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Ex Vivo Adipose Tissue Engineering by Human Marrow Stromal Cell Seeded Gelatin Sponge

LIU HONG,[1] IOANA PEPTAN,[2] PAUL CLARK,[2] and JEREMY J. MAO[1]

[1]Departments of Orthodontics and [2]Department of Bioengineering, University of Illinois at Chicago, Chicago, IL

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Abstract—The limitation of current clinical treatment for restoration extended defects of soft tissue associated with trauma, tumor resections, and congenital deformities are well known. This study demonstrates that human bone marrow stromal cells (MSCs) can be utilized to tissue engineer adipose tissue for therapeutic purposes. Adipogenic potentials of monolayer-cultured human MSCs were evaluated by biochemical measurement of an adipogenic differentiation marker (glycerol-3-phosphate dehydrogenase, G-3-PDH) and cellular morphology. After preparation by seeding human MSCs on a 3-dimensional gelatin sponge and exposure to adipogenic differentiation medium, the *ex vivo* tissue-engineered adipose constructs were assessed histomorphologically and biochemically. Lipid droplets accumulated and expanded within the constructs accompanied by a significant increase of G-3-PDH activity. The present study indicates that bone MSCs could be a cell resource in tissue engineering adipose tissue, while gelatin sponge could be a good scaffold in this approach to improve the outcome of clinical treatment.

Keywords—Human MSC, Adipose tissue engineering, Gelatin sponge.

INTRODUCTION

Extended defects of soft tissue associated with trauma, tumor resections, and congenital deformities rarely regenerate spontaneously.[23–24] The conventional clinical treatment approach is to transplant autologous grafts to restore the defects. However, limitations of this procedure include donor-site morbidities and deformities. Furthermore, transplantation is associated with unsatisfactory long-term results.[3,17,31] Although the use of biocompatible synthetic materials as autologous implants possesses some advantages over autografts, such as sufficient supply, easy manageability, and low cost, immunorejection and poor long-term results frustrate their practical application.[11] Adipose tissue is a primary component of the soft tissues. To explore a promising tool to overcome current limitations and to regenerate adipose tissue, tissue engineering has been proposed as a new approach for the restoration and regeneration of adipose tissue via application of growth factors or biocompatible scaffolds seeded with autologous cells.[2,4,7,11–15,18,25,28,31,32,34,35]

Bone marrow stromal cells (MSCs), also named mesenchymal stem cells, have been proven to display multiple differential potentials into different lineages of cells including osteoblasts, chondrocytes, myoblasts, tenocytes, and adipocytes.[6,16] Due to the minimally invasive harvesting procedure coupled with strong proliferative ability *ex vivo*, bone MSCs are considered as a promising cell resource for engineering several types of tissues. With support of appropriate biocompatible scaffolds, some prototypes of tissues have been successfully created using bone MSCs.[1,21,27] However, few studies have reported adipose tissue engineering using MSCs. In the present study, we hypothesized that adipose tissue could be engineered by MSCs seeded on scaffolds. To this end, based on qualitative and quantitative determination of the adipogenic potentials of human bone MSCs, we seeded human bone MSCs on biodegradable and biocompatible gelatin sponges and induced *ex vivo* adipogenic differentiation of human MSCs as evaluated qualitatively and quantitatively using biochemical and histomorphometrical measurements.

MATERIALS AND METHODS

Isolation of Human Bone MSCs

Human bone MSCs were isolated from fresh bone marrow of adult males and provided commercially by AllCells (AllCells Limited Liability Company, CA.). Nucleated cells of the fresh bone marrow were counted under inverted microscope, plated at a density of approximately 10^7 nucleated cells per 100-mm petri dish, and incubated in a basic culture medium composed of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C and 5% CO_2. Human MSCs were isolated from bone marrow cells by removing unattached cells via

