Histological Analysis of *In Vitro* Co-Culture and *In Vivo* Mice Co-Transplantation of Stem Cell-Derived Adipocyte and Osteoblast

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Many researchers have focused on the role of adipocytes in increasing efficient bone tissue engineering and osteogenic differentiation of stem cells. Previous reports have not reached a definite consensus on whether adipocytes positively influence *in vitro* osteogenic differentiation and *in vivo* bone formation. We investigated the adipocyte influence on osteogenic differentiation from adipose-derived stromal cells (ADSCs) and bone formation through histological analysis *in vitro* and *in vivo*. Using the direct co-culture system, we analyzed the influence of adipocytes to promote the differentiation fate of ADSCs. Using co-transplantation of ADSC-derived adipocytes and osteoblasts into the dorsal region of mice, the osteogenesis and bone quality were determined by histological morphology, radiography, and the measurement of the Ca^{2+} concentration. The adipocyte negatively affected the osteoblast differentiation of ADSCs in the *in vitro* system and induced osteogenesis of osteoblasts in the *in vivo* system through co-transplantation. Interestingly, in the co-transplanted adipocytes and osteoblasts, the bone formation areas decreased in the osteoblast only group compared with the mixed adipocytes and osteoblast group 6 weeks after transplantation. Conversely, co-transplantation and osteoblast transplantation had similar degrees of calcification as observed from radiography analysis and the measurement of the Ca^{2+} concentrations. Our results revealed that adipocytes inhibited osteoblast differentiation *in vitro* but enhanced the efficacy of osteogenesis *in vivo*. In addition, the adipocytes controlled the activity of osteoclasts in the newly formed bone tissue. Our approach can be used to reconstruct bone using stem cell-based tissue engineering and to enhance the understanding of the role adipocytes play. **Tissue Eng Regen Med 2016;13(3):227-234**

Key Words: Adipocyte; Co-culture; Co-transplantation; Osteogenesis; Adipose-derived stromal cells

INTRODUCTION

For bone tissue engineering, various studies have been pub-

lished demonstrating the bone-building capacity of progenitors, such as stem cell and other osteogenic cells [1,2]. Currently, adult stem cells, such as adipose-derived stromal cells (ADSCs) and bone-marrow-derived stem cells (BMSCs), demonstrate vast potential in bone regenerative applications because they exist in an undifferentiated state and demonstrate both self-renew and a capacity to differentiate into variable cell types [3-5]. In general, ADSCs and BMSCs differentiation fates are regulated by specific transcriptional regulators, which are present in low levels in undifferentiated cells, and differentiation toward one fate completely suppresses and induces genes associated with the other fates [6-8]. This phenomenon has been exploited in past research to regulate stem cell fate into osteoblast differentiation using defined media conditions with cytokines and growth factors or co-culture systems containing two or more cell types

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to more closely mimic the *in vivo* microenvironmental conditions [9].

Even though osteogenic differentiation and bone regeneration using stem cells brought about very interesting results with respect to their novel potential in regenerative medicine, research has been primarily focused on the osteogenic differentiation of stem cells in *in vitro* systems using co-culture and defined medium conditions [10-12]. Conversely, the influence of osteogenesis and osteogenic differentiation from neighboring cells in transplanted *in vivo* sites is not well characterized. In fact, the bone marrow niche environment includes various cell types, such as osteoblasts, adipocytes, hematopoietic stem cells and endothelial cells [6,13]. These cells may have an effect on the osteogenic differentiation of transplanted stem cells or on the osteogenesis process.

Specifically, recent studies have shown that adipocytes are an endocrine organ due to their secretion of various cytokines, such as adiponectin, leptin, and peroxisome proliferator-activated receptor- γ (PPAR- γ). And, it has been suggested that these cytokines interaction with osteoblasts regulates bone resorption and influences bone density, mass, and formation (Fig. 1) [14-16]. Interestingly, the effect of adipocytes on osteogenesis has been inconsistently reported in several previous studies. PPAR- γ , which is one cytokine secreted from adipocytes, suppresses osteogenesis by inhibiting Runx2 gene expression in osteoblasts [17,18], whereas adiponectin increases osteoclastogenesis [19,20]. By contrast, other studies have revealed that adipocytes increase osteogenesis through the action of adiponectin [21,22] and that leptin increases bone mass [23]. These reports have not reached a definite consensus on whether adipocytes positively influence osteogenic differentiation *in vitro* and osteogenesis *in*

vivo. Therefore, the roles of adipocytes should be obvious in bone tissue engineering if they are integral to efficient bone regeneration using stem cells.

