Investigative study

COLLAGENATED XENOGRAFT BIOMATERIAL INDUCES OSTEOBLAST DIFFERENTIATION MARKERS IN ADIPOSE-DERIVED STEM CELLS IN VITRO

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ABSTRACT

Bone regeneration is a complex biological process crucial for healing bone damage that involves a coordinated sequence of cellular and molecular events, including inflammation, stem cell recruitment, proliferation, differentiation, and matrix deposition. Various surgical techniques have been developed to help bone regeneration and restore tissue damaged by infections, tooth loss, neoplasms, or local trauma. The use of xenografts and alloplastic bone substitutes takes advantage of eliminating the restricted source and morbidity rate of the donor site of autologous and allogeneic grafts. In vitro, studies could help to evaluate the effectiveness of these products and can help to test the biocompatibility and biological characteristics of biomaterials. In this investigation, we studied if OsteoBiol, an animal-derived collagenated bone matrix, can promote osteoblast differentiation of adipocyte stem cells cultured in vitro. The expression levels of markers of bone differentiation were monitored at different time points by real-time Polymerase Chain Reaction. After 24 h of treatment, SPP1 was up-regulated, as were FOSL1, COL4A1, and MMP14. After 4 days of treatment, FOSL1 and COL4A1 remained increased, and COL1A1 was up-regulated. OsteoBiol promotes the expression of several important genes of osteoblast differentiation. Additional research could provide deeper insights into the underlying mechanisms and enhance the practical application of these findings in clinical settings.

KEYWORDS: *osteoblast differentiation, OsteoBiol, xenograft, adipocyte stem cells, implant dentistry, gene expression*

INTRODUCTION

Bone regeneration is a complex biological process crucial for healing bone fractures, repairing defects, and restoring bone tissue lost due to injury or disease. It involves a coordinated sequence of cellular and molecular events, including inflammation, cell recruitment, proliferation, differentiation, and matrix deposition (1).

Immediately following the trauma, the injury site is rich in blood cells from ruptured vessels and bone marrow cells, which could include both hemopoietic and adipose cells. Inflammatory cells release cytokines and growth factors that stimulate the recruitment of mesenchymal stem cells (MSCs) to the injury site (2, 3). MSCs differentiate into osteogenic cells, which synthesize and deposit new bone matrix, leading to callus formation and, eventually, bone

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remodeling. Osteoblasts and osteocytes originate from the periosteum, bone marrow, and endosteum, indicating that these three tissues contribute simultaneously to bone repair (4).

Several strategies are employed to enhance bone regeneration, including autografts, allografts, bone morphogenetic proteins (BMPs), growth factors, and tissue engineering techniques (5, 6). Autografts, harvested from the patient's own bone, remain the gold standard due to their osteogenic potential and low risk of rejection (7). Allografts, derived from cadaveric donors, provide an alternative but carry risks of immunogenicity and disease transmission. BMPs and growth factors, such as BMP-2 and platelet-derived growth factor (PDGF), promote osteogenesis and angiogenesis, accelerating bone healing (2). Tissue engineering approaches involve the use of scaffolds, cells, and signaling molecules to create biomimetic environments that facilitate bone regeneration (5).

Using xenograft bone substitutes represents a valuable and safe technique that takes advantage of availability, avoiding the need for a donor site for autologous graft retrieving (7). The animal bone can be treated with different techniques to provide a scaffold for new bone formation, which after transplant is gradually resorbed over time, allowing for integration with host bone tissue (8, 9). OsteoBiol biomaterials consist of heterologous cancellous bone produced by a process that avoids the ceramization of the hydroxyapatite crystals and preserves collagen. OsteoBiol is utilized in a variety of dental and orthopedic surgeries for bone augmentation, ridge preservation, sinus lifting, and periodontal regeneration procedures. It functions as a filling material for bone defects and contributes to the stability and success rates of dental implants by promoting osseointegration (10).

