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J Dent Res 93(1):55-61, 2014

ABSTRACT

Cell therapies utilizing mesenchymal stem cells (MSCs) could overcome limitations of traditional treatments for reconstructing craniofacial tissues. This large-scale study explored a standardized methodology for the isolation and clinical-scale expansion of alveolar bone marrow–derived MSCs (aBMSCs). We harvested 103 alveolar bone marrow samples from 45 patients using 1 of 3 standardized methodologies. Following aBMSC isolation, cells were characterized through cell-surface marker expression and lineage-specific differentiation. Long-term cultures (> 50 population doublings [PDs]) were evaluated for transformational changes through senescence, gene expression, and karyotyping. Finally, aBMSC bone-forming potential was determined *in vivo*. More than 0.5 cc of bone marrow was needed to predictably isolate aBMSCs, and, regardless of methodology for harvest, cell-surface marker expression of CD73, CD90, CD105, and Stro-1 was similar for aBMSCs, being 89.8%, 98.8%, 93.8%, and 3.2%, respectively; all cells were negative for CD11b, CD19, and CD45. aBMSCs exhibited multipotency, and karyotypes were normal up to 30 PDs, with significant cell senescence beginning following 35 PDs. Additionally, aBMSCs induced ectopic bone formation following subcutaneous transplantation into mice. These findings demonstrate a predictable approach for the isolation and safe clinical-scale expansion of aBMSCs, and thus, their clinical use could be considered for craniofacial regenerative therapies.

KEY WORDS: mesenchymal stem cells, bone marrow, cell therapy, craniofacial tissue engineering, cultured cell, bone marrow cells.

DOI: 10.1177/0022034513510530

Received August 6, 2013; Last revision September 19, 2013; Accepted October 6, 2013

A supplemental appendix to this article is published electronically only at <http://jdr.sagepub.com/supplemental>.

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Standardization and Safety of Alveolar Bone–derived Stem Cell Isolation

INTRODUCTION

A major challenge in regenerative cell therapy is identification of the most appropriate cell source. Mesenchymal stem cells (MSCs) offer great potential in regenerative medicine and tissue engineering strategies because of their multipotency, ability to mediate trophic factors, and immunomodulatory properties (Caplan, 1991; Pittenger *et al.*, 1999; Shi *et al.*, 2005; Mao *et al.*, 2006; Satija *et al.*, 2007). Though their multipotency facilitates their direct involvement in the regenerative response of a tissue through direct differentiation and replacement of damaged tissue, their release of soluble growth factors may prove to be even more significant in the regenerative process (Caplan, 2007). Additionally, the recent identification of their ability to modulate the immune response has introduced yet another important role in their use (Ren *et al.*, 2008; Kikuri *et al.*, 2010).

Historically, MSCs were first isolated and expanded from marrow aspirates of the iliac crest (bone marrow stromal cells; BMSCs) (Friedenstein *et al.*, 1978). Though isolation and expansion of MSCs from this site have become standardized, the difficulty of the bone marrow aspiration technique precludes routine therapeutic use for MSC isolation, particularly for craniofacial cell therapy. As such, it has more recently been demonstrated that MSCs can be isolated from other tissues, including fat, extracted teeth, periosteum, and alveolar bone (Zuk *et al.*, 2001; Miura *et al.*, 2003; Seo *et al.*, 2004; Matsubara *et al.*, 2005; Akintoye *et al.*, 2006; Crisan *et al.*, 2008). Though MSCs derived from these other tissues exhibit phenotypic features similar to those of bone marrow–derived MSCs, the feasibility, standardization, and predictability of isolating and expanding these cells have not been thoroughly examined in the context of their clinical utility (Rayment and Williams, 2010). We recently reported a randomized controlled clinical trial on the use of an autologous stem cell–based therapy for the treatment of localized craniofacial defects using a standardized process for cell isolation and expansion of MSCs derived from bone marrow of the iliac crest (Kaigler *et al.*, 2012). Though clinical results of this study demonstrated promise, other evidence suggests that, for craniofacial regeneration, a better cell source may be from craniofacial tissues (Matsubara *et al.*, 2005; Akintoye *et al.*, 2006).

To this end, we investigated the feasibility and standardization of a routine clinical procedure to generate MSCs from small samples of alveolar bone marrow tissue. The hypothesis underlying this investigation was that MSCs could be predictably isolated from human alveolar bone marrow (aBMSCs) and expanded *ex vivo* to clinical-scale numbers while maintaining MSC properties and genetic stability.

