Isolation of mesenchymal stem cells from the mandibular marrow aspirates

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Objective. The aim of this study was to determine if mesenchymal stem cells (MSCs) could be obtained from mandible marrow aspirates.

Study design. In 5 patients, 10 mL marrow aspirates were obtained from both the mandible and the iliac crest. Second passage MSCs were characterized by fluorescence-activated cell sorting (FACS) analysis using MSC-specific cell surface marker, and fifth-passage cells were differentiated into 3 mesenchymal tissues in vitro.

Results. Average total cell yields of MSCs were $2.8 \pm 1.8 \times 10^5$ per 10 mL marrow aspirates from the mandible. Immunophenotypes of MSCs isolated from the mandible and iliac crest were highly similar, as indicated by FACS analysis. Differentiation into mesodermal cell types, such as osteocytes, chondrocytes, and adipocytes, was successfully achieved in all MSC isolates from all aspirates.

Conclusions. The MSCs can be isolated from the mandibular aspirates, providing an alternative accessible source of MSCs for the treatment of future dental and craniomaxillofacial diseases.

Many studies have shown that mesenchymal stem cells (MSCs) are able to regenerate dental and maxillofacial tissue defects, such as a dehiscent periodontal ligament, alveolar bony defects, articular defects of the temporomandibular joints, and other maxillofacial bony defects. The results of these studies suggest that MSCs may soon play a key role in regenerative dental and craniofacial therapies.

MSCs were first identified in murine bone marrow as plastic-adherent cells that generated fibroblast colony-forming units. MSCs can proliferate in culture and differentiate into multiple cell types, such as osteocytes, chondrocytes, fibroblasts, and myogenic cells, as well as secrete potent bioactive factors that enable them to regulate the function of other types of cells. These characteristics of MSCs made them a popular cell source for regenerative medicine strategies.

Different adult tissue sources, such as bone marrow, fat, muscle, dermis, and dental tissues, for obtaining MSCs have been discovered. However, bone marrow aspirates still remain the principal source of MSCs for most preclinical and clinical studies, owing to the fact that bone marrow aspirates represent the most accessible and minimally invasive procedure to obtain MSCs. The iliac crest has been the most popular anatomic location for obtaining bone marrow aspirates. Alternative sites include the sternum and proximal ends of long bones, which also contain bone marrow.

It has been suggested that site-specific variation of MSCs in bone marrow exist and that the grafted MSCs retain the properties of the donor site after engrafting at the recipient site. Many clinical, laboratory, and developmental experiments have proven the existence of site-specific properties of progenitor cells in bone marrow. For example, the developmental origin of the craniofacial area, the maxilla, the mandible, including alveolar bone, dentin, pulp and periodontal ligament, are all formed exclusively by neural crest cells. In contrast, axial and appendicular bones such as the iliac crest are formed by mesoderm. A recent study showed that orofacial MSCs formed more bone in vivo, whereas iliac crest MSCs formed more compacted bone that included hematopoietic tissue and were more responsive in vitro and in vivo to osteogenic and adipogenic inductions. Therefore, the ideal source of MSCs for regenerating dental and craniofacial bone defects must be obtained from the maxillofacial area.

From a purely technical perspective, earlier studies have indicated that the donor site for obtaining MSCs is determined primarily by the accessibility of the bone, and the surgeon’s familiarity with the anatomy of the

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area. With this in mind, general dentists or craniomaxillofacial surgeons should feel more comfortable extracting bone marrow from maxillofacial bones, such as the maxilla or the mandible, than they would from the iliac crest or other long bones.

To date, several attempts have been made to isolate MSCs from marrow aspirates at the maxillary tuberosity of the maxilla, because that area is known to be composed of marrow-rich bone. Although MSCs could be successfully isolated from the marrow aspirates, obtaining a significant number of them could not be accomplished in every patient, because the maxillary tuberosity is quite small and the anatomy varies significantly among individuals. Therefore, MSCs isolated from the maxilla might not be consistent enough to establish it as a viable treatment option.

In contrast, the mandible is known to be composed of thicker cortical bone and less marrow than other bones, including the iliac crest, so that it can generate enough power for chewing and speaking. These unique anatomic properties led to the incorrect assumption that mandibular marrow aspirates would be an inadequate source of autologous MSCs for clinical applications. It was also thought that the intraoral approach for obtaining marrow aspirates from the mandible might increase the likelihood of generating a contaminated culture of MSCs because of oral microorganisms. However, the mandible, especially the ascending ramal area, is known to be a feasible donor site for autologous bone grafts in craniomaxillofacial surgeries and thus should have sufficient marrow for an aspiration procedure. Furthermore, the anatomy of that area is remarkably consistent in healthy patients, suggesting that marrow aspirates could be readily obtained from most patients.