Adipose-derived stem cells (ADSCs) represent a type of mesenchymal stem cell that can be harvested from adipose tissue and possess the potential to differentiate into various cell types, including adipocytes, osteoblasts, chondrocytes, and other mesodermal cells (11). As a result of their ability to be obtained through minimally invasive methods, ADSCs have been recognized as a valuable resource in the fields of tissue engineering and regenerative medicine (12, 13). In vitro, osteogenic differentiation can be simulated by supplementing the medium with ascorbic acid, b-glycerophosphate, dexamethasone, 1,25 vitamin D3, and BMP2 (14, 15). ADSCs cultured in the presence of these factors express genes that characterize osteoblast differentiation, including alkaline phosphatase, collagen type I, osteopontin, osteonectin, and Runx2 (16).

In this investigation, ADSCs were cultured in vitro with OsteoBiol, a hard biomaterial from animal bone matrix usually used in bone regeneration surgery, to verify if the biomaterial can promote stem cell differentiation toward osteogenic lineage.

MATERIALS AND METHODS

Adipocyte Stem Cells (ADSCs) isolation

Adipose tissue was extracted from the buccal fat pad (also called Bichat's fat pad) during the intervention to close oro-antral communication. It was digested for 1 h at 37°C in a solution containing 1 mg/ml collagenase type I and 1 mg/ml dispase, then dissolved in phosphate-buffered saline (PBS) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 500 µg/ml clarithromycin. The solution was filtered using Falcon strainers with 70 µm pores (Sigma Aldrich, St Louis, Mo, U.S.A.) to separate mesenchymal stem cells. Stem cells were cultivated in α-MEM culture medium (Sigma Aldrich, St Louis, Mo, U.S.A.) supplemented with 20% Fetal Bovine Serum (FBS), 100 µM 2P-ascorbic acid, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma Aldrich, St Louis, Mo, U.S.A.). The culture flasks were incubated at 37 °C and 5% CO_2, and the medium was changed twice per week.

ADSCs were characterized by immunofluorescence for the cytoskeletal component vimentin, positive mesenchymal stem cell markers CD90 and CD73, and the negative marker CD34 as described in Sollazzo et al. (17).

Cell treatment

ADSCs were seeded at a concentration of 1.0×10^5 cells/ml with a mechanically fragmented OsteoBiol sp-block (Tecnoss Dental SRL, Torino, Italy) at the concentration of 3 mg in 9 cm^2 (3 ml) wells in a DMEM culture medium supplemented with 10% serum and antibiotics. Another set of wells containing untreated cells was used as a control. The treatment was carried out at two time points: 24 h and 4 days.

The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. At the end of the treatment period, the cells were lysed and processed for total RNA extraction.

RNA isolation and gene expression quantification

Total RNA was isolated from the cells using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The pure RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

cDNA synthesis was performed starting from 500 ng of total RNA using the PrimeScript RT Master Mix (Takara Bio Inc., Kusatsu, Japan). The reaction mixture was incubated at 37 °C for 15 min and inactivated by heating at 70°C for 10 s. cDNA was amplified by real-time quantitative PCR using an ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA). All PCR reactions were performed in a 20 µL volume. Each reaction contained 10 µl of 2x qPCRBIO SYGreen Mix Lo-ROX (PCR Biosystems, Ltd., London, UK), 400 nM of each primer, and cDNA.

Custom primers belonging to the "extracellular matrix, adhesion molecule" pathway, "osteoblast differentiation," and "inflammation" pathway were purchased from Sigma Aldrich. The selected genes grouped by functional pathways are as follows: osteoblast differentiation [SPP1 (Osteopontin), SPARC (Osteonectin), RUNX2 (Runt-related transcription factor 2), ALP (Alkaline phosphatase), BGLAP (Osteocalcin), FOSL1 (FOS-like antigen 1), SP7 (Osterix), ENG (Endoglin)], extracellular matrix, adhesion molecule [COL1A1 (Collagen type I alpha1), COL4A1 (Collagen type IV alpha 1), MMP14 (Matrix Metallopeptidase 12), MMP15 (Matrix Metallopeptidase 15)], inflammation [IL6 (Interleukin 6), IL6R (Interleukin 6 Receptor)] and RPL13 (Ribosomal protein L13) as reference gene.

All experiments were performed using non-template controls to exclude reagent contamination. PCR was performed using two analytical replicates.

The amplification profile was initiated by incubation for 10 min at 95 °C, followed by a two-step amplification for 15 s at 95 °C and 60 s at 60 °C for 40 cycles. In the final step, melt curve dissociation analysis was performed.