A

exposure of harvest site

trephine bone core harvest

bone core

B

needle aspiration

marrow
aspirate

C

jawbone core

jawbone aspirate

core/aspirate
combo

Figure 1. Clinical harvesting techniques of alveolar bone marrow. aBMSCs were derived from bone cores, bone marrow aspirates, or a combination of bone core and marrow aspirate samples. **(A)** An edentulous area (space not containing a tooth) of the jawbone was exposed and a 2-mm-diameter trephine bur was used to remove bone cores of different lengths (range, 0.5-8 mm) for isolation of aBMSCs. **(B)** A needle connected to a heparinized syringe was inserted into the marrow space of the 2-mm-diameter osteotomy site prepared by the trephine bur. A 0.1- to 1-cc quantity of marrow was then aspirated from these sites for isolation of aBMSCs. **(C)** Photomicrographs of aBMSCs 7 to 10 days following initial plating show their fibroblastic, spindle-shaped morphology, similar to that of MSCs. Though there was no difference in the population-doubling time (PDT) between aBMSCs derived from the core and combination samples (from passage 1 to passage 3), PDTs for cores ($*p < .05$) and combinations ($**p < .05$) were significantly lower than those for aBMSCs derived from aspirates.

MATERIALS & METHODS

Tissue Harvest and aBMSC Isolation

To establish a standardized methodology for harvesting aBMSCs, we obtained alveolar bone marrow specimens from 45 patients undergoing routine dental implant placement, following University of Michigan Institutional Review Board approval (IRB #HUM00034368). After informed consent was obtained, we administered local anesthesia and raised a full-thickness

mucoperiosteal flap to gain access to the edentulous area where patients were having dental implants placed. To harvest the bone marrow cores, we used a 2-mm rotary trephine bur to prepare an osteotomy until the marrow space was reached (Fig. 1A). Cores were removed and placed in sterile saline. Next, a 22.5-gauge needle connected to a heparinized syringe was inserted into the marrow space, and approximately 0.5 cc of marrow aspirate was obtained (Fig. 1B). In 12 samples, the aspirate sample that was obtained was combined with the bone marrow scraped from the bone core, and these were denoted as “combination/combo”

samples. Thus, aspirate, core, and combination samples were obtained. To isolate and expand aBMSCs in culture, we processed tissue samples in the manner previously described for MSC isolation from the iliac crest, with slight modifications (see the Appendix for full details).

Population Doubling

The average population-doubling time (PDT) was calculated between passage 1 (P1) and passage 3 (P3) as t/n , where t is the duration of culture in days and n is the number of population doublings (PD), calculated according to the formula $n = (\log N_h - \log N_i) / \log 2$ (Ben Azouza *et al.*, 2012). N_h denotes the number of cells harvested at the time of counting at the end of a passage prior to subculture, and N_i refers to the number of cells initially plated at the beginning of the passage.

MSC Characterization

In accordance with the International Society for Cellular Therapy (ISCT) (Dominici *et al.*, 2006), MSCs were defined by aBMSC differentiation into chondrogenic, adipogenic, and osteogenic phenotypes (Pittenger *et al.*, 1999) and by expression of MSC markers with flow cytometry: CD45, CD90, CD73, CD105, CD34, CD19, and CD14 (Dominici *et al.*, 2006) (see Appendix).

Telomere Length, Gene Expression, Senescence, Karyotyping

The TeloTAGGG Telomere Length Assay Kit (Roche, Indianapolis, IN, USA) was used to perform the Telomere Assay (Appendix). Telomere lengths were determined by analysis of terminal restriction fragments (TRF) calculated by the following:

$$\text{TRF} = \Sigma(\text{OD}_i) / \Sigma(\text{OD}_i / L_i),$$

where OD_i is the chemiluminescent signal and L_i is the length of the TRF at position i .

- Gene expression of tumor suppressor and proto-oncogenes was also evaluated with RT-PCR (Appendix).
- Cell senescence was evaluated with a histochemical staining kit and cytogen (Sigma), and karyotyping was performed from 3 different patient samples by Cell Line Genetics (Madison, WI, USA) (Appendix).

