Allogro® acts on stem cells derived from peripheral blood

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Citation

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Abstract

Introduction
: Allogro® is a demineralized freeze-dried bone allograft, a promising material for bone grafting, which is useful as a scaffold to fill bone defects and to restore bone loss in orthopedic and maxillofacial surgery.However, how Allogro® alters osteoblast activity to promote bone formation is poorly understood.Materials and Methods: To study how Allogro® can induce osteoblast differentiation in mesenchymal stem cells, the expression levels of bone related genes and mesenchymal stem cells marker were analyzed, using real time Reverse Transcription-Polymerase Chain Reaction.Results: Allogro® causes a significant induction of osteoblast transcriptional factor like osterix (RUNX2) and of the bone related genes osteopontin (SPP1), osteocalcin (BGLAP) and alkaline phosphatase (ALPL).Conclusion: The obtained results can be relevant to better understand the molecular mechanism of bone regeneration and as a model for comparing other materials with similar clinical effects.

INTRODUCTION

The increase in orthopedic and dental prosthetic surgery is often associated with the need to restore the bone loss subsequent prosthetic failure and osteolysis resulting from wear particles of cement and polyethylene. [1] Moreover also in spinal and maxillofacial surgery is frequently necessary to restore bone stock and enhance bone healing. Autogenous iliac crest bone graft has been the “gold standard” for spinal fusion and maxillary sinus augmentation. However, bone graft harvest may lead to complications, such as chronic pain, numbness, and poor cosmesis. [2] Large bone defects still represent a major problem in orthopedics and maxillofacial surgery. Traditional bone-repair treatments can be divided into two groups: the bone transport (Ilizarov technology) and the graft transplant (autologous or allogeneic bone grafts). Three strategies are typically used to augment massive bone defect repair. The first is to engraft mesenchymal stem cells (MSCs) onto a graft or a biosynthetic matrix to provide a viable osteoinductive scaffold material for segmental defect repair. The second strategy is to introduce critical factor(s), for example, bone morphogenetic proteins (BMPs), in the form of bone-derived or recombinant proteins onto the graft or matrix directly. The third strategy uses targeted delivery of therapeutic genes (using viral and non-viral vectors) that either transduce host cells in vivo or stably transduce cells in vitro for subsequent implantation in vivo. [3] Fresh frozen allograft has been widely used for acetabular grafting in revision surgery. However there are concerns about the transmission of viral proteins and prions through this form of allograft. As a result, irradiated bone graft has been favoured in some centres. [4] Also bony allografts are used frequently in the clinic for bone defect filling. [5] In our study we used Allogro® (Ceramed, Lakewood, CO) which is a demineralized freeze-dried bone allograft. Thanks to its osteoinductivity, Allogro® is used to fill bone defects in case of non unions, delayed unions, spinal fusions, augmenting prosthetic implants and in periodontal regeneration (Figure 1 and 2).

Figure 1

Figure 1: Histology derived from 6 months sampling a human sinus lift: le photo shows the demineralized material in the central part of the graft (50x).

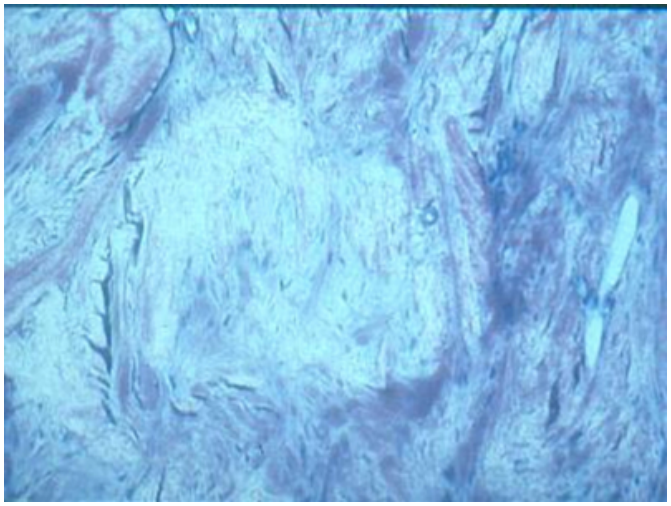
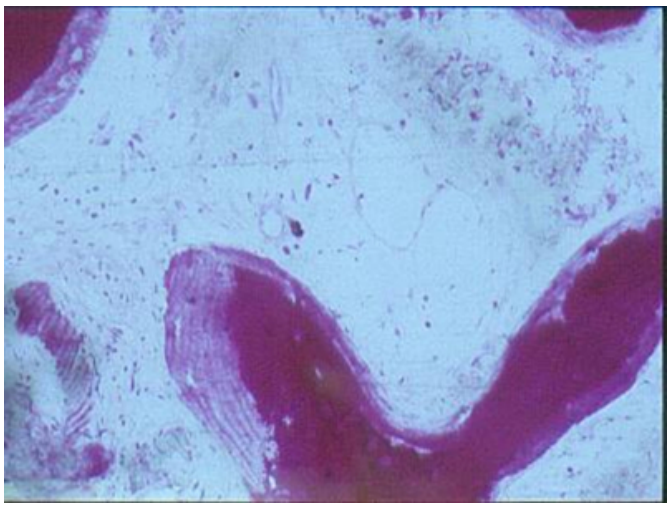


Figure 2

Figure 2: The peripheral part of the graft shows the new formed bone on the same material.



Because few reports analyze the biological effects of Allogro® $_{678}$ and none of those studies focus on the genetic effects on stem cells, the expression of genes related to the osteoblast differentiation were analyzed using cultures of stem cells derived from peripheral blood (PB-hMSCs) treated with Allogro®.

To investigate the osteogenic differentiation of PB-hMSCs, the quantitative expression of the mRNA of specific genes, like transcriptional factors (RUNX2), bone related genes (SPP1, COL1A1, COL3A1, BGLAP, ALPL, and FOSL1) and mesenchymal stem cells marker (CD105) were examined by means of real time Reverse Transcription-Polymerase Chain Reaction (real time RT-PCR).

MATERIALS AND METHODS

A) STEM CELL PREPARATION

PB-hMSCs were obtained for gradient centrifugation from peripheral blood of healthy anonymous volunteers, using the Acuspin System-Histopaque 1077 (Sigma Aldrich, Inc., St Louis, Mo, USA). Firstly, 30 ml of heparinizated peripheral blood were added to the Acuspin System-Histopaque 1077 tube and centrifugated at 1000 x g for 10 minutes. After centrifugation, the interface containing mononuclear cells was transferred in another tube, washed with PBS and centrifugated at 250 x g per 10 minutes. The enriched mononuclear pellets was resuspended in 10 ml of Alphamem medium (Sigma Aldrich, Inc., St Louis, Mo, USA) supplemented with antibiotics (Penicillin 100 U/ml and Streptomycin 100 micrograms/ml - Sigma, Chemical Co., St Louis, Mo, USA) and amminoacids (L-Glutamine - Sigma, