Original Article

Efficacy of chitinase-3-like protein 1 as an in vivo bone formation predictable marker of maxillary/mandibular bone marrow stromal cells

Naohiro Komabashiri, Fumio Suehiro*, Masakazu Ishii, Masahiro Nishimura

Department of Oral and Maxillofacial Prosthodontics, Kagoshima University Graduate School of Medical and Dental Science, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan

Article info

Article history:
Received 18 January 2021
Received in revised form 1 March 2021
Accepted 10 March 2021

Keywords:
Jaw bone marrow stromal cells
Bone formation capacity
Chitinase-3-like protein 1
Migration

Abstract

Introduction: Maxillary/mandibular bone marrow stromal cells (MBMSCs) are a useful cell source for bone regeneration in the oral and maxillofacial region. To further ensure the clinical application of MBMSCs in bone regenerative therapy, it is important to determine the bone formation capacity of MBMSCs before transplantation. The aim of this study is to identify the molecular marker that determines the in vivo bone formation capacity of MBMSCs.

Methods: The cell growth, cell surface antigens, in vitro and in vivo bone formation capacity of MBMSCs were examined. The amount of chitinase-3-like protein 1 (CHI3L1) secreted into the conditioned medium was quantified. The effects of CHI3L1 on the cell growth and osteogenic differentiation potential of MBMSCs and on the cell growth and migration of vascular endothelial cells and fibroblasts were examined.

Results: The cell growth, and in vitro and in vivo bone formation capacity of the cells treated with different conditions were observed. MBMSCs that secreted a large amount of CHI3L1 into the conditioned medium tended to have low in vivo bone formation capacity, whereas MBMSCs that secreted a small amount of CHI3L1 had greater in vivo bone formation capacity. CHI3L1 promoted the migration of vascular endothelial cells, and the cell growth and migration of fibroblasts.

Conclusion: Our study indicates that the in vitro osteogenic differentiation capacity of MBMSCs and the in vivo bone formation capacities of MBMSCs were not necessarily correlated. The transplantation of high CHI3L1 secretory MBMSCs may suppress bone formation by inducing fibrosis at the site. These results suggest that the CHI3L1 secretion levels from MBMSCs may be used as a predictable marker of bone formation capacity in vivo.

© 2021, The Japanese Society for Regenerative Medicine. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Autologous bone grafting is the gold standard for reconstructing severe bone defects; however, the method is limited by the quantity of bone that can be collected and potential paresthesia of donor sites [1]. Therefore, an innovative treatment method with less donor burden is required to reconstruct severe bone defects.

Mesenchymal stem cells (MSCs) proliferate in an undifferentiated state and differentiate into bone, cartilage, tendon, muscle, and adipose tissue [2–5]. Various bone regeneration studies using MSCs derived from the iliac crest have been reported [6,7]. While isolation and expansion of MSCs from the iliac crest have been standardized, the collection of MSCs from the iliac crest is not...
common for dentists. Compared with iliac crest MSCs, the collection of maxillary/mandibular bone marrow stromal cells (MBMSCs) from the jaw bone marrow does not require a major surgical procedure, especially for dentists. MBMSCs and iliac MSCs have comparable osteogenic differentiation potential; however, MBMSCs have a weaker potential to differentiate to chondrocytes and adipocytes because of their different stem properties [8,9]. MBMSCs have been a candidate cell source for bone regenerative medicine in the maxillofacial region [10], and several bone regeneration studies using MBMSCs have been reported [11,12]. The population of platting bone marrow cells is heterogeneous; some MBMSCs have progenitor properties and multi-potent differentiation capacity, whereas others are devoid of this capacity [13]. Therefore, to use MBMSCs for bone regeneration therapy, it is necessary to determine their capacity to form bone in vitro at an early culture stage.

Various methods are used to measure the osteogenic differentiation potential of MSCs, such as alizarin red staining, ALP activity quantification, and gene expression level measurements of RUNX2, Osterix, and osteopontin [14,15]. Conversely, mRNA levels of osteogenic markers or various cell surface marker expressions in vitro are not predictable for the in vivo bone formation capacity of iliac MSCs [16]. Cell sorting of iliac bone marrow stromal cells or adipose tissue-derived stromal cells using flow cytometry can collect MBMSCs with high osteogenic differentiation potential [17,18]. However, the number of colony forming stromal cells collected from jaw bone marrow is small and it is difficult to isolate high bone-forming capacity cells by cell sorting in clinical settings. In addition, cell culture for cell transplantation therapy is expensive; therefore, it is necessary to find a simple marker to determine the high bone-forming capacity of cells in vivo at an early stage of culture.

