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Bone Differentiation from Human Lipoaspirate

MOHAMED M. ABD EL-AAL, M.D., M.S.[1]; SUNIL THOLPADY, M.S.[2]; GREGORY A. HELM, M.D., Ph.D.[3,4] and ROY C. OGLE, Ph.D.[3,4]

The Department of Plastic and Reconstructive Surgery[1], Ain Shams University and the Departments of Cell Biology[2], Neurosurgery[3] and Biomedical Engineering[4], University of Virginia Health Sciences Center.

ABSTRACT

Stem cells have a unique ability to renew themselves and to become a host of diverse cell types. Recent studies have demonstrated the presence of multipotential mesenchymal stem cells in fat that have osteogenic potentials. In this study, human adipose tissue was obtained from elective liposuction procedures. Stem cells were isolated through a series of washes in PBS and digestion with collagenase. The isolated cells were plated onto 24-wells tissue culture plates. The cells were divided into three groups; cells treated with BMP-9 virus (I), cells exposed to a *ß*-galactosidase expressing virus (II), and untreated cells (III). At the end of first and second weeks, cells were stained for markers of bony differentiation. RT-PCR was also performed to confirm the presence of bone specific markers. Cells treated with the BMP-9 expressing adenovirus formed bony nodules when viewed microscopically after one week while the other two groups did not. BMP-9 treated cells also demonstrated elevated levels of calcium, alkaline phosphatase, osteopontin, and osteonectin when compared to cells that were treated with *ß*-gal virus or no treatment. Bone specific mRNA (bone osteonectin and osteopontin) was also present at the end of first and second weeks. In this study, we demonstrate the rapid differentiation of fat derived stem cells to osteoblasts by infection with an adenovirus expressing BMP-9. Fat derived stem cells hold great promise in the treatment of clinical problems requiring cellular replacement and may be used to accelerate the healing of bony defects of the skeletal system. The ease of their isolation and purification add to their attractiveness as a potential future therapy.

INTRODUCTION

Primary or secondary large bony skeletal defects after trauma, tumors resection, fractures, osteomyelitis poses a real challenge for most of surgeons, especially plastic surgeons in head and neck region. These defects need to be reconstructed by hard tissues to restore the anatomical and functional continuity. In the past, multiple-stage reconstruction operations were the routine procedure for the treatment of such defects. Current treatments depend on the use of autografts, which are either non-vascularized or vascularized grafts with or without combined soft tissue for coverage or lining **[1-4]**. More recently, different bone graft substitutes used to eliminate the postoperative donor site complications and to decrease the length of the procedure. Yet, these substitutes still carry the dangerous of infection, rejection and exposure with the entire subsequent unfavorable cosmetic outcome **[5-9]**. Distraction osteogenesis appeared in the last decade as an alternative therapy for the treatment of such defects without the need of external grafts. However, the difficulty and the length of these open reconstruction procedures and the unrealistic expectations of the results showed that all the above-mentioned techniques are not without considerable morbidity **[10-12]**.

Clinical basic research now is aiming for the development of new techniques to regenerate and reconstruct bone defects without the need of lengthy open reconstruction operations. Different osteoconductive materials, such as poly-alpha-hydroxy acids and hydroxyapatites are used to enhance bone regeneration in areas of bony defects **[13]**. Guided bone regeneration techniques by using osteopromotive membranes that prevent migration of scar tissue between the healing bony edges were also experimentally tested with relatively good results **[14]**.

The use of Bone morphogenic proteins (BMPs) to induce bone formation is another important approach for the treatment of critical-sized bony defects. Bone morphogenic proteins have been shown to induce new bone formation by attracting and stimulating the differentiation of primitive mesenchymal cells into chondrocytes and osteocytes. Different bone morphogenic proteins (2, 7, and 9) showed an efficient role to promote new bone formation both in vitro and in vivo **[15-21]**.

Delivery of bone morphogenic proteins using gene therapy has potential advantages over direct

protein delivery, such as lower coast, longer expression of factors, and more controllable physiological levels [22,23]. Two approaches have been described for gene insertion, inside the body "in situ" or outside the body "ex vivo" [24,25]. The ex vivo approach require harvesting cells from patient or donor sources, expanding their population in tissue culture, transfecting the cells with the gene an re-implanting the cells at the treatment site [26].