Address correspondence to Liu Hong, MD, PhD, Department of Orthodontics, University of Illinois at Chicago, MC 841, 801 S. Paulina Street, Chicago, IL 60612-7211. Electronic mail: liuhong1@uic.edu

the first time medium exchange 5 days after the plating. Human MSCs were incubated to proliferate until 70–80% confluent. The cells were trypsinized and subcultured as Passage-1 with an initial density of 2×10^5 cells per dish. The cells used in the present study were Passage-5.[5]

Adipogenic Differential Potential of Monolayer Cultured Human MSCs

Human MSCs were incubated on 6-well plates at an initial density of 10^5 cells/well in basic medium. Upon reaching 80% confluence, the basic medium was replaced with adipogenic medium composed of basic medium with adipogenic supplements including 50 nM of Dexamethasone, 10 mM of insulin, and 5 mM of isobutyl-methylxanthine. Initiation of adipogenic differentiation was considered as day-0. The medium was exchanged twice a week. On defined days, the cells were collected and evaluated qualitatively and quantitatively as measured by biochemical and morphological methods.

Preparation of Tissue-Engineered Adipose Construct

Sterile gelatin sponges commercially provided (Gelfoam@, Pharmacia & Upjohn, Kalamazoo, MI) were used as scaffolds in the present study. The characteristics of the gelatin sponges were observed by scanning electron microscopy (SEM) (Hitachi, S-3000N). The gelatin sponge was trimmed into $4 \times 4 \times 4$ mm^3 cube, and prewetted with basic medium for 1 h. Tissue-engineered adipose constructs were prepared by immersing the prewetted sponges into a prepared human MSC suspension with a cell density of 3×10^6 cell/ml in a tube. A slight vacuum was created in the tube by using a 20-ml syringe to accelerate cell seeding evenly throughout the sponges. Then the mixture of sponges and cell suspension was incubated at 37°C for 2 h.[9] The constructs of cell-seeded sponges subsequently were transferred to a petri dish containing adipogenic medium. The medium was exchanged twice a week. At defined time points, tissue-engineered adipose constructs were collected and subjected to biochemical and histomorphometric measurement.

DNA Content and Glycerol-3-Phosphate Dehydrogenase (G-3-PDH) Activity

The proliferation rates of human MSCs under adipogenic differentiation were quantitatively determined by measurement of DNA. On the defined day, either monolayer-cultured cells or tissue-engineered constructs were washed twice with phosphate buffered saline (PBS, pH 7.4) and immersed in 0.5 ml of 1% Triton-X100 for 15 min. The Triton-X100 solution along with either scraped monolayer-cultured cells or tissue-engineered construct was transferred to a small vial followed by homogenization using sonication (Dismembrator Model 100, Fisher Scientific.). The DNA concentration of cell lysate was measured fluorometrically using Hoechst dye 33258 (Bio-rad Laboratories). The fluorescent optical density of each sample was measured using a fluorometer having an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The amount of DNA in each sample was determined by using a prepared standard curve.[29]

The extent of adipogenic differentiation was assessed biochemically by measurement of G-3-PDH activity spectrophotometrically.[28] The cell lysate was measured for G-3-PDH activity in a final concentration of 100-mM triethanolamine-HCl (pH 7.5), 2.5-mM EDTA, 0.12-mM NADH, 0.1-μM β-mercaptoethanol, and 0.2-mM dihydroxyacetone phosphate. The absorbance at 340 nm was monitored in a spectrophotometer at 30°C at 60-s intervals for 3 min. 1 mU of enzyme activity was defined as the amount catalyzing the oxidation of 1-nM NADH/min.[22,19]

Histomorphometry

Oil-red-O staining, a specific staining for intracellular lipid accumulation, was used to assess morphologically the adipogenic differentiation of human MSCs. At defined time points, monolayer cultured cells were fixed by 10 wt% formalin for 15 min and subjected to the staining. The tissue-engineered adipose constructs were fixed by 10% formalin solution and frozen cut into 10 μm-thick sections at the middle of each construct. The sections were stained with Oil-red-O followed by histomorphometrical analysis to quantitatively measure the extent of adipogenic differentiation. Microphotographs were taken on each stained section of tissue-engineered construct using a microscope connected to a digital image analyzer. The red-stained areas representing lipid accumulations in each section were measured by image analysis software (*Imagepro Plus*), and the extent of adipogenic differentiation was indicated by the percentage of red-stained area within the section.

