous with some widely spreaded out cells (Figure 2A). There was another difference that the culture with autologous serum was much more sensitive to trypsin-EDTA than the cells grown in FBS when detaching from the plastic surface. This observation is the same phenomenon with the observation of Shahdadfar *et al.* (*24*).

To confirm that the expanded plastic adherent cells are the mesenchymal stem cells, immuno-histological staining was also performed for CD90 (Figure 2B). CD90 was positive for the cells expanded with both FBS or AS supplemented medium.

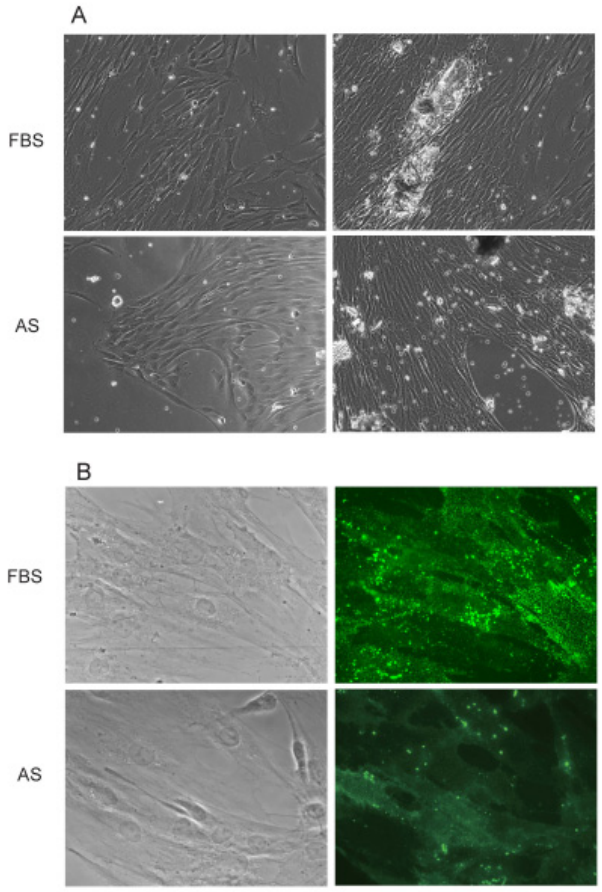

Figure 2. Morphology and immuno-histological staining of hMSC. (**A**) Phase-contrast photomicrograph of the cells expanded with 10% FBS or 10% AS (autologous serum). Original magnification, ×100. (**B**) Cells were stained by anti-CD90 mAb and Alexa 488 labeled secondary antibody. MSCs expanded in 10% FBS or 10% autologous serum supplemented medium are shown. Original magnification, ×400.

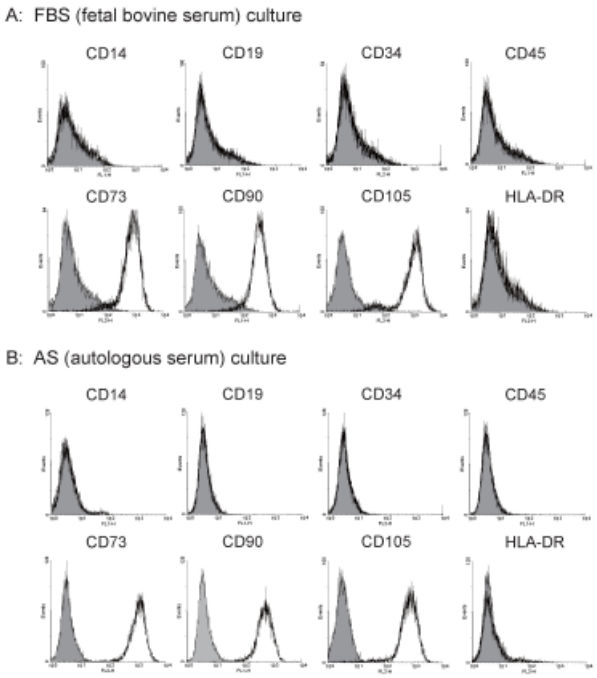

Figure 3. Phenotype of cultured human bone fragments derived mesenchymal stem cells (hMSCs). MSCs expanded in 10% FBS supplemented medium (**A**), or 10% autologous serum (**B**) were examined for their surface antigens by flow cytometric analysis. The histograms shaded in gray are negative controls, and the histograms without any color are the samples stained with respective antigens.