Stem cells have been reported to be present in embryonic tissue and in postnatal tissues as well [27-31]. Stem cells are capable to differentiate into tissues in which they reside [32-37]. Recent studies have shown that stem cells can cross the lineage boundaries and can differentiate into cell types of another tissue [38-40]. For example, bone marrow derived stem cells can differentiate into neurons and glia [41,42], hepatic oval cells [43], provides cells for neovascularization [44,45], and restores cartilage, muscle, bone, and fat [46-51].

Within the last few years some researchers focused on human adipose tissue as an alternative source of autologous adult stem cells that is obtainable in large amounts, under local anesthesia, with minimal discomfort and donor site morbidity. Recent studies have shown that fat derived stem cells can differentiate into osteoblasts, chondrcytes and muscle cells using different medias [52-56].

The experiment reported in this study describes the capacity of differentiation of human stem cells derived from human adipose tissue into mature osteoblasts by using BMP-9 in vitro. That may open the door for further studies to apply the same principle to reconstruct critical sized defects with fat derived stem cells transfected by bone morphogenic proteins as powerful bone inducers.

MATERIAL AND METHODS

Cell harvest:

Liposuction aspirate after tumescent liposuction procedure from the trunk of 35 years female lady was brought to the lab under complete aseptic conditions. Stem cells harvested as previously described [53]. Liposuction aspirate washed with copious amount of saline several times to remove the red blood cells. Collagenase added to lipoaspirate for 30 minutes at 37ºC to digest connective tissue components and release cells. All mixture filtered to get rid of the stromal part. The filtered centrifuged at 1200g for 10 minutes to get rid of supernatant and the cells were plated on cell culture plates in 10% fetal bovine serum in Dulbecco modified Eagle medium (DMEM). Cells were incubated in a 95% air -5% CO^2 humidified environment. When cells reach 100% confluent in cultured plates, cells were released with 0.05% trypsin (DIFCO, Becton-Dickinson Labware, Franklin Lakes, NJ) in Ca^{+2},Mg^{+2}-free Dulbecco's phosphate buffered saline (GIBCO) containing 0.0744% ethylenediamine tetraacetic acid (EDTA, Sigma), centrifuged at 100g for 20min, and the supernatant aspirated. The cell pellet was replated in 10% bovine serum in DMEM in more plates. The above process has been repeated for cell expansion till the 6^{th} passage. Cell numbers were determined with a hemocytometer.

Adenovirus constructs:

The first generation adenoviral vector containing the *ß*-galactosidase (*ß*-gal) gene under the control of the cytomegalovirus (CMV) promoter was used as a control virus (Ad-*ß*-gal, a gift of Dr. Chinghai Kao, University of Virginia Cancer Center, Charlottesville, VA). A first generation adenoviral vector with human BMP-9 gene under the control of CMV promoter (Ad-BMP-9) was used in the treatment group (a gift from Genetics Institute).

Osteogenic differentiation:

Cells at 85-90% confluence in 24-well plates were divided into three groups. Group I and II were infected with adenovirus BMP-9 and adenovirus *ß*–galactosidase at 2,10 and 50 plaque-forming unit (pfu) per cell over a night respectively. Group III was left uninfected. DMEM was changed in the three groups every other day for two weeks.

Bone differentiation assay:

Osteogenesis was assessed after one and two weeks with immunohistochemical staining (for osteonectin, osteopontin), Alkaline phosphatase activity and Von Kossa staining. Cell numbers also were determined at first day and after 1 and 2 weeks.

Reverse transcriptase-polymerase chain reaction analysis:

The ability of virus to differentiate fat derived stem cells to osteoblasts in vitro was examined also by Reverse transcriptase-polymerase chain reaction analysis for osteonectin and osteopontin. RNA was isolated from processed fat derived stem cells from the three groups after 1 and 2 weeks.

RESULTS

Morphological changes:

Microscopic examination of adenoviral BMP-9 infected cells (group I) showed increase in cell density and change in cell morphology from the third day. The cells become shorter with loss of the fibroblast appearance to become more cubical or rounded cells. The cells also aggregated in groups around small islands of mineral deposition (three-dimensional extracellular matrix) that started to be seen within the first week and steadily increased throughout the study period. Neither dramatic increase in cell density nor other morphological changes were noted in adenovirus *β*-galactosidase infected cells (group II) or uninfected cells (group III).