MSCs secrete various factors that influence osteogenesis, angiogenesis, and cell migration in auto or paracrine manners [19,20]. In addition, MSC conditioned medium promotes high bone formation in vivo [21]. We hypothesized that the in vivo bone formation capacity of MBMSCs for transplantation could be predicted in advance by quantifying the factors secreted by MBMSCs into culture supernatant. The purpose of this study is to identify in vivo bone formation predictor markers released in MBMSC culture conditioned medium.

2. Methods

2.1. Human MBMSC culture from maxillary/mandibular bone marrow

Human bone marrow was collected from maxillary/mandibular bone according to a protocol approved by the ethical committee at Kagoshima University. Bone marrow aspirates were collected as previously reported [22]. Briefly, all samples were collected non-invasively using a GC biopsy needle (GC, Tokyo, Japan) and syringe via a hole drilled in the maxilla or mandible during dental implant surgery. Collected bone marrow aspirates (approximately 150 μl) were immediately suspended with 80 μl Heparin Sodium Injection (5000 units/5-ml, MOCHIDA PHARMACEUTICAL CO LTD, Tokyo, Japan). A portion of the collected bone aspirate was analyzed using an automated hematolyzer analyzer (MEK-6358, Nikoh Kohden, Tokyo, Japan), and the white blood cell number and white/red blood cell ratio (W/R ratio) was counted. Bone marrow aspirates from seven donors were seeded at a density of approximately 5 × 10^4 white blood cells/cm² on tissue culture plates and the proliferated cells were used as MBMSCs. The medium was alpha modified Eagle’s minimum essential medium (α-MEM, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic (Thermo Fisher Scientific) (growth medium). MBMSCs were incubated at 37 °C in a humidified incubator and 5% CO₂/95% air atmosphere for 5–7 days with careful handling until cells adhered to the plate. The adhered MBMSCs were fed with fresh growth medium every 3 days. Cell passaging was performed prior to confluence. MBMSCs were suspended with trypsin and EDTA (0.25%; Thermo Fisher Scientific) and seeded at a density of 5 × 10^3 cells/cm² on 100 mm tissue culture dishes (Corning, NY, USA) in 10 ml of growth medium. MBMSCs obtained from the third to fifth passages were used for the following experiments.

Cell growth was examined using a cumulative cell growth curve. MBMSCs were seeded at a density of 5 × 10^3 cells/cm² on 12-well tissue culture dishes and maintained in 1 ml of growth medium until confluent. Just before approaching confluence, MBMSCs were suspended with trypsin and EDTA and counted. The remaining cells were reseeded at a density of 5 × 10^3 cells/cm² for the next passage. This process was continued until the MBMSCs growth stopped.

2.2. Cell growth analysis

Human umbilical vein endothelial cells (Lonza, Basel, Switzerland) (HUVECs) were cultured in endothelial cell basal medium-2 (Lonza), supplemented with EGM-2MV BulletKit (Lonza). Normal human dermal fibroblasts (NHDf, Lonza) and MBMSCs were cultured in growth medium. Each cell was seeded in a 96-well plate at 1000 cells/well and cultured with their growth medium for 6 h and the medium was changed to include CH3LI (0.01, 0.1, 1, 10, or 100 ng/ml). Seventy-two hours later, Premix WST-1 Cell Proliferation Assay System (Takara Bio Inc., Shiga, Japan) was added at 10 μl/well and incubated at 37 °C for 40 min. The absorbance of the solution at 450 nm was measured using a spectrophotometric microplate reader (Multiskan FC; Thermo Fisher Scientific).

2.3. Flow cytometric analysis

MBMSCs were analyzed by flow cytometry to detect cell surface antigens previously reported as criteria for identifying MSCs [23]. MBMSCs were harvested with trypsin and EDTA, centrifuged at 1500 × g for five minutes, and resuspended at 1 × 10^5 cells/ml in PBS containing 0.5% bovine serum albumin, 2 mM EDTA, and FcR Blocking Reagent (Miltenyi Biotec, Bergisch Gladbach, Germany). Aliquots were then incubated with individual antibodies: anti-human CD73 (phycoerythrin (PE)-conjugated mouse IgG1; BioLegend, San Diego, CA, USA), anti-human CD90 (fluorescein isothiocyanate (FITC)-conjugated mouse IgG1; BioLegend), anti-human CD105 (FITC-conjugated mouse IgG1; Ancell Corp., Bayport, MN, USA), anti-human CD14 (FITC-conjugated mouse IgG1; BioLegend), anti-human CD34 (PE-conjugated mouse IgG1; BioLegend), and anti-human CD45 (FITC-conjugated mouse IgG1; BioLegend). FITC-conjugated mouse IgG1 (BioLegend) and PE-conjugated mouse IgG1 (BioLegend) were the isotype controls. Flow cytometric analysis was performed with Guava easyCyte 5 (Merck, Darmstadt, Germany), and data analysis was performed using Guava Express Pro (Merck).