Statistical Analysis

All quantitative data were expressed by *mean* $\pm$ *standard* deviation. Student *T* tests were used to compare the extent of adipogenic differentiation in treatment groups using adipogenic medium and controls with basic medium. p Values <0.05 were considered as significant.

RESULTS

Adipogenic Differential Potential of Monolayer-Cultured Human MSCs

Human MSCs with morphology similar to fibroblasts were isolated from human bone marrow. After 1 week of incubation in 6-well plates with adipogenic differentiation medium, the cells morphologically resembled adipocytes, exhibiting rounded appearance. Intracellular lipid droplets,

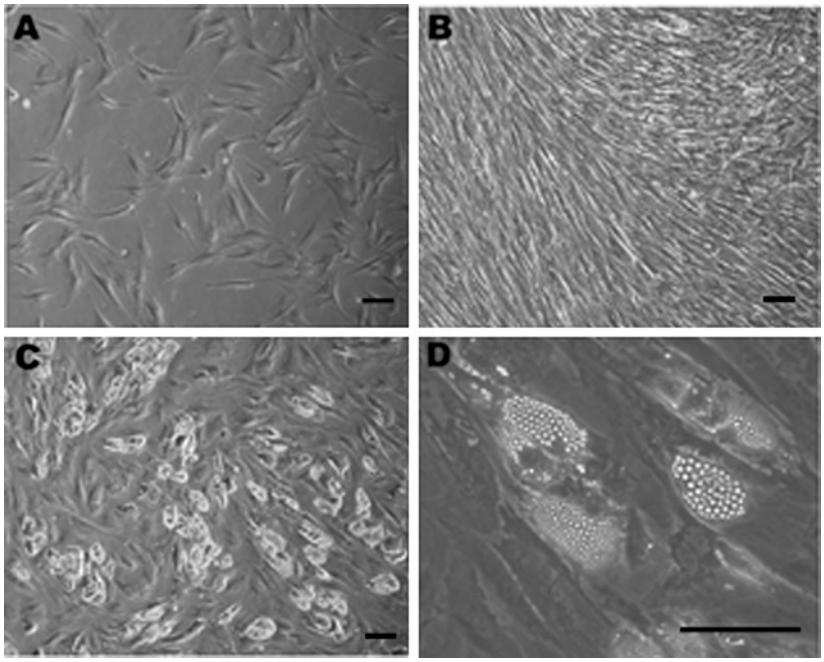

FIGURE 1. Morphology of adipogenic differentiating human bone MSCs. (A) human bone MSCs. (B) Confluence of MSCs 3 weeks after incubation in basic medium. (C) Human MSCs after 3 weeks exposure to adipogenic medium. (D) Intracellular lipid vesicles in adipogenic differentiating human MSCs. Phase contrast microscopy. *Bar* = 100 μm.

specific ultrastructural features of adipocytes, were observed under a higher magnification of inverted microscopy. In contrast, control MSCs exposed to the nondifferentiation medium retained their fibroblast-like spindle shapes (Fig. 1). The proliferation rate of human MSCs slowed after exposure to adipogenic differentiation medium, as compared to control cells exposed to basic medium [Fig. 2(A)]. Seemingly, the MSCs proliferated more slowly once the cells initiated adipogenic differentiation. The amount of G-3-PDH activity from cell lysate of the human MSCs increased with exposure time to adipogenic medium. The cellular differentiation efficiency was improved with time also, calculated by normalizing G-3-PDH activity to unit gram of DNA. Ten-fold higher activities of G-3-PDH were determined in cells of the adipogenic differentiated group after 3 weeks as compared to control cells exposed to basic medium [Fig. 2(B)]. A positive reaction of Oil-red-O staining, specifically demonstrating accumulations of lipid droplets, was observed in the monolayer cultured human MSCs under adipogenic differentiation. The intracellular lipid accumulation of the adipogenic differentiated cells seemingly increased with time of incubation. In contrast, no positive reaction of Oil-red-O staining was found in the controlled groups even after 6 weeks (Fig. 3).