Proliferation and differentiation was more rapid and involved all cells in the plates with high virus concentrations. However, cells treated with high virus titers (50-pfu/cell) showed signs of cytotoxicity after day 7 and 50% of these cells were detached by 2 weeks. In contrast, cells treated with low titers (2-pfu/cell) were not significantly increased in number and with less cell toxicity and take off.

Bone specific markers:

Osteogenic differentiation was detected at one and two weeks in BMP-9 adenovirus treated group (I). This was indicated by positive alkaline phosphatase staining (Fig. 1-$A_{1,2}$), positive osteonectin and osteopontin monoclonal antibodies (Fig. 1-$B_{1,2}$). Cells infected with Ad-BMP-9 expressed alkaline phosphatase activity, and osteonectin and osteopontin production in a dose and time dependent fashion as demonstrated by increased staining intensity. Calcium deposition and formation of extracellular mineralized nodular structure in group I was confirmed by positive Von Kossa staining (Fig. 1-$C_{1,2}$). None of the two control groups (II and III) showed staining above the baseline.

PCR:

Reverse trascriptase-polymerase chain reaction (PCR) was used also to confirm the osteogenic differentiation within the first and second week. Consistent with immunohistochemistry data, reverse trascriptase-polymerase chain reaction analysis confirmed the expression of osteonectin and osteopontin in Ad-BMP-9 treated cells with more increased expression at 2 weeks (Fig. 2).

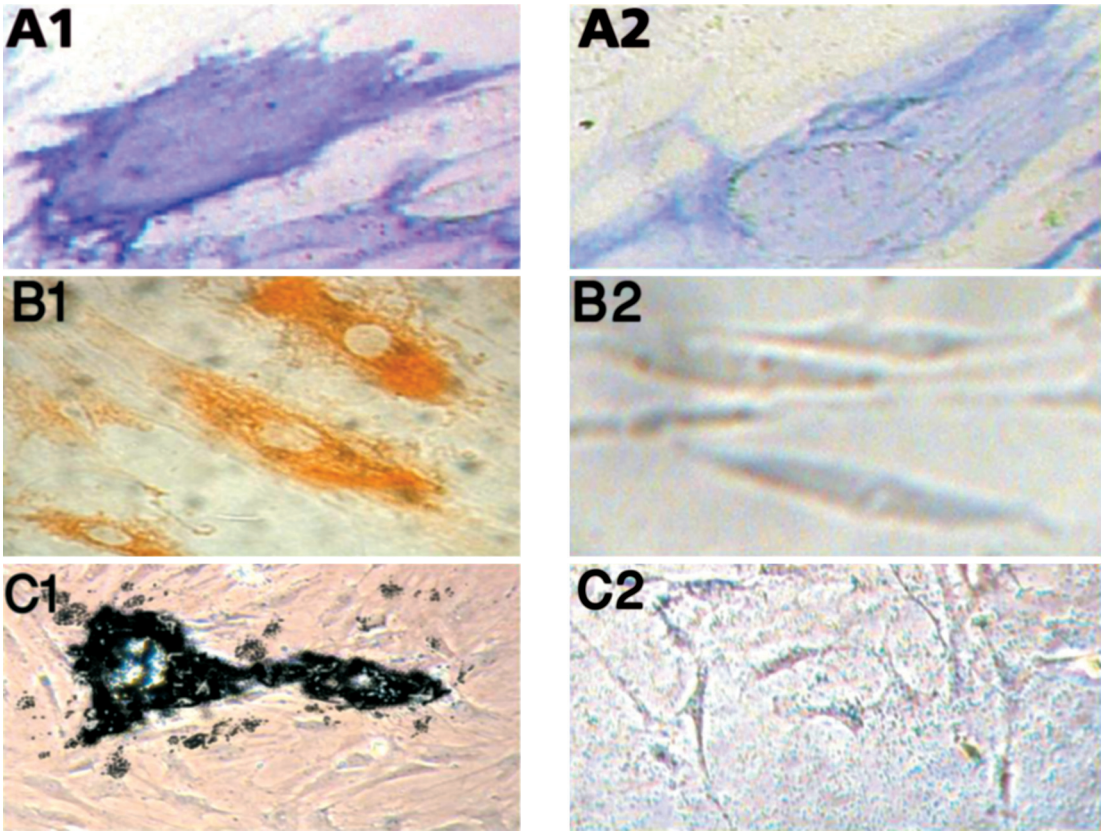
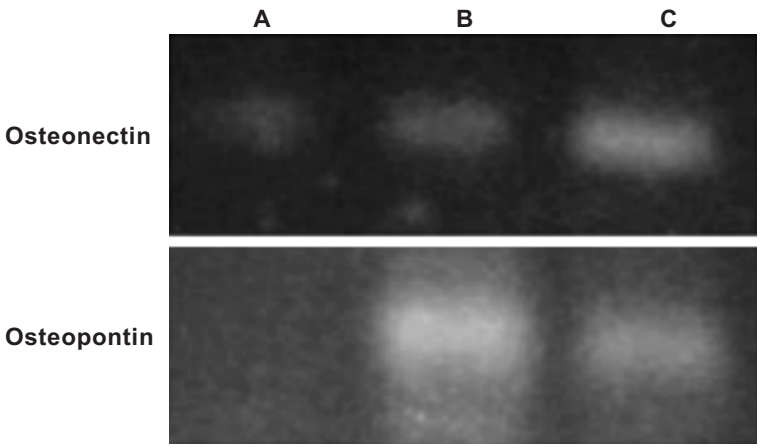