2.4. Osteogenic differentiation

MBMSCs were seeded at a density of 5 × 10^3 cells/cm² in 24-well tissue culture dishes and were fed growth medium until confluent. The MBMSCs were maintained in osteogenic induction medium (α-MEM supplemented with 10% FBS, 0.1 μM Dexamethasone, 50 μg/ml l-Ascorbic acid 2-phosphate, and 10 mM β-Glycerophosphate). MBMSCs were fed osteogenic induction medium every 2–3 days.
2.5. Alkaline phosphatase (ALP) activity assay

Osteogenic differentiated MBMSCs were washed twice with physiological saline, then 0.2% Triton-X100 was added to the culture well and MBMSCs were disrupted on ice for 10 s using a Q55 Sonicator (Qsonica, Newtown, CT, USA). Lysed MBMSCs were centrifuged at 12,000 × g for 5 min. The supernatant was incubated with 16 mM PNPP (SIGMA FAST 2.6. Alizarin red staining activity per minute per milligram protein.

The ALP activity was normalized against the amount of protein in the same well and the value was expressed as ALP activity per minute per milligram protein.

2.6. Alizarin red staining

Osteogenic differentiated MBMSCs were washed twice with a solution containing 10 mM Tris–HCl, pH 7.6, and 0.9% NaCl and then fixed in 95% ethanol at room temperature for 1 h. MBMSCs were stained with 1% Alizarin-red S (Merck) for 10 min at room temperature.

2.7. Experimental animals and transplantation of MBMSCs

The animal selection, management, and surgical procedure were approved by the animal experiment ethics committee of Kagoshima University (Approval Number D20013). Twenty-one, 4-week-old male C57BL/6J immunodeficient mice (KBT Oriental., LTD, Saga, Japan) (SCID mice) were used in this experiment. MBMSCs from 7 samples were transplanted into three mice (N = 3). The mice were anesthetized with a combination of inhalation anesthesia with isoflurane (1%–2%) and intraperitoneal injection of xylazine (0.2%, 0.1 ml). After anesthesia, the mice were cleansed with povidone iodine. After an approximately 1 cm skin incision, muscle dissection, and periosteal elevation, the parietal bone was carefully exposed. The graft was then implanted under the parietal peristeum and the skin and periosteum were sutured with absorbable thread. During surgery, xylazine (2%, 0.1 ml) was administered locally to relieve postoperative pain. In addition, 6 mg/kg of gentamicin was administered intraperitoneally to prevent postoperative infection. After 8 weeks, the mice were euthanized and the transplant site was collected for histological observation.

Graft materials were prepared as follows. Each solution of 5 × 10^5 MBMSCs was mixed with 25 mg β-tricalcium phosphate (β-TCP) (Olympus Terumo Biomaterials Corp., Tokyo, Japan). After incubation at 37 °C for 60 min, the mixture was centrifuged at 35 × g for 1 min and the supernatant was discarded. The pellet of β-TCP with adhered cells was mixed with 3% atelocollagen (KOKEN CO., LTD, Tokyo Japan) and incubated at 37 °C for 30 min for gel formation. These mixtures were transplanted under the periosteum of the parietal bone of the immunodeficient mice (KBT Oriental., LTD, Saga, Japan).

2.8. Histological examination and histometric analysis

Eight weeks after transplantation, mice were euthanized by cervical dislocation after anesthesia with isoflurane. The transplanted site of parietal bone was collected and the soft tissue was removed and fixed in 4% paraformaldehyde for 24 h at 4 °C. Tissue was treated with 25% formalin formate demineralization solution for 1 day, dehydrated with 70% ethanol, and embedded in low-melting-point paraffin. The samples were cut into 5 μm sections with a rotating microtome. Sections were subjected to hematoxylin–eosin and Masson’s trichrome staining. Immunohistological analysis for angiogenesis was performed with CD31 immunostaining (DAB, Anti-CD31 (ab182981): abcam, Cambridge, UK). The in vivo bone formation capacity was expressed as the percentage of bone area per tissue area (BA/TA) in each visual field of transplanted region, excluding the recipient bone and β-TCP. BA/TA was measured using Image-J (National Institutes of Health, Bethesda, MD, USA) and the results are represented by the means ± SD from three different sections of the same sample.

Angiogenesis at the transplanted site was expressed as the number of running blood vessels per unit area, excluding the recipient bone and β-TCP. The number of running vessels was counted in three randomly chosen fields from each section and the mean ± SD was calculated.