The purpose of this study was to investigate the influence of adipocytes on osteogenic differentiation *in vitro* and osteogenesis *in vivo*. To that end, the top and bottom co-culture system was used to analyze the influence of adipocytes on the osteogenic differentiation fate of ADSCs. The adipocytes and osteoblasts derived from ADSCs were co-transplanted to determine the effect of the adipocytes on new bone formation in the dorsal region of mice.

MATERIALS AND METHODS

Isolation and culture of ADSCs

Human subcutaneous adipose tissues were obtained via elective liposuction from patients who had given informed consent. Only adipose tissue that would otherwise have been discarded was used for ADSCs isolation with the approval of the Medicine Ethics Committee of Konkuk University (IACUC No. KU13125-1). The enzymatic digestion method was used to isolate ADSCs from the adipose tissues [24]. Briefly, the adipose tissue was minced and washed with phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA) until the blood was removed. The minced tissue was then digested with PBS containing 2 mg/mL collagenase type II (Sigma-Aldrich) and 0.2% bovine serum albumin for 45 min at 37°C with shaking. Subsequently, digested adipose tissue was filtered using a 100- μ m filter and centrifuged to remove the floating adipocytes from the stromal-vascular fraction. The ADSCs isolated from the adipose tissue were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% (v/v) FBS, 1% (v/v) GlutaMAX, and 1% (v/v) penicillin-streptomycin in 5% CO₂ at 37°C.

ADSC cultured on porous membranes and differentiation

The ADSCs (1×10^5 cell/well) were seeded on inverted transwell cell culture inserts fitted for six-well plates and were cultured for 24 h. Following the adhesion of the ADSCs to the bottom surface of the insert, the plate was turned upright and incubated in adipogenic induction media (Gibco BRL, Gaithersburg, MD, USA) for 14 days. ADSCs grown in basal medium were used as controls. Standard oil red O stain was used to identify adipogenic differentiation of the ADSCs. Subsequently, ADSCs were seeded inside the insert. Following an additional 24 h incubation, the medium was refreshed with osteogenic induction media (Gibco BRL) for 14 days. After 30 total days of ADSC culture, fully osteogenic differentiated cells were identified using

Figure 1. A schematic overview of the influence of adipocytes on osteoclasts and osteoblasts. Adipocytes release many adipokines, such as leptin, adiponectin, PPAR- γ , which regulate osteoblast and osteoclast differentiation. PPAR- γ : peroxisome proliferator-activated receptor- γ .

standard alizarin red staining.

Co-transplantation of osteoblasts and adipocytes differentiated from ADSCs

To obtain osteoblasts and adipocytes derived from ADSCs, ADSCs were cultured with osteogenic and adipogenic induction media for 14 days, respectively. For co-transplantation of osteoblasts and adipocytes derived from ADSCs, immune-deficient mice (BALB/c-nu, 8-week-old females; SLC, Tokyo, Japan) were anesthetized with an intramuscular administration of ketamine hydrochloride (50 mg/kg; Yuhan, Seoul, Korea) and xylazine hydrochloride (5 mg/kg; Bayer Korea, Seoul, Korea). The osteoblasts (5×10^6 cells) were mixed with adipocytes (5×10^6 cells) in 1 mL of type I collagen hydrogel (Sigma-Aldrich, St. Louis, MO, USA) and injected into the subcutaneous dorsum of the mice. Transplantation of the osteoblasts (1×10^7 cells) suspended in 1 mL of type I collagen hydrogel without adipocytes served as the control. The fifteen mice were evenly divided into three experimental groups. All animal care and experimental procedures were performed with the approval of the animal care committee of Konkuk University (IACUC No. KU13125-1). Four and six weeks after injection, the mice were sacrificed, and all implants were retrieved. Bone formation was measured by histological analysis and plain radiogram (soft X-ray; Softtex, Sofron Co., Tokyo, Japan). The amount of calcium deposited in the implants was examined as described previously [25]. For histology, each sample was fixed in 10% (v/v) neutral-buffered formalin for 2 days, decalcified with a histological decalcifying agent (National Diagnostics, Atlanta, GA, USA) for 1 day, washed with tap water, and then processed for histology. The five tissue sections per sample obtained in 5- μ m thickness were stained with Goldner's trichrome and observed under a light microscope (Olympus IX70; Olympus, Okaya, Japan) to measure the bone formation area. The percentage of bone area and adipose area that occupied the implants was calculated (bone area/total area $\times 100$ and adipose area/total area $\times 100$).