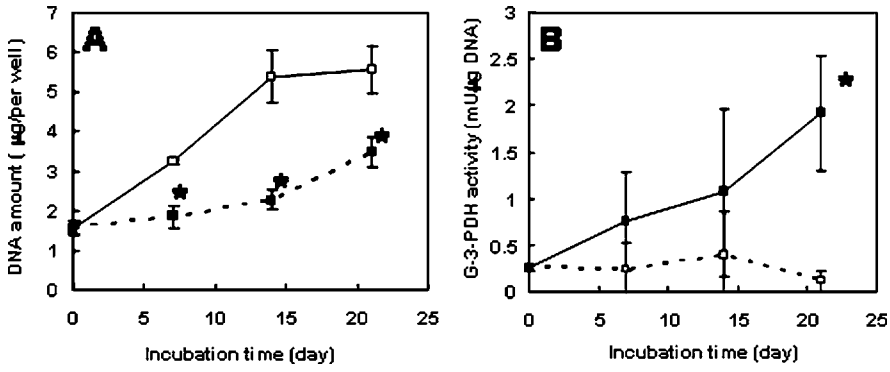

FIGURE 2. Quantitative assessment of adipogenic differentiating human MSCs. Comparison of DNA content (A) and efficiency of G-3-PDH of human MSCs (B) incubated in basic medium (□) and adipogenic medium (■). Each group consisted of three samples. *: $p < 0.05$ compared to the control group.

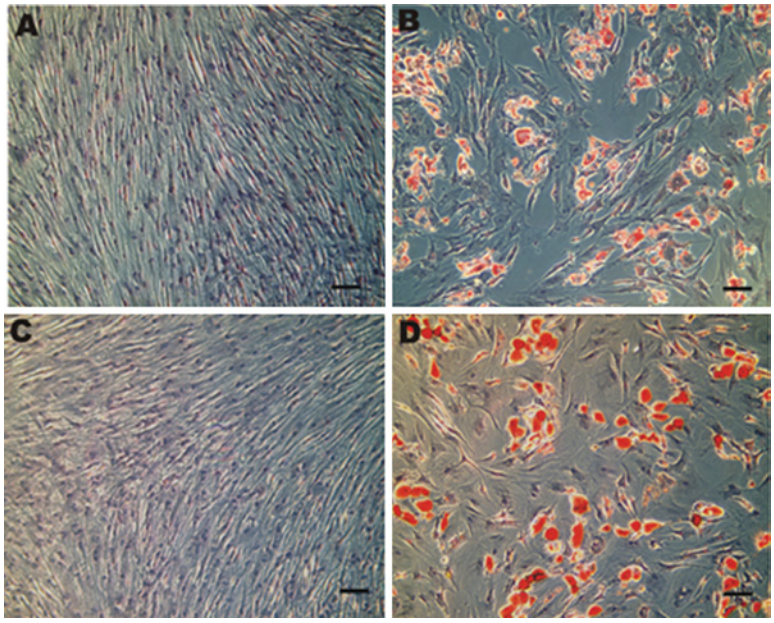

FIGURE 3. Qualitative assessment of adipogenic differentiation of human MSCs. Adipocyte specific Oil-red-O staining on human MSCs after 3-and 6-week incubation in basic medium (A and C, respectively) and adipogenic differentiation medium (B and D, respectively). Positive reaction expressed by red staining indicates lipid formation, a specific morphological characteristic of adipogenic differentiation. *Bar* = 100 μm.