2.9. Antibody array of the culture supernatant

MBMSCs were seeded at a density of 5 × 10^3 cells/cm^2 on 100 mm tissue culture dishes and maintained in growth medium until confluent. The growth medium was changed to serum-free medium (α-MEM supplemented with 1% antibiotic–antimycotic) and cultured for 24 h. The MBMSC culture supernatant was collected, and subjected to a Proteome Profiler Human Angiogenesis Array Kit (R&D Systems, Minneapolis, MN, USA), Human Chemokine Array Kit (R&D Systems), and Human XL Cytokine Array Kit (R&D Systems), according to the manufacturer’s protocols.

2.10. Quantification of the secreted protein

The above culture supernatant were assessed by a Human Chitinase 3-like 1 (CHI3L1) Quantikine ELISA Kit (R&D Systems), Human CXCL1/GRO alpha Quantikine ELISA Kit (R&D Systems), Human CCL2/MCP-1 Quantikine ELISA Kit (R&D Systems), Human CCL7/MCP-3 Quantikine ELISA Kit (R&D Systems), and Human IL-8/CXCL8 Quantikine ELISA Kit (R&D Systems) following the manufacturer’s protocols. The absorbance of the solution at 450 nm and 570 nm was measured using a spectrophotometric microplate reader and the readings at 570 nm were subtracted from the readings at 450 nm. Because the number of cells in each MBMSC at the time of confluence was different, the secretion amount of each factor was normalized per 1 × 10^4 cells.

2.11. RNA isolation and real-time reverse transcription polymerase chain reaction

MBMSCs were seeded at a density of 5 × 10^3 cells/cm^2 on 100 mm tissue culture dishes and maintained in growth medium until confluent. Total RNA was isolated with ISOGEN (NIPPON GENE, Tokyo, Japan). Reverse transcription was performed using ReverTra Ace qRCR RT Master Mix (TOYOBO, Osaka, Japan) (mixed Oligo dt Primer and Random Primer) following the manufacturer’s protocol. Quantitative real-time PCR was performed using the CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA) and the THUNDERBIRD SYBR Green PCR kit (Toyobo). The sequences of all the primers used are shown in Table 1. The expression level of each mRNA was normalized to the expression level of GAPDH.

2.12. Cell migration assay

Cell migration assays were performed using Transwell 24-well tissue culture plates made of polycarbonate membranes (Corning) with 8 μm pores. MBMSC, HUVECs, and NHDFs were seeded in the inner chamber of the transwell plate at a concentration of 1 × 10^5 cells/100 μl. The inner chambers were placed in an outer
2.13. Statistical analysis

Data are expressed as the mean ± standard deviation. Statistical analysis was performed using IBM SPSS Statistics Version 26. Comparison between two groups was performed using Student’s t-tests. Statistical analysis for multiple comparisons among groups was performed using one-way ANOVA, and the Tukey post hoc correction was applied.

3. Results

3.1. Characterization of MBMSCs

Maxillary/mandibular bone marrow samples were obtained from seven donors and the cell growth capacity, in vitro osteogenic differentiation potential, and expression profiles of cell surface markers were evaluated. There was inter-sample variation in cell proliferating capacity, and all samples proliferated to at least 1 x 10^6 cells in 60 days of culture (Fig. 1A). ALP activity indicating the early stage of osteogenic differentiation were measured 7 days after osteogenic induction, and alizarin red staining indicating the calcification of the cell layer occurred 14 days after osteogenic induction. MBMSC1, 2, 3, and 4 showed high ALP activity and MBMSC1, 2, 3, and 4 showed obvious calcium deposition (Fig. 1B and C). In the flow cytometric analysis, all MBMSCs showed cell surface antigen expression profiles similar to those previously reported as MSCs (positive for CD73, CD90, and CD105 and negative for CD14, CD34, and CD45) (Table 2).

3.2. Bone formation capacity of MBMSCs

MBMSCs and β-TCP/collagen mixtures were transplanted under the periosteum of the parietal bone of immunodeficient mice and 8 weeks later, the transplant site was collected and histologically analyzed. Newly formed bone was observed continuously from recipient bone; however, the amount of formed bone varied among each MBMSC transplanted group (Fig. 2A). As a result, MBMSCs were divided into a high bone formation group (MBMSC3, 4, 5, 7) and a low bone formation group (MBMSC1, 2, 6) (Fig. 2B).