Statistical analysis

Quantitative data were expressed as the means $\pm$ standard deviations. Statistical comparisons were carried out using Student's t-test (SAS software, SAS Institute Inc., Cary, NC, USA). A probability level of less than 0.05 was considered statistically significant.

RESULTS

Effect of adipocytes on osteogenic differentiation in the co-culture system

As shown in Figure 2, the ADSCs were subjected to the porous membrane culture technique as previously described

[9,26]. In this method, the ADSCs were seeded on the inverted transwell inserts to grow homogeneously on the bottom of the insert. Following the initial 24 h incubation, the inserts were inverted and cultured with adipogenic induction medium for 14 days. The adipogenic differentiation of ADSCs was analyzed using oil red O staining for lipid droplets. The plated ADSCs attached to the bottom of the insert and began to grow successfully, which resulted in normal ADSC morphology (Fig. 2A). The results of the oil red O staining showed that ADSCs cultured in the adipogenic medium intensely produced lipid droplets (Fig. 2B). To examine whether the adipocytes that differentiated from the ADSCs were effective for the osteogenic differentiation of ADSCs, undifferentiated ADSCs were seeded on the top surfaces of the insert membranes with adipogenic differentiated cells and undifferentiated ADSCs on the bottom of the insert membranes and cultured with osteogenic medium for 2 weeks. The osteogenic differentiation of ADSCs was analyzed using alizarin red staining for the presence of a calcific deposition. Before alizarin red staining, the cells on the bottom of the porous membrane were eliminated using a cell scraper to avoid a complicating factor (cells on the bottom) for a more controlled comparison of the two culture system types. The results of the alizarin red staining showed that ADSCs co-cultured with ADSCs on the top and bottom of transwell membrane were intensely stained compared with the top of ADSCs co-cultured with the bottom of adipocytes (Fig. 2C and D), which demonstrated that the top of ADSCs co-cultured with bottom of ADSCs produced more calcific deposition. These results indicate that adipocytes in a direct co-culture system inhibited osteogenic differentiation of ADSCs.

Effect of adipocytes on osteogenesis in co-transplantation

The ADSCs successfully differentiated into osteoblast-like cells and adipocyte-like cells *in vitro*. The ADSC-derived osteogenic cells cultured under the osteogenic differentiation condition had a morphology similar to that of osteoblasts and stained positively for mineralization with alizarin red staining. The ADSC-derived adipogenic cells cultured under adipogenic differentiation conditions had lipid droplets and stained positively for oil red O staining. To evaluate the osteoblast-like cells/adipocyte-like cells mixture on the osteogenesis *in vivo*, adipocyte-like cells and/or osteoblast-like cells were inoculated in a collagen gel and were injected in a dorsal subcutaneous site in mice (Fig. 3A). The dorsal subcutaneous site, which is a well known ectopic bone, is several advantages for successful bone regeneration test by osteogenic cells [27,28]. In addition, the time scale of *in vivo* bone healing and critical concentration have been well established [29,30]. Thus, we choose the dorsal subcutaneous site

to analyze the effect of adipocytes on osteogenesis.