Therefore, we hypothesized that mandibular bone marrow aspirates are an accessible and productive source of autologous MSCs for future dental and craniomaxillofacial cell therapies. To confirm this hypothesis, MSCs from mandibular marrow aspirates were isolated, cultured, characterized, and differentiated with standard methods. The yield of MSCs, their immunophenotypic profile, and their differentiation potential from mandibular marrow aspirates were compared with those same parameters from iliac marrow aspirates within the same individual. The results were then scrutinized to determine if mandibular bone marrow MSCs could replace more standard sources of MSCs in future cell therapies.

MATERIAL AND METHODS

Aspiration of bone marrow from the mandible and iliac crest

All procedures were conducted with informed consent and an Institutional Review Board–approved protocol. Bone marrow aspirates were taken from both the mandible and the iliac crest of patients undergoing routine orthognathic surgery (n = 5; age range 18-42 years). Before the mandible aspiration, the internal and external oral areas were aseptically scrubbed and the inferior alveolar nerve was blocked with 0.1% lidocaine. To facilitate insertion of the aspiration needle into the mandible, a small step incision of 1 cm was made on the oral mucosa along the external oblique ridge of the mandible, and a small hole was drilled into the marrow portion with the use of a small round bur. After confirming that blood was flowing out of the hole, an 18-G marrow aspiration needle was inserted into the marrow cavity of the ascending ramal area of the mandible. The approximate depth of insertion was determined at the most abundant area of the marrow, avoiding damage to the inferior alveolar nerve by referring to a 3-dimensional computerized tomographic scan image of the anatomic areas obtained before aspiration. For the iliac marrow aspiration, the anterior iliac crest was sterilized in a routine manner, and the needle was directly inserted into the marrow cavity by passing through the skin, muscle, and overlying cortical bone. In both the mandible and the iliac bone, the marrow was manually aspirated and the aspiration time recorded from the start of the aspiration to when the volume reached 10 mL. The collected marrow aspirates were immediately transported to the laboratory for MSC isolation.

Isolation of MSCs from mandible and iliac crest bone marrow

Mononuclear cells of mandibular and iliac marrow aspirates were separated with the use of a Ficoll-Hypaque solution (density 1.077 g/cm^3; Sigma, St. Louis, MO), maintained in an α–minimum essential medium (α-MEM; Gibco BRL, Carlsbad, CA) supplemented with 15% fetal bovine serum (FBS; Invitrogen, La Jolla, CA) and seeded at 5 × 10^5 cells/cm^2 on tissue culture plastic. Nonadherent cells were removed after 3 days and the cells washed with phosphate-buffered saline solution (PBS; Gibco BRL, Carlsbad, CA). The medium was exchanged twice weekly. At 40%-50% confluence, the fibroblast-like colonies were harvested with 0.25% trypsin–EDTA (HyClone, Logan, UT) and seeded in a culture flask for expansion with ~1:10 splitting ratio.

Immunophenotyping of MSCs

Second-passage cells were trypsinized, washed, and resuspended in PBS at a concentration of 1 × 10^5 cells/mL. Cells were immunolabeled with the following mouse monoclonal antihuman antibodies: HLA-ABC, HLA-DR, CD14, CD29, CD34, CD44, CD45, CD73, CD90, CD105, and CD166 (Becton Dickinsin [BD],
San Jose, CA). Mouse isotype antibodies served as controls (BD). The cells were then washed with PBS and fixed in 1% paraformaldehyde (Sigma). Fluorescence-activated cell sorting (FACS) data were collected using a FACScan flow cytometer (BD) with minimum of 10,000 events.

**Proliferation assays**

At passage 1, MSCs from the mandible (n = 5) and iliac marrow aspirates (n = 5) were cultured at 1 × 10^5 cells/cm² in a 35-mL flask. Cell morphology was observed to determine whether cellular senescence had occurred, and pictures were taken over the next 7 days under phase-contrast microscopy. At 1, 3, and 7 days of culture, cells were harvested and counted with a hemocytometer.