3.3. Relationship between protein secretion and gene expression level of MBMSCs in vitro with bone formation capacity in vivo

Comprehensive protein analysis was performed using the conditioned medium from MBMSC3, 4, 5 (high bone formation group) and MBMSC6 (low bone formation group). Analysis using three different antibody array kits showed that CHI3L1, GROα, MCP-1, MCP-3, and IL-8 measurements differed between the two MBMSC groups with different bone formation capacity. All these protein amounts were less in the MBMSCs group with high bone formation capacity and more in the MBMSC group with low bone formation capacity (Fig. 3). The amount of specific protein in the MBMSC conditioned medium was then quantified by ELISA. The amount of secreted CHI3L1 was high in MBMSC1, 2, 6 and low in MBMSC3, 4, 5, 7, which indicates that the MBMSC group with high bone formation capacity secreted less CHI3L1, and the MBMSC group with low bone formation capacity secreted more CHI3L1 in the culture stage (Fig. 4A). Table 3 shows the amount of CHI3L1 secreted from MBMSCs during culture and the BA/TA ratio. Among the other four proteins tested (GROα, MCP-1, MCP-3, and IL-8), no correlation between the secreted protein amount and the bone formation capacity was observed (Fig. 4B–E). Similarly, CHI3L1 mRNA expression was negatively correlated with bone forming potential (Fig. 5A), while the other gene expressions did not show any correlation (Fig. 5B–E).
3.4. Effects of CHI3L1 on the cell growth and osteogenic differentiation of MBMSCs

To examine the effect of CHI3L1 on MBMSC growth, cells were incubated with different concentrations of CHI3L1 (0.01–100 ng/ml) for 72 h. Treatment with CHI3L1 did not affect the MBMSC growth (Fig. 6A). To examine the effect of CHI3L1 on the osteogenic differentiation capacity of MBMSCs, ALP activity assay and alizarin red staining were performed. In all MBMSCs, there were no difference in the ALP activity and the degree of calcification regardless of the CHI3L1 concentration (Fig. 6B and C).

3.5. Effects of CHI3L1 on cell growth and cell migration of other cells

Cell growth assays of HUVECs and NHDFs were performed to determine the paracrine effect of secreted CHI3L1 from transplanted MBMSC to surrounding recipient cells. Treatment with CHI3L1 for 72 h showed no significant difference in HUVEC cell growth at any concentration. Alternatively, treatment with 1–100 ng/ml CHI3L1 significantly promoted NHDFs growth (Fig. 7A).

A migration assay was performed to evaluate the migration effect by CHI3L1 on HUVECs and NHDFs. The quantitative analysis showed that CHI3L1 significantly promoted HUVECs migration, peaking at 0.1 ng/ml (Fig. 7B and C). The migration effect of CHI3L1 was observed on NHDFs in a dose dependent manner (Fig. 7D and E). The migration effect by CHI3L1 was not observed in the MBMSCs at any experimental dose (data not shown).

3.6. Histological analysis at the transplantation site

The transplant sites of collagen fibers were evaluated using Mason's trichrome staining. The low bone formation groups (MBMSC1 and 6) showed strong collagen fiber staining near the recipient bone (Fig. 8A) and the high bone formation group (MBMSC3 and 5) was less fibrotic (bone and β-TCP are slightly blue) (Fig. 8A). Immunohistochemical analysis was performed using CD31 antibody to observe angiogenesis. The MBMSC1 transplanted site had significantly more running vessels stained with CD31 than the MBMSC3 and five transplanted sites (Fig. 8B and C). These results show that fibrosis and angiogenesis occurred actively at the site where MBMSCs releasing high concentrations of CHI3L1 were transplanted.

4. Discussion

In this study, the in vivo bone formation capacity of MBMSCs was inconsistent with the results of in vitro MBMSC osteogenic differentiation. Alternatively, since the CHI3L1 secretion pattern from MBMSCs was correlated with the in vivo bone formation capacity of MBMSCs, CHI3L1 may be an in vivo bone formation predictor marker of MBMSCs.

As previously mentioned, human MSCs cultures are composed of a heterogeneous mixture of cells at various stages of differentiation and with distinct osteogenic potentials [24]. Consistent with this report, the MBMSCs used in our experiments showed sample specific variability in the cell growth and osteogenic differentiation.
Indeed, the osteogenic differentiation capacity of MBMSCs collected from other parts of the same donor was different (data not shown). Therefore, the differences in osteogenic differentiation capacity in vitro and/or bone formation capacity in vivo for each MSC are because of differences in the cell composition of the collected MSCs rather than individual differences.

The osteogenic potential of MSCs derived from human iliac crest, femur, and vertebrae declines with aging [25]. The decreased bone formation and fracture repair rate observed with aging is caused by a decrease in the number and/or bone formation capacity of MSCs, and changes in the secretion of cytokines such as interleukin-1β from MSCs [26,27]. Therefore, the factors secreted from MBMSCs may regulate the osteogenic differentiation of MBMSCs in vivo.