The implants were evaluated by histological analysis and plain radiogram 4 and 6 weeks after transplantation. From the histology of the implants, new bone and adipose formation occurred in both groups (Fig. 3), but new adipose formation was more extensive in the co-transplantation group than in the osteoblast-like cells only transplantation group (Fig. 3D). The co-transplantation group showed a 2-fold greater adipose area than the osteoblast transplantation group at 4 and 6 weeks. The findings from the Goldner's trichrome staining showed that the co-transplantation and osteoblast-like cells transplantation groups had similar degrees of new bone formation at 4 weeks (Fig. 3C). However, the morphology of newly formed bone in the co-transplantation group was different from the new bone in the osteoblast-like cells transplantation group (Fig. 3B). The osteoclast density was significantly higher in the osteoblast-like cells-only transplantation group than in the co-transplantation group. By contrast, the osteoblast density in the co-transplantation

group was significantly higher than in the osteoblast-like cells transplantation group. After 6 weeks, new bone formation had not increased in either group, but the new bone area significantly decreased in the osteoblast-like cells transplantation group compared with the new bone area formed after 4 weeks. Four weeks after transplantation, soft X-ray and measurement of the Ca^{2+} concentration showed that implants from both groups resulted in moderate mineralization (Fig. 4). Six weeks after transplantation, implants from both groups resulted in extensive mineralization. Notably, both groups had similar degrees of mineralization in the implants.

DISCUSSION

Many studies have reported that adipocytes can be made to influence the cellular function and metabolism of a variety of cells [31]. Generally, adipocytes are a large cell population within the human body [32], and they secrete various cytokines,

Figure 2. Influence of adipocytes on the osteogenic differentiation of ADSCs in a co-culture system. To set up the co-culture system, the undifferentiated ADSCs (1×10^5 cell/well) were seeded on inverted transwell inserts fitted for six-well plates and were cultured for 24 h. To analyze the adipogenic differentiation, oil red O staining of ADSCs cultured on the bottom of a porous membrane with basal media as control (A) and adipogenic induction media (B). To analyze the effect of adipocyte on the osteogenic differentiation, alizarin red staining of ADSCs cultured inside the insert with undifferentiated (C) and adipogenic-differentiated ADSCs with osteogenic induction media (D). ADSCs: adipose-derived stromal cells.

such as adiponectin, leptin, and PPAR- γ [33,34]. Several studies have reported that adipokines exert their bone metabolic effects by controlling the activity of osteoblasts and osteoclasts or by controlling the osteogenic differentiation of stem cells [19,33]. Although there are a number of studies related to bone metabolism using adipokines, the effect of adipocytes on the osteogenic differentiation of stem cells and bone regeneration *in vivo* has not been studied in direct co-culture conditions or with the co-transplantation of adipocytes. It was unknown whether adipocytes would have the same effects on bone metabolism as observed in adipokine studies. To clarify, the effect of adipocytes on osteogenic differentiation and osteogenesis was tested in co-culture and co-transplantation with adipocytes.

First, we tested whether the osteogenic differentiation of ADSCs was affected by adipocytes. The effect of adipokines on osteogenic differentiation has been a controversial issue for several years. For example, Liu et al. [35] reported that activated PPAR- γ

and adiponectin decreased Runx2 expression in osteoblastic cells. It is well known that Runx2 is an osteogenic transcription factor that plays a central role in regulating osteogenic differentiation and skeletal morphogenesis [36]. However, Chen et al. [37] reported that adiponectin enhances osteogenic differentiation in human ADSCs by activating APPL1-AMPK signaling. The osteogenic differentiation occurred in a time- and dose-dependent manner. Treatment with 1 $\mu\text{g/mL}$ adiponectin was the most effective concentration. In our study, osteogenic differentiation in the adipocyte-co-cultured ADSCs was significantly decreased compared with the osteogenic differentiation of the control group. In the co-culture system, adipocytes would secrete various cytokines, such as adiponectin, leptin, and PPAR- γ . Subsequently, ADSCs can be influenced by secreting various cytokines. In this study, despite not knowing the defined amount and types of secretions from the adipocytes contained in the culture medium, we deliberately used a co-culture system to

Figure 3. Histological analysis of osteogenesis through the co-transplantation of adipocytes and osteoblasts. A conceptual diagram of the animal co-transplantation experiment (A). Histological osteogenesis analysis using Goldner's trichrome staining. The bone is stained green and the osteoclasts were indicated by red arrow (B). Bone formation area (C) and adipose formation area (D) in the implants. * $p < 0.05$.