**In vitro differentiation**

Induction of osteogenic, adipogenic, and chondrogenic differentiation was performed according to previously published methods. Briefly, to induce osteogenic differentiation, the MSCs were cultured at 37°C with 5% CO2 in osteogenic medium, consisting of α-MEM supplemented with 10% FBS, 100 nmol/L dexamethasone (Sigma), 10 nmol/L β-glycerol phosphate (Sigma), and 50 μmol/L L-ascorbic acid 2-phosphate (Sigma). The medium was replaced twice a week for 3 weeks. Deposition of extracellular calcium matrix by differentiated cells was examined by Von Kossa stain.

To induce chondrogenic differentiation, ~2 × 10^5 MSCs were placed in a 15-mL polypropylene tube and centrifuged at 500g for 5 minutes. The pellet was cultured at 37°C with 5% CO2 in chondrogenic medium, consisting of high-glucose Dulbecco modified Eagle medium (DMEM) supplemented with 500 ng/mL bone morphogenetic protein 6 (R&D Systems, Minneapolis, MN), 10 ng/mL transforming growth factor β3 (Sigma), ITS+ Premix (6.25 μg/mL insulin, 6.25 μg/mL transferring, 6.25 mg/mL selenious acid, 1.25 mg/mL BSA, and 5.35 mg/mL linoleic acid, at 1:100 dilution; BD), 100 nmol/L dexamethasone (Sigma), 50 μg/mL ascorbate-2-phosphate, 40 μg/mL 1-prolene (Sigma), and 100 μg/mL pyruvate (Sigma). The medium was replaced twice a week for 4 weeks. Deposition of extracellular calcium matrix by differentiated cells was examined by Von Kossa stain.

To induce adipogenic differentiation, cells were cultured at 37°C with 5% CO2 in adipogenic medium consisting of high-glucose DMEM supplemented with 10% FBS, 1 μmol/L dexamethasone (Sigma), 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX, Sigma), 0.2 mM indomethacin (Sigma), and 10 μmol/L insulin (Sigma). The medium was replaced twice a week for 3 weeks. The cells were examined for the presence of neutral lipid vacuoles that stained with oil red O (Sigma).

**Statistical analysis**

All results in this study are presented as mean ± SD. Unpaired t test was used to compare the significance of experimental groups. A P value of <.05 was considered to be statistically significant.

**RESULTS**

**Aspiration of bone marrows from the mandible and iliac crest**

Bone marrow aspirates were successfully collected from both the mandible and iliac bone. Total aspiration time for 10 mL of marrow bloods was ~10 minutes for the mandible and 2 minutes for the iliac crest. The time difference might be attributable to the relative marrow content in each bone. Two of the mandible aspirates required additional holes to allow for the necessary volume to be collected.

In the collection of mandible aspirates, potential complications include damage of the inferior alveolar nerve or dental root, infection due to contamination with saliva, and temporary syncope due to anxiety during the aspiration procedure. Of the 10 cases in the present study, no complication was reported. Therefore, the intraoral approach, which is regarded to be semisepctic, appeared to be safe for collecting mandibular marrow aspirates. However, our sample size was too small to make any definitive statement on this matter.

**Isolation of MSCs**

After removing red cells from the marrow aspirates, mononuclear cells and MSCs from each bone marrow sample were counted (Table I). The iliac marrow aspirates produced ~3 times more mononuclear cells initially (32.3 ± 8.8 × 10^5/10 mL) compared with those from mandibular marrow aspirates (12.1 ± 4.8 × 10^5/10 mL; P < .05). The adherent cells from both samples had a fibroblastic morphology that was maintained over 7 days of culture (Fig. 1). Although a higher number of MSCs were obtained from the iliac marrow aspirates, the percentages of MSCs in the mononuclear cell population obtained from the mandible and the iliac crest were similar (3.35 ± 2.7% and 3.69 ± 2.49%, respectively; P = 0.44). The pattern of proliferative activities in both mandibular and iliac MSCs appeared to be similar (Fig. 2). Significant increase in the number of MSCs at culture day 3 and day 7 was observed in both samples. At culture day 3, mandible and iliac MSCs
washed 3.14 \times 10^5 \text{ cells and } 3.04 \times 10^5 \text{ cells, respectively. At culture day 7, mandibular and iliac MSCs were expanded to } 10.51 \times 10^5 \text{ cells and } 10.32 \times 10^5 \text{ cells, respectively (Fig. 2).}

The success rates for the isolation of bona fide MSCs from both mandibular and iliac marrow aspirates were 100% based on previously established MSC criteria.\textsuperscript{34}

**Immunophenotypes of MSCs**

Flow cytometry was not able to distinguish any differences between mandibular and iliac marrow derived MSCs (Table II). MSCs from both origins were negative for CD14, CD34, CD45, and HLA-DR, which are hematopoietic stem cell markers, and positive for CD29, CD44, CD73, CD90, CD105, CD166, and HLA-ABC, which are positive makers of MSCs (Table II).