CHI3L1, which was identified as an in vivo bone formation predictor marker of MBMSCs, is also known as YKL-40. CHI3L1 is a 39 kDa glycoprotein secreted by various cells such as macrophages, neutrophils, fibroblasts, vascular smooth muscle cells, and endothelial cells [28]. CHI3L1 is involved in normal and tumorigenic angiogenesis [29]. CHI3L1 is also expressed in normal bone marrow and its expression is induced in inflammatory disease and degenerative diseases such as rheumatoid arthritis, asthma, and tumors [28,30–33]. Multiple myeloma patients with elevated serum CHI3L1 have more severe bone destruction, including increased

Fig. 2. Bone formation on mice parietal bone with the transplants (MBMSCs, β-TCP, and atelocollagen). (A) The bottom side of the image shows the orientation of the recipient parietal bone. The transplant site was harvested at 8 weeks for analysis. High-magnification images of hematoxylin–eosin staining show the black boxes in low-magnification. * shows the bone replacement material and the arrows shows newly formed bone. Scale bars: 400 μm. (B) Bone area/tissue area (BA/TA) ratio (mean ± SD, n = 3).
bone resorption activity and a shorter time to bone disease progression [34]. In a mouse model of osteomyelitis, the inhibition of increased CHI3L1 in the bone marrow resulted in increased expression of proteins involved in Notch signaling and decreased inflammation, resulting in a significant reduction in cortical bone destruction [35]. Alternatively, CHI3L1 secretion is increased by IL-6 induced osteogenic differentiation of human MSCs [36]. CHI3L1 might be act as a negative feedback factor for bone formation. Although several reports have been described for CHI3L1 and bone formation, the effect of increased CHI3L1 on the regeneration of bone with MSCs has not been clarified.

Using real-time RT-PCR and ELISA, we have clarified that MBMSCs synthesized and secreted CHI3L1, and there are large inter-sample and/or sample specific variability in its amount. Although it has been reported that CHI3L1 is not synthesized in undifferentiated iliac MSCs [37], some undifferentiated MBMSCs synthesized and secreted CHI3L1 in this study. MBMSCs have lower chondrogenic and adipogenic differentiation capacity [9].

Fig. 3. Antibody arrays of the MBMSC culture supernatant. Protein levels in the supernatant of MBMSC culture determined by antibody-based protein arrays of (A) angiogenesis, (B) chemokine, and (C) cytokines.
and greater osteogenic differentiation capacity than iliac MSCs [38,39]. In addition, MBMSCs and iliac MSCs have been reported to have different gene expression according to DNA microarray analysis [40]. Differences in the amount of synthesized CHI3L1 may be because of differences in the inter-sample properties of MBMSCs and iliac MSCs. Comparing the relationship between the CHI3L1 secretion amount and the amount of new bone formation, MBMSCs that secrete a high level of CHI3L1 had low bone formation capacity, and MBMSCs that secrete a low level of CHI3L1 had high bone formation capacity in vivo. This result showed that the amount of CHI3L1 secreted into the conditioned medium negatively correlates with the in vivo bone formation capacity of the transplanted MBMSCs. We could not find clear correlation between the expression of four proteins (GROα, MCP-1, MCP-3, and IL-8) and the in vivo bone formation capacity of MBMSCs, so they cannot be in vivo bone formation predictor markers of MBMSCs.

MBMSC growth and osteogenic differentiation were not affected by CHI3L1. Therefore, the bone formation capacity by MBMSCs themselves would not be affected by secreted CHI3L1 from MBMSCs. Although treatment with CHI3L1 did not affect HUVEC growth, it promoted NHDF growth in a dose dependent

Table 3
Bone area/tissue area (BA/TA) ratios and CHI3L1 secretion.

| Expression level of CHI3L1 (pg/ml/10,000 cells) | BA/TA (%) |
|-----------------------------------------------|----------|
| MBMSC1 249 ± 18.6                            | 0.24 ± 0.09 |
| MBMSC2 829 ± 148.1                           | 1.84 ± 0.15 |
| MBMSC3 11 ± 25.0                             | 35.2 ± 5.48 |
| MBMSC4 0.09 ± 4.0                            | 14.12 ± 1.24 |
| MBMSC5 35 ± 8.6                              | 25.69 ± 1.74 |
| MBMSC6 646 ± 115.0                           | 1.21 ± 0.24 |
| MBMSC7 0.02 ± 0.0                            | 23.62 ± 2.10 |
manner. Migration of HUVECs and NHDFs were also enhanced by CHI3L1. The effects of CHI3L1 on the growth of NHDFs and migration of HUVECs and NHDFs are consistent with previous reports [41-43]. Furthermore, CHI3L1 infiltrates macrophages and induces the transcription levels of pro-inflammatory cytokines such as IL-6, IL-8, IL-12, IFN-γ, TNF-α, CXCL9, CXCL11, and IL-18 from macrophages, epithelial cells, osteoclasts, and other cells [44]. These pro-inflammatory cytokines promote fibrosis [45]. In addition, increased IFN-γ suppresses osteoclast differentiation [46] and then the imbalance between bone resorption and bone formation leads to abnormal bone remodeling [47]. CHI3L1 secreted by MBMSC had no effect on MBMSC itself; however, CHI3L1 might have a positive effect on in situ vascular endothelial cells and fibroblasts. Indeed, CHI3L1 enhanced the migration of vascular endothelial cells and fibroblasts and promoted angiogenesis and fibroblast growth in our experiments. It is assumed that CHI3L1 released from transplanted MBMSCs induced angiogenesis and fibrosis rather than bone formation.