Ex Vivo Adipose Tissue Engineering of Gelatin Sponge Seeded with Human MSCs

The highly porous gelatin sponge with pore size of 200–400 μm was used in the present study [Fig. 4(A)]. After gelatin sponges seeded with human MSCs were cultured in adipogenic medium for 4 weeks, lipid droplets were observed under an inverted microscope [Fig. 4(B)]. Adipogenic differentiated cells, characterized by intracellular lipids, were evenly distributed within the constructs. More extensive accumulation of lipid droplets was also found

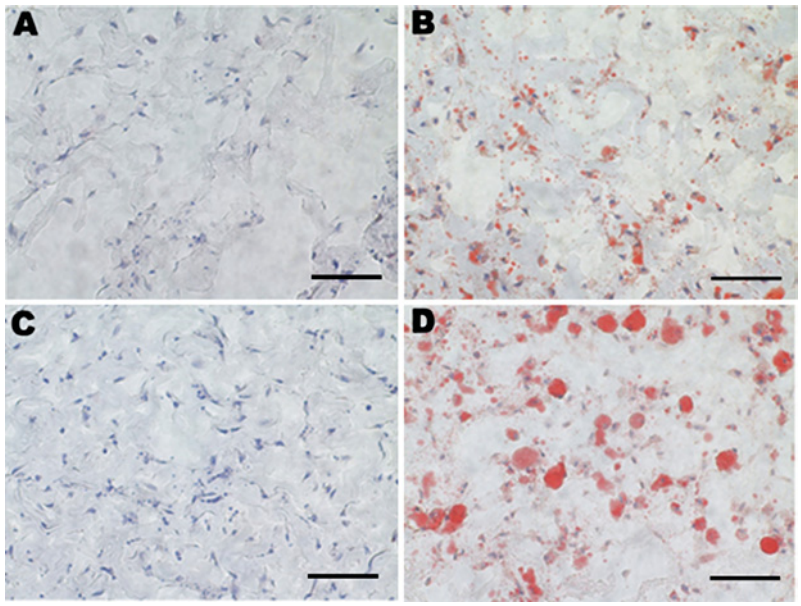

FIGURE 5. Microphotograph of histological sections of *ex vivo* adipose tissue-engineered constructs. Oil-red-O staining is on histological section of human MSC seeded gelatin sponges 4-and 6-week incubation in basic medium (A and C, respectively) and adipogenic differentiation medium (B and D, respectively). Positive reaction was only found in the adipogenic differentiated constructs (B and D). *Bar* = 100 μm.

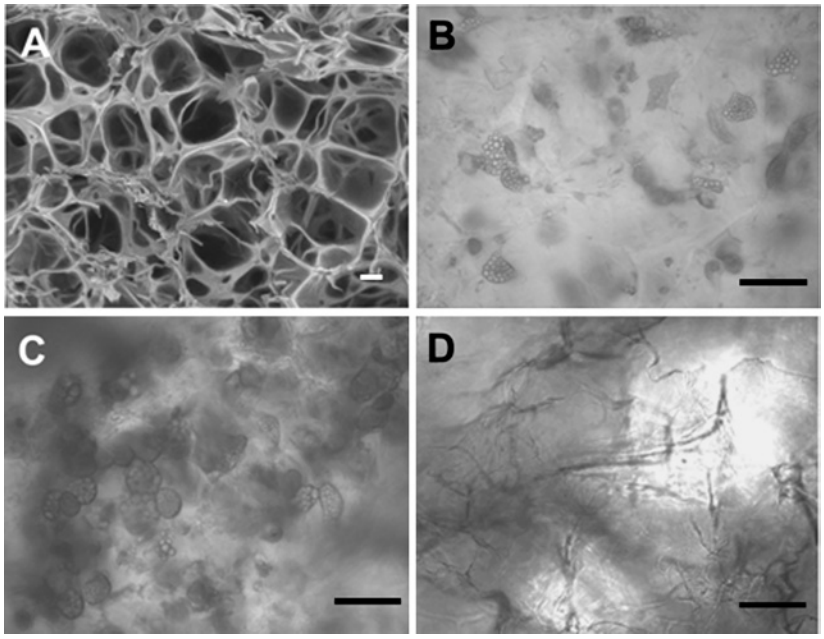

FIGURE 4. Morphology of adipogenic differentiating tissue-engineered construct consisting of human MSC seeded gelatin sponges. (A) microarchitecture of gelatin sponges characterized by SEM. Lipid vesicles occurred in tissue-engineered construct exposed to adipogenic medium 4 (B) and 6 weeks (C). Negative observation of tissue-engineered construct in basic medium after 6 weeks (D). Phase contrast microscopy, *Bar* = 100 μm.