Fig. (1): A_1, Positive alkaline phosphatase staining in group (I), A_2, Negative alkaline phosphatase staining in group (II & III). B_1, Positive osteonectin and osteopontin monoclonal antibodies in group (I), B_2, Negative osteonectin and osteopontin monoclonal antibodies in group (II &III). C_1, Positive Von Kossa staining in group (I), C_2, Negative Von Kossa staining in group (II &III).



A- Undifferentiated.
B- Osteogenic media 1 week.
C- Osteogenic media 2 weeks.

Fig. (2): Reverse tramscriptase-polymerase chain reaction (PCR) confirm the expression of osteonectin and osteopontin in Ad-BMP-9 treated cells (I) with more increased expression at 2 weeks.

DISCUSSION

This study clearly demonstrates a new successful Osseo-differentiation of human fat derived stem cells using BMP-9 adenoviral gene therapy in vitro. Microscopic examination after 1 and 2 weeks showed the morphological changes of primitive fibroblast like cells to more osteoblastic like appearance. That was proved by increased alkaline phosphatase activity, and formation of islands of mineralized extracellular matrix. Histoimmunochemical staining and reverse transcriptase-polymerase chain reaction analysis also revealed the expression of bone specific markers (osteonectin and osteopontin) in a time dependent faison. This study supports the osteogenic capability of derived stem cells by a new powerful induction approach using gene therapy that may have potential clinical applications in the future. However this work needs a lot of basic and clinical research to solve gene therapy current limitations.

Current studies are trying to construct second-generation viral vectors with low immune response to overcome the immune reaction problems **[18]**. BMP gene therapy can be used also to induce bone, cartilage, ligaments and tendons **[18,57]**. The use of BMP gene therapy techniques may improve the reconstruction of craniofacial defects and can minimize the need for open surgical approaches involving the use of autografts, allografts, or other bone substitutes.

BMP-9 adenoviral vector has shown to play an important role in chondrogenesis and osteogenesis in vitro and in vivo animal models as in thigh and paraspinal musculature of athymic nude rats **[19-22,58]**.

Significant bony healing was noticed in different experimentally created bony defects after direct application "in situ" of BMP adenoviral vectors without overgrowth to the surrounding normal soft tissues **[22]**. The second approach that is currently investigated is the "ex vivo" gene therapy **[25,26]**. By this technique the cells are transfected with the desired gene in tissue cultures and subsequently implanted into the treatment region. These cellular implants will continue the expression of the carried gene and local secretion of a variety of osteogenic growth factors that signal other pleuripotential cells to migrate to the anatomic site, which will lead to more osseous-differentiation and bone formation.