3.3. *Immunophenotypes of the MSC-like cells*

Cell surface markers of the expanded cells were examined by flow cytometric analysis. Both cells that were expanded with FBS (MSC1) or autologous serum (MSC4) had the same phenotype for the antigens examined. More than 95% were positive for CD73, CD90, and CD105, which showed separated fluorescence peak from the negative controls. On the other hand, the cells were less than 2% positive for CD14, CD19, CD34, CD45, HLA-DR, in which the stained fluorescence peaks completely overlapped with negative controls (Figures 3A and 3B). These phenotypes represent mesenchymal stem cells (MSCs) (*25*), and these results strongly suggest that the proliferated plastic adherent cells are indeed hMSCs. The difference between the supplemented serum in culture medium was also seen here, similarly to the optical observation, that the range of forward scatter (FS) and the side scatter (SS) of FBS culture was wider than AS culture (data not shown). This indicates the FBS cultured cells vary in their size and the state of intracellular granules and nucleus, compared to the AS cultured cells. Separating BD (bone derived) cells and CR (collagenase released) cells in preparation did not show any difference in the characteristics of the surface markers of which indicate the cells to be MSCs.

3.4. *MSC-like cells had the abilities to differentiate*

To fulfill the minimal criteria for MSC, ability to differentiate into multiple mesenchymal lineages of expanded human mesenchymal stem cells were examined for MSC1 (FBS culture) and MSC4 (AS culture) utilizing Human Mesenchymal Stem Cell Functional Identification kit (R&D Systems).

Osteogenic differentiation was induced by adding osteogenic supplement in 10% FBS + αMEM medium for 3 weeks. The cells were fixed by ice-cold ethanol and stained with Alizarin Red S to indicate the calcium accumulation. The red staining of calcium were seen in both MSC1 and MSC4, but the amount differed in a great extent (Figure 4A) and the negative controls cultured without differentiation supplement showed no staining at all (data not shown). The cells expanded in FBS medium showed the calcium accumulated staining to cover most of the cells after 3 weeks (about 80%) in differentiation medium (Figure 4A, *left*). On the other hand, the cells expanded in AS medium showed very little staining (about 10-20%) after 3 weeks of

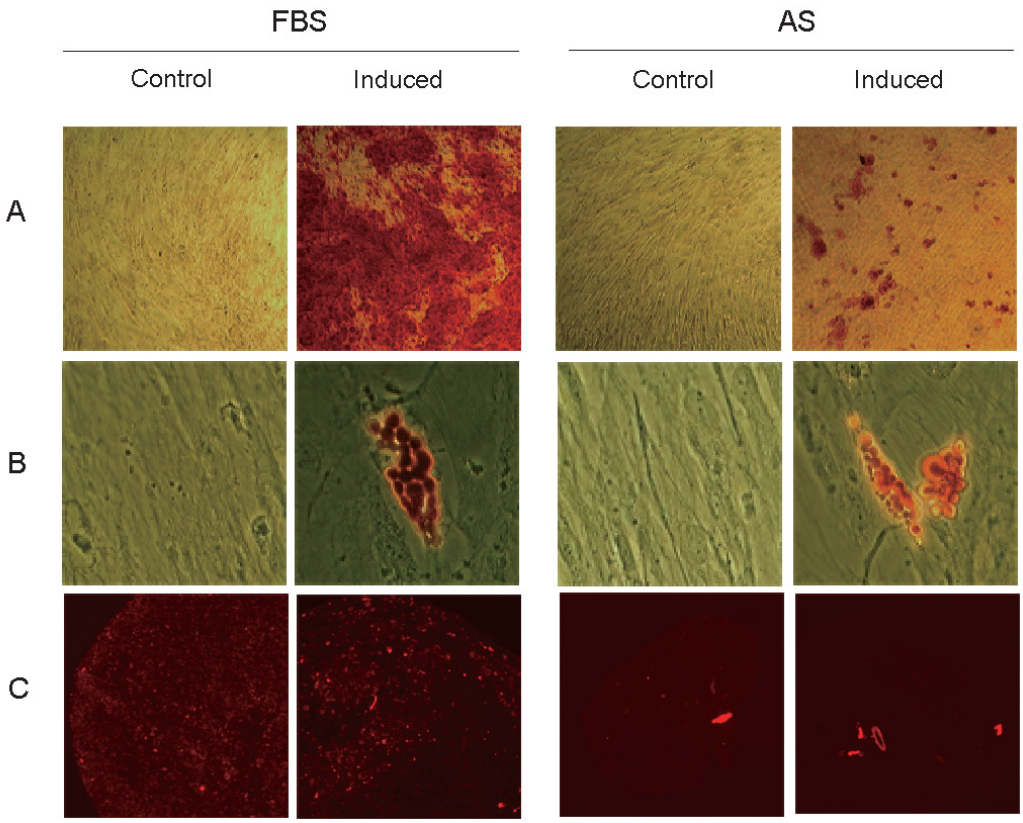

Figure 4. Differentiation ability of hMSCs. hMSCs induced to differentiate in osteogenic direction (**A**) were stained with Alizarin Red S, in adipogenic direction (**B**) were stained with Oil-red O. Original magnification, ×40, ×100, respectively. The cells induced in chondrogenic direction (**C**) were immuno-histochemically stained by anti-human aggrecan antibody as a primary antibody. Original magnification, ×100.

differentiation medium culture (Figure 4A, *right*), but the accumulation did increase after 2 more weeks of culture (data not shown). These results imply AS culture differentiate more slowly than FBS culture and do not jeopardize the ability to differentiate.