Statistical analysis

The relative gene expression was quantified with the delta/delta Ct calculation method (18), using the reference gene RPL13 to normalize gene expression levels. The gene expression levels change of treated cells were calculated as fold-changes relative to untreated cells; fold change was considered biologically relevant when the expression doubled, i.e. fold changes ≥ 2, or halved, i.e. fold changes ≤ 0.5.

RESULTS

ADCSs were phenotypically characterized using immunofluorescence. Fig. 1a shows cytoskeletal filaments stained with vimentin. The cell surfaces were positive for mesenchymal stem cell markers CD90 (Fig. 1b) and CD73 (Fig. 1c) and negative for markers of hematopoietic origin CD34 (Fig. 1d).

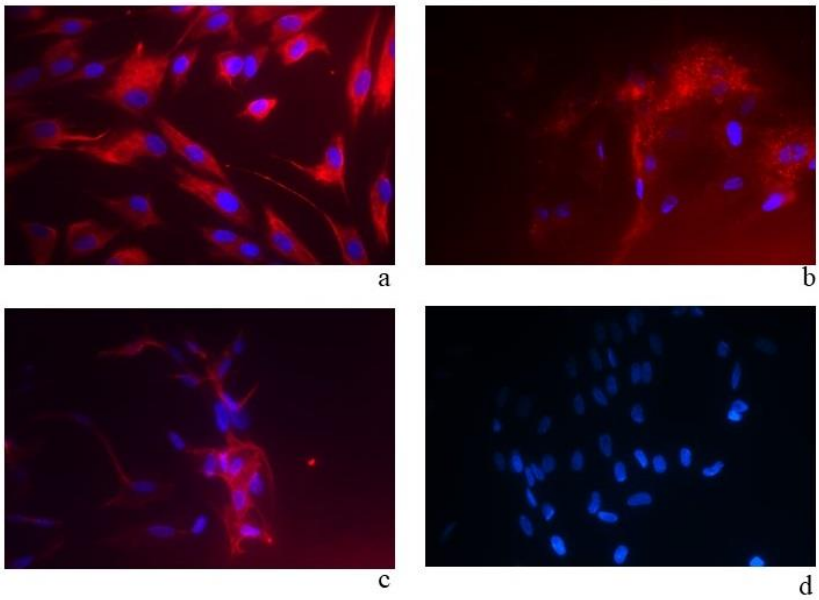

Fig. 1. *DPCSs by indirect immunofluorescence (Rhodamine). Immunofluorescence staining of vimentin (**a**), mesenchymal stem cell marker CD90 (**b**), CD73 (**c**), and hematopoietic markers CD34 (**d**). Nuclei were stained with DAPI. Original magnification x40.*

The expression level of genes involved in osteoblast differentiation was monitored in ADSCs grown with OsteoBiol xenograft biomaterial by quantitative real-time PCR and compared with untreated cells. The expression level variation of transcription factors, extracellular matrix, and inflammation pathways was measured after 24 h and 4 days of treatment as fold change levels. Several genes showed more than a two-fold increase in expression level. Indeed, after 24 h of treatment, SPP1, FOSL1, and COL4A1 and MMP14 were up-regulated. After 4 days of treatment, FOSL1 and MMP14 were further increased, and COLL1A1 was up-regulated.

DISCUSSION

ADSCs possess self-renewal capacity and can differentiate into multiple cell lineages, including adipocytes, osteoblasts, chondrocytes, and myocytes. Multipotent differentiation potential, together with their abundance in the easily accessible fat tissue, has been granted to ADSCs by the scientific community because they represent a promising tool in regenerative medicine and tissue engineering.

In this study, we utilized an in vitro ADSC culture to assess the osteoinduction capacity of OsteoBiol, a commonly used biomaterial in bone regeneration surgery. OsteoBiol is a collagenated bone matrix derived from animal cancellous bone, employed by surgeons as a filling material for bone defects or as a scaffold to facilitate bone growth. Our aim was to determine whether exposure to OsteoBiol can stimulate osteoblast differentiation of ADSCs. To test this, we monitored the expression levels of a selected panel of genes at 4 hours and 4 days post-treatment. It was observed that collagen type I and type IV, two structural extracellular matrix proteins, were over-expressed in ADSCs cultured on the OsteoBiol biomaterial.