The advantages of the ex vivo technique are that no viral particles or DNA complexes are injected directly into the patient with a higher efficiency of cell transduction **[59]**. Furthermore, the ex vivo approach has the advantage of expressing osteogenic morphogen gene and supplying more bone precursor cells, which may be of limited number at some treatment regions **[60]**. For example, it is clear that the number of mesenchymal stem cells present throughout the body decrease with age, chronic debilitating diseases, or due to local tissue degeneration as after radio- or chemotherapy. Therefore, the introduction of genetically premodified stem cells to secrete osteogenic morphogens into the treatment site is considered a great advantage in gene therapy **[26]**.

Previous studies proved that stem cells could be isolated from both embryonic tissues and different adult organs **[27-30]**. Bone marrow considered one of the most popular sources of mesenchymal stem cells (MSCs) that have the ability to differentiate into osteoblasts, chondroblasts, and fibroblasts **[46-47]**. Williams et al. have shown also that cells isolated from skeletal muscles possess adipogenic, osteogenic, chondrogenic, and myogenic

differentiation **[61]**. Other studies isolated these multipotential cells from other sources as epidermis **[62]**. However, the above sources of adult pluripotent stem cells have potential limitations as; painful procedure, donor site morbidity, functional impairment, and low number of mesenchymal stem cells that often requiring ex vivo expansion before differentiation **[52,54,63]**. Recent studies showed that the processing of human liposuctioned fat results in a population of multilineage cells, called processed lipoaspirate cells, that can be differentiated into adipogenic, osteogenic, chondrogenic, and myogenic cells **[54,64]**.

Fat can be obtained in big quantities with a relatively safe procedure (liposuction), under local anesthesia, with minimal discomfort, and less donor site morbidity. Zuk et al. showed that processed lipoaspirate (PLA) cells can be maintained in vitro for long periods with stable population doubling and low level of senescence **[54]**. Their results also showed that the majority of PLA cells are of mesodermal or mesenchymal origin with low levels of pericytes, endothelial cells, and smooth muscle cells. Adipose tissue may be an important new alternative source of multipotent stem cells for future tissue engineering therapies.

Recent studies showed the osteogenic potential of fat derived stem cells by culturing these cells in osteogenic media containing Dulbecco modified Eagle medium with 10% fetal bovine serum, 0.1 µm dexamethazone, 10 mM *β*–glycerol phosphate, and 50 µg/ml ascorbic acid-2-phosphate **[64]**. The period of differentiation, however, is over two weeks as evidenced by endogenous alkaline phosphatase activity and the formation of mineralized nodular structures confirmed by von Kossa staining. The use of gene therapy as a power bone inducer may be able to overcome the time needed and other limitations of conventional bone differentiation using osteogenic medias. For example BMP gene expression at treatment site could be sustained for long periods of time as adenoviral vectors can provides gene expression for several weeks. Furthermore, recent development of inducible expression systems that may make it possible to control the expression of BMP-gene, which could be useful to control bone deposition over time.

Another important advantage of transfection of cells using BMP gene therapy is the simplicity of the protocol, as it requires only 24 hours of cultivation in vitro with the virus before implanting cells in the treatment site. On contrary, osteogenic differentiation using osteogenic media required at least two weeks before the clinical usage of these cells. We also found that low titers (2-pfu/cell) of expression of BMP-9 was sufficient to drive the fat derived stem cells to bone in half the time of the normal differentiation protocol. We were able to show that all bone specific markers that we looked for were expressed much more quickly than in similar studies reporting the usage of osteogenic media.

These results are important for many reasons. First, this is the first description of an alternative protocol for the differentiation of fat cells into bone than is usually used. This indicates that there are more than one, and quite possibly several methods for the induction of differentiation in the fat derived stem cells. The second important reason is the reduction in the time for differentiation that's means reduction in the time for implantation. This would make the dream of bedside therapy more realizable, if cells could be taken out of a patient, purified, infected with a virus, and put back into the patient. This would save time, money and probably lead to better patient outcomes.

The preliminary data presented in this study with the theoretical advantages of the ex vivo approach of gene therapy using fat derived stem cells supports the future clinical applications of this technique for the treatment of various craniofacial defects.

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