Figure 4. Soft X-ray and Ca^{2+} concentration of the implants. Representative high-resolution X-ray images showing bone density compared between co-transplantation group and osteoblast-like cell only group after 4 and 6 weeks (A) and the quantitative data of calcium deposition in the implants shown in (B). * $p < 0.05$.

contain the complicated systemic factors as opposed to controlling one adipokine factor, to achieve a more controlled comparison of the effect of adipocytes on osteogenic differentiation. Our study is the first to find that adipocytes inhibit the osteogenic differentiation of ADSCs.

Finally, we investigated the effect of adipocytes on *in vivo* bone formation. The new adipose formation areas in the implants were significantly larger in the co-transplantation group containing adipocytes than in the osteoblast-only transplantation group. The greater propensity for adipose formation was likely caused by the co-transplanted adipocytes. Four weeks after transplantation, both groups showed similar degrees of new bone formation. Interestingly, the morphology of the newly formed bone was different between the groups. In the co-transplantation group, the newly formed bone was surrounded by osteoblasts. Some osteoblasts that were captured by the osseous matrix become osteocytes. By contrast, many osteoclasts were found in lacunae that were situated in the newly formed matrix in the osteoblast-only transplantation group. Six weeks after transplantation, the new bone area was significantly decreased in the osteoblast-only transplantation group compared with the same group at 4 weeks. Interestingly, from the view on the effects of adipocyte's cytokines, our results shown the opposite results between *in-vitro* and *in-vivo* condition. In this regard, we think that the secretion level of cytokines from adipocytes will bring out change in *in-vitro* and *in-vivo* condition. In the previous study, Soukas et al. [38] reported that expression of numer-

ous genes between *in-vivo* and *in-vitro* adipocytes was differed by one or more transcriptional cellular programs, which were not fully understood. Specifically, the PPAR- γ , which inhibited the osteogenic differentiation from ASCs and MSCs and promoted osteoclastogenesis, was up-regulated in *in-vitro* conditions and down-regulated in *in-vivo* conditions. The leptin, which promoted the osteogenic differentiation from ASCs and MSCs and inhibited the osteoclastogenesis, was up-regulated in *in-vivo* condition and down-regulated in *in-vitro* condition. In addition, several reports have demonstrated that the PPAR- γ and leptin regulated the RANKL/OPG/RANK signaling pathway, which is important to control of osteogenic differentiation and osteoclastogenesis [39,40]. Therefore, several previous reports agree with our data that adipocytes are associated with reduced osteoclast activity in newly formed bone tissue. Our results demonstrate that adipocytes have potential as a supplementary strategy to regulate osteogenic differentiation of ADSCs *in vitro* and to control the activity of osteoclasts in the newly formed bone tissue.

Basically, the purpose of our present study was to analyze the effect of co-transplantation of adipocytes on the osteoblastogenesis using co-transplantation. However, various unknown adipocyte factors will regulate the unexpected signaling pathways, when was the co-transplantation with osteoblast and adipocyte [41]. These factors is important for analyzing the absolute effects of adipocyte on new bone formation and energy homeostasis [42]. Thus, additional study was needed about gene and

protein levels of generating new bone tissue for analysis of molecular effect of adipocyte.

In addition, our results show that both groups had similar degrees of mineralization, but that had the difference of bone formation area. These results can imply that the cell density of adipocyte controlled the bone mineral density in the bone remodeling and tissue engineering. Generally, in human body, the bone is the five type by shape. Also, each type of bone have different bone mineral density. The sponge bone is one of the two types of bone tissue found in the ends of long bones, as well as in the pelvic bones and ribs. The sponge bone has a high surface area to mass ratio because it is less dense [43]. On the other hand, the skull bone has a high bone density [44]. Therefore, our study provide the basic information of efficient bone remodeling to apply to various bone character.

In conclusion, this study demonstrates that adipocytes inhibit osteogenic differentiation of ADSCs *in vitro* but enhance the efficacy of osteogenesis *in vivo*. Our approach can be used in reconstructing bone using stem cell-based tissue engineering and in understanding the role of adipocytes in osteoblast differentiation and osteogenesis through co-transplantation.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

All animal care and experimental procedures were performed with the approval of the animal care committee of Konkuk University (IACUC No. KU13125-1).

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