**Multilineage differentiation potential**

The ability of MSCs from both anatomic locations to differentiate into mesenchymal lineages was tested using fifth-passage cells from mandibular (n = 5) and iliac (n = 5) marrow. MSCs were induced to differentiate along osteogenic, chondrogenic, and adipogenic lineages under specific cultured conditions. After 3 weeks of induction, no differences in the osteogenic differentiation capacity were detected, 100% for each group (Fig. 3, A and D). No mineralized matrix was observed in the cells that were cultured on regular growth medium.

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**Table I.** Establishment of number of mesenchymal stem cells (MSCs) cultured from mandible- or ilium-derived mononuclear cells

<table>
<thead>
<tr>
<th>No.</th>
<th>Age/ gender</th>
<th>Volume (mL)</th>
<th>Mononuclear cells ( \times 10^6 )</th>
<th>MSCs (passage 1) ( \times 10^5 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mandibular marrow (5 samples)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>22/M</td>
<td>10</td>
<td>4</td>
<td>3.15</td>
</tr>
<tr>
<td>2</td>
<td>28/F</td>
<td>10</td>
<td>5</td>
<td>1.075</td>
</tr>
<tr>
<td>3</td>
<td>45/F</td>
<td>10</td>
<td>6.4</td>
<td>1.3</td>
</tr>
<tr>
<td>4</td>
<td>18/M</td>
<td>10</td>
<td>30</td>
<td>3.1</td>
</tr>
<tr>
<td>5</td>
<td>23/M</td>
<td>10</td>
<td>15</td>
<td>5.5</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td></td>
<td>12.1 ± 10.9</td>
<td>2.8 ± 1.8</td>
</tr>
<tr>
<td>Iliac marrow (5 samples)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>22/M</td>
<td>10</td>
<td>45.6</td>
<td>8.75</td>
</tr>
<tr>
<td>2</td>
<td>28/F</td>
<td>10</td>
<td>23.2</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>45/F</td>
<td>10</td>
<td>12.8</td>
<td>3.3</td>
</tr>
<tr>
<td>4</td>
<td>18/M</td>
<td>10</td>
<td>60</td>
<td>37.55</td>
</tr>
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<td>5</td>
<td>23/M</td>
<td>10</td>
<td>20</td>
<td>2.4</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td></td>
<td>32.3 ± 19.7</td>
<td>13.4 ± 14.4</td>
</tr>
</tbody>
</table>

The number of total mononuclear cells was counted before initial seeding procedure. The MSCs from mandibular and iliac marrow were calculated on culture day 7.

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Fig. 1. In vitro expansion of mandibular or iliac marrow–derived mesenchymal stem cells (MSCs). At culture day 7, mandible or iliac marrow–derived mononuclear cells were attached and growing well. The mandible-derived MSCs (A, C) and iliac-derived MSCs (B, D) show similar morphology. Original magnification \( \times 100 \).
After 4 weeks of chondrogenic induction, no significant differences were detected. There was no difference in the percentage of cells positive for safranin O between mandibular marrow (60%) and iliac marrow (60%) derived cells (Fig. 3, B and E).

After 3 weeks of adipogenic induction, 100% (n = 5) of both mandibular and iliac marrow samples consisted of cells with an adipogenic phenotype (Fig. 3, C and F). No adipogenic phenotype was induced in control cells that were cultured on standard growth medium.

**DISCUSSION**

Cellular therapies have emerged as leading candidates for regenerative treatment of a variety of diseases, including dental and craniofacial disease, for example, defective tissue regeneration of defective periodontal ligament or maxillofacial bones. In particular, MSCs, a population of adult-derived adherent cultured cells, have shown great promise in numerous clinical trials. Bone marrow remains the most easily accessed source of autologous MSCs for cell-based therapies.