In the case of low level CHI3L1 secretion into the conditioned medium by undifferentiated MBMSCs, transplanted MBMSCs showed high bone formation in vivo. Within the limitations of this study, CHI3L1 could be used as a negative marker to determine the bone formation capacity of MBMSC. However, because CHI3L1 is not the only factor that affects bone formation in vivo, it is necessary to consider the other factors that may also be involved in bone formation regulation. Furthermore, to clinically apply CHI3L1 as an in vivo bone formation predictor marker of MBMSCs, it is necessary to elucidate the mechanism by which CHI3L1 inhibits bone formation in vivo.

**Fig. 5.** Expression of protein genes in MBMSCs. Total RNA was extracted from MBMSCs and the mRNA levels of (A) CHI3L1, (B) GROα, (C) MCP-1, (D) MCP-3, and (E) IL-8 were determined by quantitative RT-PCR (mean ± SD, n = 3).
Fig. 6. Effect of CHI3L1 on cell growth and osteogenic differentiation potential of MBMSCs. (A) MBMSCs were treated with CHI3L1 (0.01–100 ng/ml) or saline for 72 h, and then a WST-1 assay was performed (mean ± SD, n = 3). (B) The alkaline phosphatase (ALP) activity was measured on Day 7 after induction of osteogenic differentiation with the addition of CHI3L1 (mean ± SD, n = 3). (C) Alizarin red staining of MBMSCs. Matrix calcification on Day 14 after induction of osteogenic differentiation with the addition of CHI3L1 was visualized with alizarin red staining.
Fig. 7. Effect of CHI3L1 on human umbilical vein endothelial cells (HUVECs) and normal human dermal fibroblasts (NHDFs). (A) Cell growth capacity of HUVECs and NHDFs with 0–100 ng/ml of CHI3L1 by the WST-1 assay at 72 h (mean ± SD, n = 3). (B) Representative photomicrographs of migrating HUVECs treated with CHI3L1 (0.01–100 ng/ml) or saline for 6 h. (C) Quantitative analysis of the migrating cells is shown (mean ± SD, n = 3). Scale bars: 200 μm. (D) Representative photomicrographs of migrating NHDFs treated with CHI3L1 (0.01–100 ng/ml) or saline for 6 h. (E) Quantitative analysis of the migrating cells (mean ± SD, n = 3) *P < 0.05, **P < 0.01, ***P < 0.001 (VS. 0 ng/ml). Scale bars: 200 μm.
5. Conclusions

The results of this study indicate that MBMSCs, which secret a small amount of CHI3L1 into conditioned medium, showed high in vivo bone formation capacity. Conversely, MBMSCs that secreted a large amount of CHI3L1 into the conditioned medium showed low in vivo bone formation capacity. The amount of CHI3L1 in the MBMSC culture conditioned medium might be useful as an in vivo bone formation predictor marker of MBMSCs.

Declaration of competing interest

None declared.

Acknowledgments

The authors thank GC for providing the puncture needle; and the patients in Kagoshima University Hospital Special Clinic for Oral Implantology, for the bone marrow samples. This work was supported by Grants-in-Aid for Scientific Research (19K10212, 20H03881) from the Japan Society for the Promotion of Science. We thank Ashleigh Cooper, PhD, from Edanz Group (https://en-author-services.edanz.com/ac) for editing a draft of this manuscript.