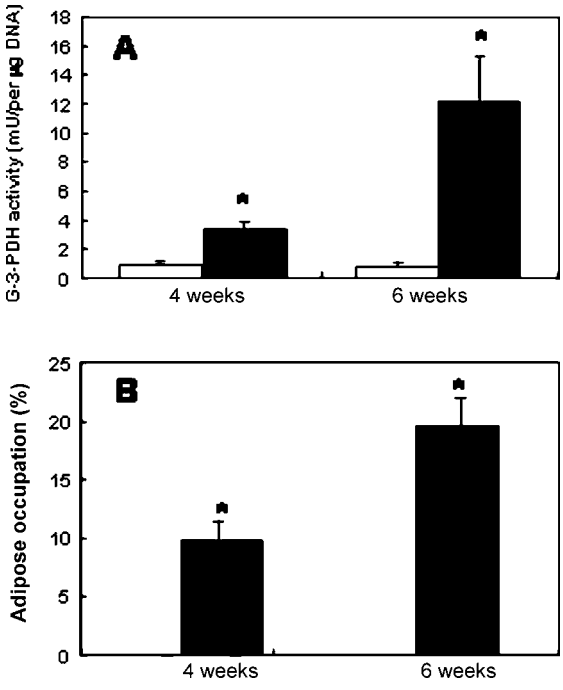

FIGURE 6. Quantitative assessment of extent of adipogenic differentiation of human MSC seeded gelatin sponges in tissue engineered constructs. G-3-PDH activity (A) and histomorphological adipose volume (B) of tissue-engineered constructs by human MSCs seeded on gelatin sponges were compared between the adipogenic differentiation group (closed column) and control groups with basic medium (open column). Each group consisted of three samples, *: $p < 0.01$ compared to the control groups.

after 6 weeks of differentiation time [Fig. 4(C)]. In contrast, no lipid droplets were observed in the control construct sponges seeded with MSCs cultured in basic medium [Fig. 4(d)]. Histological staining by Oil-red-O revealed that extensive triaceglyceral containing lipid droplets accumulated in the constructs of MSCs seeded in gelatin sponge 4 and 6 weeks after adipogenic differentiation. The extent of the accumulation seemingly increased with extended incubation time [Fig. 5(B) and 5(d)]. No positive reaction of Oil-red-O staining was found in the constructs of control groups [Fig. 5(A) and (C)].

Figure 6 quantitatively summarizes the biochemical and histomorphological measurements of the extent of adipogenic differentiation of adipose tissue-engineered constructs after 4 and 6 weeks incubation. Both G-3-PDH activities and efficiency of G-3-PDH activity of the tissue-engineered adipogenic constructs were significantly higher than control constructs of hMSC seeded gelatin sponges without differentiation. Furthermore, the amounts increased with extended incubation time. Parallel to the finding in histological examination and the measurement of G-3-PDH activities, the area of accumulation of lipid droplets within the tissue-engineered constructs under adipogenic differentiation was significantly higher than those without differentiation. The accumulation of lipid also tended to increase following extended incubation time. Six weeks after incubation in adipogenic medium, approximately 20% volume of tissue-engineered constructs was occupied by lipid droplets.