MSCs from the mandible for regenerating dental and craniofacial tissue regeneration may have several clinical advantages. First, the mandibular approach might enable dentists or craniomaxillofacial surgeons to more easily collect marrow aspirates for a clinical use because of their familiarity with the mandible. Better patient compliance in the outpatient office might be achieved because the aspiration procedure will be performed at the similar area where a main dental or maxillofacial treatment would be conducted. These could facilitate increase popularity and wider use of MSCs in the field. Second, the site-specific character of the isolated MSCs from the mandible may contribute to a better clinical outcome in dental and craniomaxillofacial regeneration. The mandible contains less bone marrow but offers protection for vital structures, such as air sinuses, dentition, and the neurovascular bundle. In contrast, the ilium, a part of the pelvic girdle, is physiologically adapted for support of body weight, contains more red marrow, and contributes more to hematopoiesis. These features representing site-specific skeletal characteristics suggest the existence of a unique cell population and support the rationale for using the mandible as a source of marrow aspirates for craniomaxillofacial cell therapies.

One of the critical factors for a successful clinical application of MSCs is to obtain enough cells. In the present study, the initial yield of the MSCs from mandibular aspirates was 3 times lower than that of iliac aspirates. A possible reason for this might be the lower volume of marrow in the mandible compared with the ilium. For certain clinical applications (i.e., engineering of artificial cartilage), low initial cell yield might make the mandible less attractive. This is because these cases require a large quantity of cells to maintain cell-to-cell contact within the reconstructed recipient site. Interestingly, the relative percentages of MSCs among the mononuclear cells isolated from the mandible (1%-7%, 7-fold range) and the iliac bone (0.07-7%, 100-fold range) are much higher than the average value reported previously, because it is generally accepted that MSCs represent only 0.01%-0.001% of the total nucleated cells within the bone marrow. This suggests that the mandible may represent a more predictable and consistent source of MSCs compared with the iliac bone.
The problem of low cell yield might be resolved in several ways. As we did in 2 cases in our study, the simplest of these would be to collect more marrow aspirates from additional sites in the mandible, although that requires additional surgical trauma. Another solution would be extensive in vitro expansion of the MSC population before use. In fact, 3 days of culture were sufficient to expand MSCs obtained from the mandible to reach the same cell number as that initially obtained from the iliac (data not shown). Also, recent advances in culture techniques and the use of extracellular matrices carrying supportive growth factors and cytokines may also mitigate the problem of lower initial cell yield.

Akintoye et al. reported that MSCs obtained from the mandibular marrow bones showed proliferative activity superior to those from iliac marrow. They suggested that this higher proliferative activity was derived from more blood supply and faster turnover rate of the mandibular marrow cells than of the ilium. However, in our study, proliferative potential of the MSCs from the 2 bones did not show significant difference. This apparent inconsistency between the 2 studies may be due to the fact that Akintoye et al. used marrow bone chips whereas we did marrow aspirates as a source of the mandibular MSCs. Further study is needed to clarify this disparity.

In this study, long-established criteria were used to define MSCs from the marrow blood aspirates. However, differences in the definition of MSCs do exist. To bring uniformity to this issue, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed minimal criteria to define human MSCs. First, MSCs must be plastic-adherent when maintained in standard culture conditions. Second, MSC must express CD105, CD73, and CD90 integrin and adhesion molecules and lack expression of CD45, CD34, CD14, hematopoietic related antigens, and HLA-DR surface molecules. Third, MSCs must differentiate to osteoblasts, adipocytes, and chondroblasts in vitro. Although these criteria will probably require modification as new knowledge unfolds, this minimal set of standard criteria will foster a more uniform characterization of MSCs.

In the present study, individual variation in the yield of MSCs was observed in both bones (Table I). For
clinical applications, variations in cell yield among individuals should also be considered. Muschler et al. reported a significant age-related decline in the number of nucleated cells harvested per aspirate for both men and women. They also observed that the number of osteoprogenitor cells harvested per aspirate decreased significantly with age for women, but not for men. Consistent with that study result, we observed individual variations in MSC yield among individuals by age and gender, although these differences proved to be not statistically significant. Therefore, considering these individual differences in cell yield, further studies are needed to establish an optimal protocol for acquiring a sufficient number of MSCs from mandibular aspirates to achieve consistent clinical outcomes.

In summary, the present study indicates that MSCs can be isolated and expanded from the mandibular marrow blood aspirates, which provides an alternative and accessible source for the collection of autologous MSCs for use in regenerative dental and craniofacial therapies.

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