References

[1] Younger EM, Chapman MW. Morbidity at bone graft donor sites. J Orthop Trauma 1989;3(3):192–5.
[2] Caplan AI, Bruder SP. Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. Trends Mol Med 2001 Jun;7(6):259–64.
[3] Deans RJ, Moseley AB. Mesenchymal stem cells: biology and potential clinical uses. Exp Hematol 2000 Aug;28(8):875–84.
[4] Friedenstein AJ, Piatetzky-Shapiro II, Petrakova KV. Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol 1966 Dec;16(3):381–90.
[5] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999 Apr 2;284(5411):143–7.
[6] Risbud MV, Shapiro IM, Guttapalli A, Di Martino A, Danielson KG, Beiner JM, et al. Osteogenic potential of adult human stem cells of the lumbar vertebral body and the iliac crest. Spine (Phila Pa 1976) 2006 Jan 1;31(1):83–9.
[7] Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J Cell Biochem 1997 Feb;64(2):295–312.
[8] Igarashi A, Segoshi K, Sakai Y, Pan H, Kanawa M, Higashi Y, et al. Selection of common markers for bone marrow stromal cells from various bones using real-time RT-PCR: effects of passage number and donor age. Tissue Eng 2007 Oct;13(10):2405–17.
[9] Matsubara T, Suardita K, Ishii M, Sugiyama M, Igarashi A, Oda R, et al. Alveolar bone marrow as a cell source for regenerative medicine: differences between alveolar and iliac bone marrow stromal cells. J Bone Miner Res 2005 Mar;20(3):399–409.
[10] Egusa H, Sonoymaya W, Nishimura M, Atsuta I, Akiyama K. Stem cells in dentistry – part I: stem cell sources. J Prosthodont Res 2012 Jul;56(3):151–65.
Phinney DG, Kopen G, Righter W, Webster S, Tremain N, Prockop DJ. Donor-derived extracellular vesicles derived from human bone marrow stromal cells heterogeneity. Stem Cell Rev Rep 2011 Sep;7(3):560–8.

Gan L, Liu Y, Cui D, Pan Y, Zheng L, Wan M. Dental tissue-derived human mesenchymal stem cells and their potential in therapeutic application. Stem Cells Int 2020 Sep 1;2020:8966472.

Wang X, Xing H, Zhang G, Wu X, Zou X, Feng L et al. Restoration of a critical mandibular bone defect using human alveolar bone-derived stem cells and porous nano-HA/collagen/PLA scaffold. Stem Cells Int 2016;2016:8741641.

Peveen-Fischer M, Levin S, Zipori D. The origins of mesenchymal stromal cells. J Cell Physiol 2005 Jan;202(1):41–8.

Davies OG, Cooper PR, Shelton RM, Smith AJ, Scheven BA. Isolation of adipose tissue-derived mesenchymal stem cells using a quantitative proteomics approach. Stem Cell Res 2014 Jan;12(1):153–65.

Marom R, Shur I, Solomon R, Benayahu D. Characterization of adhesion and differentiation markers of human bone marrow stromal cells. J Cell Physiol 2005 Jan;202(1):41–8.

Rosen CJ, Verault D, Steffens C, Cheleuitte D, Glowacki J. Effects of age and estrogen status on the skeletal IGF regulatory system. Studies with human bone marrow mesenchymal stromal cells. J Cell Biochem 1999 Dec 1;75(3):424–8.

Aquino M, Katagiri W, Yoshimi R, Inukai T, Hibi H, Ueda M. Conditioned media from human bone marrow mesenchymal stromal cells promote angiogenesis in a rat myocardial infarction model. J Mol Med (Berl) 2014 Apr;92(4):387–97.

Park YT, Lee SM, Kou X, Karabucak B. The role of interleukin 6 in osteogenic and neurogenic differentiation potentials of dental pulp stem cells. J Endod 2019 Nov;45(11):1342–8.

Bian S, Zhang L, Duan L, Wang X, Sun Y, Possick JD, Pan L, Nicolae R et al. Effect of variation in CHI3L1 on serum YKL-40 level, risk of asthma, and lung function. N Engl J Med 2008 Apr 17;358(16):1682–91.

Recklies AD, Ling H, White C, Bernier SM. Inflammatory cytokines induce production of CHI3L1 by articular chondrocytes. J Biol Chem 2005 Dec;280(50):41213–21.

Ober C, Tan Z, Sun Y, Possick JD, Pan L, Nicolae R et al. Effect of variation in CHI3L1 on serum YKL-40 level, risk of asthma, and lung function. N Engl J Med 2008 Apr 17;358(16):1682–91.

Miyazaki N, Komabashiri, F. Suehiro, M. Ishii et al. Regenerative Therapy 18 (2021) 38.

Osugi M, Katagiri W, Yoshimi R, Inukai T, Hibi H, Ueda M. Conditioned media from human bone marrow mesenchymal stromal cells promote angiogenesis in a rat myocardial infarction model. J Mol Med (Berl) 2014 Apr;92(4):387–97.

Lieder R, Sigurjonsson OE. The effect of recombinant human interleukin-6 on osteogenic and adipogenic differentiation of dental pulp-derived mesenchymal stem cells. Biorens Open Access 2014 Feb 1;3(1):29–34.

Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K et al. T-cell-dependent regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma. Nature 2000 Nov 30;408(6812):600–6.

Prins HJ, Braat AK, Gawlitta D, Dhert WJ, Egan DA, Tjissen-Slump E et al. In vitro induction of alkaline phosphatase levels predicts in vivo bone forming capacity of human bone marrow stromal cells. Stem Cell Res 2014 Mar;12(2):428–40.