To induce differentiation in adipogenic direction, semi-confluent cells were cultured with or without adipogenic supplement in 10% FBS + αMEM medium for 3 weeks. The cells were then fixed and stained with Oil Red O, which stained the lipid vacuoles within the cells. As shown in Figure 4B, both MSC1 and MSC4 showed accumulation of lipid vacuoles within the cells that apparently indicate the cells have differentiated to adipocytes. These vacuoles were absent in negative controls (data not shown). The rate of differentiation was roughly 10-50% and was not stable in each experiment in which may be due to several culture conditions such as the freshness of the differentiation supplements and the passage number of the MSC.

Chondrogenic differentiation was induced in 15 mL conical tubes in a form of pelleted micromass. Although the number of cells at start was the same, the size of the pellets differed between MSC1 (FBS culture) and MSC4 (AS culture) that MSC4 formed much smaller mass. The pellets were examined after 3 weeks of culture by immuno-cytochemistry of human aggrecan, or collagen II to show the characteristic of chondrocyte. Despite the results of osteogenic and adipogenic differentiation, chondrogenic differentiation was not clear. MSC1 (FBS culture) showed human aggrecan (Figure 4C) or collagen II staining (data not shown) in both culture with or without differentiation supplements. The pattern of the staining was different in which the culture without the supplements showed overall dim staining while the culture with the supplements had some bright positive staining section and dark negative part (Figure 4C, *left*). On the other hand, MSC4 (AS culture) did not show much expression of aggrecan or collagen II, in both cells cultured with or without the differentiation supplements (Figure 4C, *right*).

3.5. *MSCs proliferate and migrate well in fibrin clots*

To explore the potential of hMSC in clinical use, expanded cells or bone fragments were cultured in fibrin clots, Bolheal. Instead of using Bolheal as it is prepared for fibrin glue in clinical use, we diluted fibrinogen and thrombin to make more soft gel for culturing the bone dusts or MSCs so that the cells will have enough space to grow and migrate. The

concentration of fibrin was decided based on the studies previously reported (*26*,*27*).

From a bone fragment buried inside Bolheal, growing cells were seen after 12 days of culture. Cells grew outward from the bone fragment in a bundle (Figure 5A) and gradually made a swirl like pattern inside Bolheal. It took about 4-5 weeks for cells grown from a bone fragment to fill the Bolheal gel (100 μL in volume) and expanded cells finally started to appear outside the fibrin clot (Figure 5B).

4. Discussion

Our study showed that the bone dust-derived cells cultured in FBS and also in autologous serum were plastic-adherent and expressed CD73, CD90, CD105, and were negative for CD14, CD19, CD34, CD45 and HLA-DR. In addition, these cells differentiated to osteogenic and adipogenic direction. These evidences strongly suggest that the obtained cells are mesenchymal stem cells, and the bone dusts, the waste produced during the TLIF procedure, could be a practical candidate for the source of MSCs. This report is the first to show that the possibility of using the bone dusts as the source of MSCs. We have also shown that these cells grew well in clinically available serum glue (Bolheal), suggesting it as a possible scaffold to utilize the bone dusts or expanded MSCs as autologous bone grafts.

In the aspect of the future application, this study demonstrated the possibility of using the bone dusts as the source of stem cells in TLIF procedure. Harvesting bone dusts from bone marrow was simple and safe because the process in which provided them was a routine part of TLIF surgery, and caused no complication or postoperative discomfort to the patients. The bone dusts are immediately available during surgery for the transplant as a source of stem cells, without multi-step treatment that is required for bone marrow aspirant. The TLIF surgeons prefer shorter surgical time and the immediate availability of the graft material is an important factor for adopting the techniques. The harvesting and processing of the bone dusts in current technique should satisfy the TLIF surgeons with these regards.