In vivo Bone Formation

The bone-forming capacity of 19 different aBMSC populations (8 aspirates, 7 cores, 4 combinations) was evaluated qualitatively in a subcutaneous mouse model, as previously described (Nör *et al.*, 1999; Kaigler *et al.*, 2005). Collagen/calcium phosphate sponges (CopiOs™, Zimmer, Warsaw, IN, USA) loaded with aBMSCs were implanted, and analysis of bone formation was performed 4 wks following implantation. Four implants containing no cells were also implanted as a control. Tissue sections were categorized in 1 of 3 categories based upon whether or not bone could be identified in the retrieved implant: (1) no bone; (2) minimal bone - bone could be detected but in < 50% of the tissue section; and (3) abundant bone - bone present in > 50% of the tissue section (see Appendix for details).

Statistical Analysis

Statistical analysis was performed with the use of Instat software (GraphPad Software, San Diego, CA, USA). All data were reported as mean $\pm$ standard deviation, unless otherwise noted. Statistically significant differences were determined by two-tailed Student t tests, and statistical significance was defined as $p < .05$.

RESULTS

aBMSC Isolation from Marrow Tissue

We collected 103 bone marrow samples from 45 patients and, of these, 93 were able to generate aBMSCs (Appendix Table 1). There was a significant difference in the expansion success rates between cells derived from aspirates (82%) and those derived from either core (97.5%; $p = .02$) or combination (100%; $p = .04$) samples (Appendix Table 2). Following 1 to 2 wks of initial plating of the samples, cells could be identified which had morphological characteristics similar to those of fibroblastic, spindle-shaped MSCs (Fig. 1C). As measured by PDT, cell proliferation of aBMSCs derived from aspirate, core, and combination samples was evaluated, and it was demonstrated that, in early culture (passages 1-3), the proliferation was at least twice as fast for core ($p < .001$) and combination ($p < .001$) samples relative to aBMSCs derived from marrow aspirates (Fig. 1C).

MSC Characterization

Following isolation and cell expansion (up to 5 passages or < 30 population doublings), all aBMSCs derived from core, aspirate, and combination samples expressed high levels of CD73 (> 97%), CD90 (> 95%), and CD105 (> 90%) (Fig. 2A). There were no differences noted in expression of these markers among the tissues harvested (core/aspirate/combo) (Fig. 2B). Stro-1 expression was also evaluated, and its expression was no different among core ($4.6 \pm 1.9\%$), aspirate ($6.2 \pm 2.2\%$), and combination ($3.5 \pm 1.9\%$) samples. For 5 different aBMSC populations from each tissue type, cell-surface marker expression of CD11b, CD19, CD34, and CD45 was also evaluated, and there was no expression of these markers following cell expansion, confirming their identity as MSCs (data not shown). Additionally, cell-surface marker expression was evaluated from 5 randomly selected aspirate and core samples immediately after the tissue was harvested, prior to expansion (Appendix Table 3). Analysis of these data indicates significant enrichment of CD73, CD90, and CD105 cell populations following expansion of aBMSCs in culture.

MSC multipotency was confirmed through lineage-specific gene expression and phenotypic differentiation toward different cellular lineages following culture under adipogenic, chondrogenic, and osteogenic conditions (Appendix Fig.).

Genetic Stability following Clinical-scale Expansion: Telomere Length, Gene Expression, Cell Senescence, Karyotype

To evaluate the safety of aBMSC expansion, we first evaluated telomere length, since it has been shown that telomere length can indicate aging of MSCs (Baxter *et al.*, 2004). The mean

Figure 2. Cell-surface marker expression of aspirate, core, and combination samples demonstrated high expression of MSC markers following expansion. **(A)** aBMSC populations consistently yielded high proportions of cells which were positive for CD73, CD90, and CD105 and negative for CD45 (bottom panel shows negative controls for top panel). **(B)** Following cell expansion, there was no difference in the cell-surface marker expression of CD73, CD90, or CD105 among aBMSCs derived from aspirate, core, and combination samples.

telomere lengths from 3 different aBMSC cultures derived from aspirate, core, and combination samples are shown in Fig. 3A. It was determined that in early- (P1-P4; PD < 25) and middle-passage (P5-8; < 50) aBMSCs, there was no significant shortening (< 1 kbp) in mean telomere length. To evaluate transformational alterations in gene expression, we measured the expression of tumor-associated genes over time. Tumor suppressor genes p53, ARF, DBCCR1, and the proto-oncogene c-myc were evaluated and demonstrated no significant changes in gene expression over time (Fig. 3B). Additionally, evaluation of cell senescence demonstrated that aBMSCs were not transformed, since there were significantly ($p < .05$) greater proportions of cells which became senescent over time (Figs. 3C, 3D). Finally, normal karyotypes, exhibiting no clonal or numerical abnormalities, were seen following long-term culture of aBMSCs (> 50 PDs) from three different patients (Fig. 3E).