The SPP1 gene was overexpressed early, only 4 hours after OsteoBiol administration. The SPP1 gene encodes for Osteopontin, which is recognized as a marker of bone differentiation due to its expression during the early stages of osteoclast and osteoblast progenitor differentiation (19). Osteopontin is a phosphorylated glycoprotein secreted by osteoblasts into the mineralizing extracellular matrix during bone development (20). The highest expression of this protein is seen in mature osteoblasts at sites of bone remodeling (21).

Osteopontin plays a crucial role in bone mineralization and the attachment of osteoclasts to the mineral matrix (22, 23). Osteoclast integrins binding to osteopontin activates signaling pathways that enhance osteoclast activity (24). Through this mechanism, OPN facilitates the resorption phase of bone remodeling, essential for removing old or damaged bone and regulating bone density. Osteopontin is also involved in various physiological and pathological processes, such as immune response and inflammation (25).

Collagen Type I is the primary structural protein in the extracellular matrix of bone. Type I collagen is not exclusively expressed by osteoblasts but is also produced by fibroblasts; however, collagen Type I remains a useful marker for osteoblast differentiation when expressed together with other bone markers (26). Collagen Type IV is a major basement membrane component that separates epithelial and endothelial cells from connective tissue (27). Several experimental evidences suggest that collagen type IV is not only produced by epithelial cells, but it is expressed in other tissues, where is involved in tissue genesis, differentiation, homeostasis, and remodeling (28). For example, collagen type IV appears to play a significant role in the differentiation of stem cells towards osteoblasts and adipoblasts. In these cells, the inhibition of miR-214-5p promotes the survival of osteoblasts and extracellular matrix production by targeting COL4A1 (29). Another investigation showed that miR-214-5p may weaken bone marrow stem cells' osteogenic differentiation by downregulating COL4A1. Additionally, miR-214-5p may promote adipogenic differentiation by downregulating the TGF-β/Smad2/COL4A1 signaling pathway (30).

Another line of evidence associates COL4A1 to different skeleton pathologies. A genomic region at 13q34, including COL4A1 and COL4A2 (collagen type IV alpha-1 and alpha-2 subunits), was significantly linked with forearm bone mineral density in a genome-wide linkage scan (31). A significant COL4A1 gene expression level was found in human osteoporosis fracture bone compared to bone from individuals with osteoarthritis or no bone pathology (32).

The Matrix metalloproteinase family of proteins (MMP) is involved in the digestion of extracellular matrix proteins during normal physiological processes such as embryonic development and tissue remodeling. Since MMP14 appears to play a multifaceted role in regulating various signaling pathways and cell fate decisions critical for bone formation and remodeling, this protein could be considered an additional marker of osteoblast differentiation (33). Research has shown that deleting the membrane-anchored MMP14 in mesenchymal progenitors redirects cells' fate from osteogenesis to adipo- and chondrogenesis (34). The same treatment did not have the same effect in committed osteoblasts (34). Interestingly, MMP14 seems to regulate the differentiation of mesenchymal stem cells into bone-producing osteoblasts in 3-dimensional collagen matrices (35). Moreover, MMP14 is essential for osteoblast survival during the osteoblast-to-osteocyte transition and is required for proper lacunae formation (36). Parathyroid hormone stimulates

osteocyte proliferation by activating the Wnt pathway and increasing MMP14 expression levels, which in turn appear to regulate soluble RANKL production, thus controlling bone resorption (37). Finally, MMP14 can regulate osteoclastogenesis. Suppressing MMP14 expression in osteoblasts had the effect of increasing the numbers and activity of osteoclasts (37, 38).

CONCLUSIONS

Xenografts are considered valid alternatives to autografts. Indeed, animal-derived biomaterials are available in large quantities, while advancements in processing techniques have significantly lowered the risk of immunogenic reactions and infection transmission. OsteoBiol is a collagenated exogenous bone-derived biomaterial successfully used in regenerative medicine. In this investigation, we showed that ADSCs cultured in vitro with OsteoBiol increased the expression levels of genes considered markers of osteodifferentiation. Indeed, these undifferentiated cells over-expressed SPP1, COL1A1, COL4A1, and MMP14 at different time points when cultured with OsteoBiol. However, additional research is necessary to clarify the specific mechanisms involved in the cell signaling pathways underlying the current results.

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