This study also demonstrated that the bone dust-derived cells proliferated well in the Bolheal. We need to further examine that the cells proliferated in Bolheal are also MSCs, and then the ability to differentiate in osteogenic direction within Bolheal. Then, *in vivo*

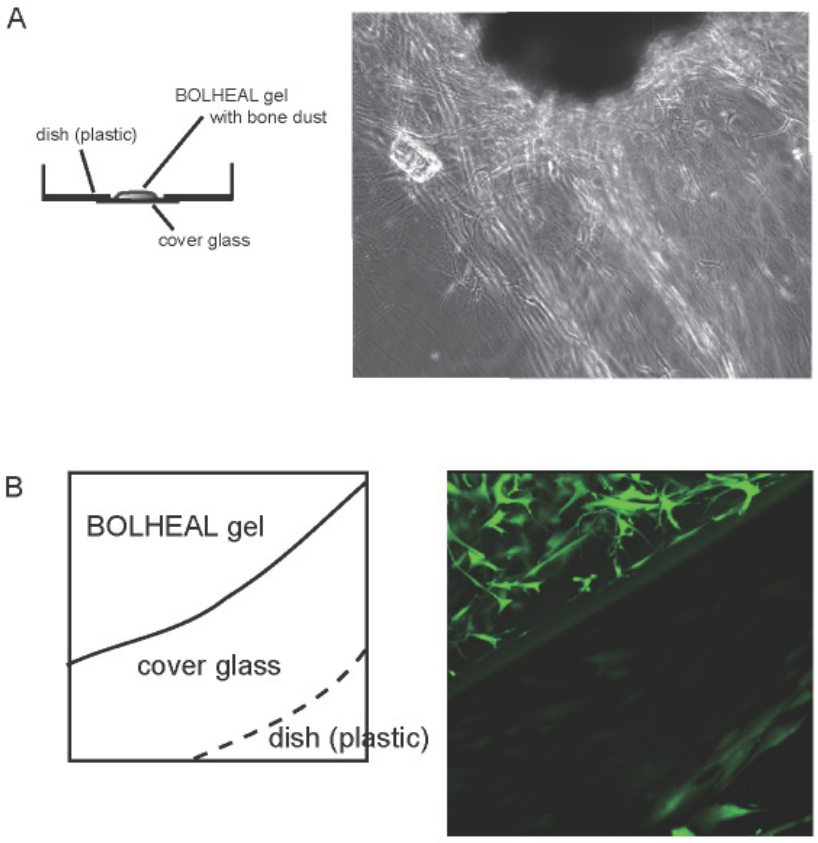

Figure 5. hMSCs culture from the bone fragments in a fibrin clot. Bone fragment was cultured in Bolheal gel with 10% FBS + DMEM medium. (**A**) Cells growing outward from the bone fragment on day 14. (**B**) Bolheal gel culture was stained with Calcein AM and observed by confocal microscope on day 59. Grown cells have come out from Bolheal onto cover glass and further grown to the dish area.

studies in experimental animals are needed to confirm the ability of the graft, Bolheal and the bone dusts or cultured MSCs, to differentiate in osteogenic direction in physiological environment. Our data indicate that the differentiation of AS cultured cells to osteogenic direction was slower or less efficient than FBS cultured cells. This observation needs to be studied more carefully and precisely, on the assumption that xenogenic materials have to be eliminated.

Appropriate osteoinductive materials are also required to be evaluated by not only the physiological effects but also the cost of the material(s). We are considering Vitamin D3 (*28-31*), Resveratrol (*32*), or 17-beta estradiol (*33*) as alternatives for expensive BMP-2.

The yield of stem cells from bone marrow aspirant is known to be inconsistent. The number of mononuclear cells harvested from the bone marrow aspirant in which the stem cells reside has been reported to be 1-2.9% of the collected cells by magnetic devices (*34*) and estimated to be 0.01-0.0001% by a density gradient preparation (*35*). Initial large cell population is a prerequisite for the survival of the transplanted cells. Therefore, keeping the bone dusts in Bolheal provides not only an appropriate scaffold for cell growth but also a safe harbor for survival. In addition, Bolheal can be mixed with osteoinductive materials for the stem cell to differentiate to bone producing cells, the osteoblast cells.

The other aim of current study was to prepare the bone dust-derived stem cells for transplantable grade by eliminating xenogenic material. Firstly, the bone dusts were cultured in autologous serum at concentration of 5, 10, and 20%. The cells proliferated in all of the three concentrations, although the proliferation rate was lower in the medium with 5% autologous serum. The elimination of collagenase treatment of the bone dusts is also in favor of keeping the bone graft non-xenogenic and since we assume to use the bone dusts immediately during the spine fusion surgery in the future application, we also attempted to culture some bone dusts without collagenase digestion. Plastic adherent cells expanded well in the same manner as the collagenase digested bone dusts did, and the characterization of these cells has to be examined. Although the serum glue, Bolheal, is known to be minimally immunogenic, immunological reaction against the serum glue can still be significant (*36,37*). In order to make it completely xenogenic, and allogenic material-free, purified thrombin could be replaced with chemically synthesized material, such as thrombin receptor activating peptide (TRAP) (*38*).

In this study, we have shown a possibility of making another protocol for the TLIF procedure in which Bolheal will be the osteoconductive material and the bone dusts from bone marrow waste for the source of stem cells.

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