In vivo Bone Formation

Four implants were placed into each mouse, and 4 wks after implantation, implants were removed from the mice and processed for histological evaluation. H&E staining provided histological evidence of bone formation in tissue specimens from each of the cell types implanted (derived from core, aspirate, and combination samples). High magnification of representative sections of the implants show vascularized tissue with areas of woven bone

trabeculae (Fig. 4A). In implants containing no cells, there was no evidence of bone formation. Various degrees of bone were present in the implants (Fig. 4B), and of the 87 aBMSC samples retrieved following implantation (8 samples were not retrieved due to the loss of 2 animals), evidence of bone formation was noted in 80 of them (Fig. 4C). Of the 7 samples where no bone was detected, 4 of them were transplanted aBMSC populations from aspirates, 1 was from a core, and 2 were from combination samples.

DISCUSSION

MSCs from the iliac crest have been considered the “gold standard” against which all other more recently identified MSCs have been compared. However, from a feasibility and therapeutic standpoint, procuring cells by this approach works against the principles of cell therapy, which aim to regenerate defective tissues from relatively small fractions of tissue (Langer and Vacanti, 1993). In the present study, 3 reproducible clinical techniques were used for the isolation of MSCs from alveolar bone (aBMSCs). Though core and combination samples were the most predictable for generating aBMSCs, aBMSC expression of CD73, CD90, and CD105 was no different among tissue samples. aBMSCs

exhibited multipotency, genetic stability over time, and the ability to form *de novo* bone when implanted subcutaneously.

Other studies have demonstrated that osteogenic cells can be isolated from alveolar bone tissues by various methodologies (Mailhot and Borke, 1998; Matsubara *et al.*, 2005; Akintoye *et al.*, 2006; Cicconetti *et al.*, 2007; Jo *et al.*, 2007). Yet, only one of these has defined the cell populations as MSCs according to the ISCT (Dominici *et al.*, 2006), and none has described a standardized, predictable methodology for tissue harvest with a large population of patients. Matsubara *et al.* (2005) isolated BMSCs from marrow aspirate specimens taken during various dental procedures. The overall success of BMSC expansion from aspirates was 70%, yet greater success was achieved with cell expansion from aspirates collected during tooth extraction or reconstructive surgeries, compared with aspirates obtained during dental implant placement. In our study, using the standardized protocol described herein, seven clinicians were able to isolate aBMSCs from osteotomy sites created prior to dental implant placement in 45 patients. Additionally, we were able to define specific isolation parameters for success, in that, when harvesting bone cores > 2 mm in length or collecting > 0.5 cc of marrow aspirate, we could predictably isolate aBMSC populations with 100% success and expanded them to clinical-scale numbers in 2 to 3 wks. Higher expansion success associated with these tissue volumes could be due to greater total cell numbers harvested, in that, when > 6.4×10^8 cells were collected in bone marrow aspirates, aBMSCs could be predictably isolated for

Figure 3. Genomic stability following clinical-scale expansion of aBMSCs. Telomere length, gene expression, senescence, and karyotyping all demonstrated that, following long-term culture of aBMSCs, no transformational changes occurred in cells. **(A)** Southern blot of telomere restriction fragments from 3 populations of aBMSCs at early (E, < 25 population doublings) and middle/intermediate (M, 25-50 population doublings) stages of expansion demonstrated no changes in mean telomere length over time. **(B)** Following RT-PCR, a representative photograph of aBMSC gene expression of the tumor suppressor genes p53, ARF, DBCCR1, and the proto-oncogene c-myc. At early (PD15), middle (PD34), and late (PD52) stages of cell expansion, no changes in gene expression occurred over time (PD = population doubling). **(C)** Photographs showing representative staining of aBMSCs undergoing senescence in early (PD < 25), middle (PD 25-50), and late (PD > 50) stages of cell expansion. **(D)** Quantitative evaluation of cell senescence demonstrated that as cells are expanded and passaged in culture, the percentage of cells undergoing senescence significantly increases from early to middle passages (* denotes $p < .05$), and from middle to late passages (** denotes $p < .05$). This indicates that aBMSCs are not bypassing their normal growth cycle through expansion. **(E)** Following long-term culture (> 50 PDs), aBMSCs exhibited a normal karyotype (representative of 3 aBMSC populations karyotyped).