DISCUSSION

The present study demonstrated that human bone MSCs possess a strong capacity to differentiate into adipocyte lineage under adipogenic conditions. Gelatin sponges as a scaffold effectively supported the adipogenic differentiation of MSCs. This work verified the feasibility of adipose tissue engineering using adipogenic differentiated bone MSCs seeded on a gelatin sponge. This technique may represent a new option for clinical reconstruction and correction of soft tissue defects. However, further studies are required, especially *in vivo* and long-term studies, before the technique can be applied practically for human clinical and cosmetic therapy. The generation and maintenance of an engineered adipose tissue *in vivo* has been shown to highly rely on vascularization.[14] Therefore, a high stimulation of angiogenesis induced by growth factors within constructs may be required to promote and maintain the *in vivo* outcome of adipose tissue engineering.[4,14]

Cell phenotype selection for seeding on scaffolds is a critical one among multiple complicated factors to determine the feasibility of adipose tissue engineering for clinical application.[2,23,24,31] Even though adipose tissue consists mainly of adipocytes, mature adipocytes are difficult to use in adipose tissue engineering due to their characteristic low proliferation. Furthermore, viability of transplanted adipocytes highly relys on immediate re-vascularization to provide nutrients to support their high metabolic rate, which is technically difficult in practice. Several studies recently exploited cells from enzymatic digestion of adipose tissue, called preadipocytes or adipocyte precursor cells to tissue engineer adipose tissue.[11,12,25,34,35] The preadipocytes, potentially isolated via a less invasive procedure called liposuction, exhibited high proliferation rate and adipogenic differentiation potentials.[19,23,24] Excellent results were obtained in several experiments, but the long-term result still needs to be improved.[11,25,26] The seeded cells from enzymatic digestion of fat tissues were a heterogeneous cell mixture containing various cell phenotypes such as fibroblast, preadipocyte, and endothelium. Recent studies found that cells, isolated from adipose tissue by similar techniques to previously reported preadipocyte isolation procedures, possess multiple differentiation potentials much like mesenchymal stem cells. They were able to be differentiated into several cell lineages using specific lineage media.[10,20,37] Therefore, adipose tissue-derived multifunctional MSCs are believed to contribute, at least in part, to adipogenic differentiation potential of the cell mixture digested from adipose tissue. However, it was demonstrated recently that adipose tissue-derived cells have lower differentiation potentials than those of bone-marrow MSCs in osteogenesis and chondrogenesis.[8] However, the adipogenic potential of adipose-derived cells may be higher than the other differentiation lineages because of the presence of more adipogenic committed preadipocytes. Therefore, adipose-derived cells are one of the choices as seeding cells for adipose tissue engineering. Adipogenic potentials of human bone MSCs were quantitatively and qualitatively confirmed by the biochemical and morphological measurements in the present study. A high adipogenic differentiation ability of human MSCs seeded on gelatin sponges demonstrated that bone marrow derived MSCs could become another cell resource for adipose tissue engineering.[31] Combined with a promising scaffold, adipose tissue engineering based on MSCs might be exploited for the treatment of soft tissue deficiency. Although it is still unclear which seeding cell candidate would be more suitable for the adipose tissue engineering, bone MSCs might be more useful in underweight or child patients who don't have a large enough amount of fat for preadipocyte collection.

A scaffold for adipose tissue engineering should be characterized by soft tissue-like mechanical properties, biocompatibility, degradability, and ability to enhance vascularization. Gelatin refined from collagen possesses high biocompatibility and have been extensively applied in food manufacture and medical application.[36] Gelatin sponges also have been developed as a carrier or a scaffold for growth factor release and cell seeding in various tissue engineering studies.[30,33] Because of its soft tissue-like mechanical properties, gelatin sponge might be a more suitable scaffold for adipose tissue engineering as compared to a stiff scaffold such as poly lactic-co-glycolic acid (PLGA) sponge. Gelatin sponge possesses similar properties with PLGA sponge in facilitating angiogenesis. The pore size of 200–400 μm of gelatin sponges used in the present study would benefit cell migration and angiogenesis formation.[14] Because the biodegradation rate of gelatin sponges can be controlled by the extent of chemical crosslinking, gelatin sponge with an optimal degradation rate to maximize results of tissue-engineered adipose would be investigated in ongoing studies.[33] Collectively, its characteristics determined that the gelatin sponge could be a promising scaffold for adipose tissue engineering. Gelatin sponge incorporated with bone MSCs could be a good candidate in adipose tissue engineering for clinical applications.

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