Figure 4. *De novo* bone formation *in vivo*. aBMSCs from aspirate, core, and combination samples were placed on collagen/calcium phosphate sponges and implanted subcutaneously into immunocompromised mice. Four wks following implantation, implants were removed and qualitatively assessed for bone formation. **(A)** H&E staining showed histological evidence of bone formation in representative tissue specimens from sponges containing aBMSCs derived from core, aspirate, and combination samples, and no aBMSCs. High-magnification images show vascularized tissue with areas of woven bone trabeculae (magnification, 200x; arrows denote regions of bone). **(B)** Low-magnification images are shown of the implants illustrating the global distribution of the tissue throughout the sponges (magnification, 40x). **(C)** In evaluations of 19 aBMSC populations (8 aspirate, 7 core, 4 combination; $n = 5$ implants per condition) retrieved following implantation, the degree to which there was bone formation was scored according to the following qualitative scale: (1) no bone, (2) minimal bone (< 50% of tissue in implant is bone), or (3) abundant bone (> 50% bone in implant).

expansion. This is an important consideration if this approach is to be advanced for clinical cell therapy.

Another safety consideration in cell therapy is that of long-term *ex vivo* cell culture. We have previously demonstrated that, following 2 wks of *ex vivo* expansion of iliac crest—derived bone marrow progenitor and stem cells, 1.5×10^7 cells are sufficient to reconstruct localized craniofacial defects (Kaigler *et al.*, 2013). In the present study, these cell numbers were generated 2 to 3 wks following aBMSC isolation. It has been well documented that, following long-term cell culture, MSCs can undergo transformational changes (Baxter *et al.*, 2004; Kim *et al.*, 2009; Duggal and Brinchmann, 2011), yet investigation of the genetic stability of long-term cultures of alveolar bone—derived cells is lacking. Poloni and colleagues evaluated fetal MSCs and found that, relative to bone marrow—derived MSCs, fetal MSCs had greater expansion potential in culture and did not undergo transformational changes in long-term cultures (up to 70 PDs) (Poloni *et al.*, 2011). These results are in agreement with those of our study, in that we did not observe abnormal changes in gene expression, karyotype, or senescence in long-term cultures (> 50 PDs). This finding holds significance in that it is important to establish that cell expansion does not result in genetic instability and is safe, prior to the development of clinical protocols that use these cells. Additional safety analyses, including *in vivo* evaluations of tumor formation, are planned as next steps toward clinical investigation.

In our study, we also evaluated age, location of the jaw, previous bone grafting of the tissue harvest site (3–9 mos prior to harvest), the surgeon who harvested the tissue, and the amount of tissue harvested as variables that could potentially influence aBMSC parameters. The only parameter affected by these variables was PDT, and it was affected by the location of the site and the history of grafting in the site (Appendix Table 4). In early cultures (< passage 2; < 10 PDs), sites which had been previously grafted had a lower PDT than those that had not, and cells isolated from the upper jaw had a lower PDT than those from the lower jaw. Both of these variables have a common feature in that more marrow spaces are present in these regions, and, as such, these

regions are associated with high cellularity. The upper jaw is inherently comprised of bone which is more cancellous relative to that of the lower jaw (Misch, 1989). Similarly, regions of the jaw actively undergoing the regenerative process following bone grafting are comprised of less mature bone with high cellularity. Hence, it is not surprising to see that aBMSCs from the upper jaw and from previously grafted sites initially had a higher proliferation rate (lower PDT).

An important step in advancing cell therapy approaches toward clinical protocols is the standardization of methodologies; key points of standardization include origin of the cells, procurement method, characterization, expansion potential, and safety following expansion. As noted previously, several studies have reported findings which begin to address some of these points, yet, to our knowledge, there are no other studies which have evaluated all of these feasibility parameters on a large scale. Our study is the first to define specific criteria necessary for predictable isolation and safe clinical-scale expansion of aBMSCs using universally accepted guidelines established by the ISCT. Additional pre-clinical studies are needed for further investigation of the regenerative potential and safety of aBMSCs using longer time points and comparisons of bone-regenerative outcomes of stem cells derived from other skeletal tissues (*i.e.*, iliac crest, dental pulp). These are necessary next steps in preparation for clinical investigation of their regenerative capacity.

ACKNOWLEDGMENTS

The authors acknowledge, in addition to co-authors D.K. and S.M., the other five clinicians who participated in the harvesting of alveolar bone tissue samples. The authors also acknowledge the University of Michigan Flow Cytometry Core and University of Michigan School of Dentistry Histology Core. This study was funded by the Burroughs Wellcome Fund (CAMS) and the University of Michigan Faculty Grants Program (OVPR). The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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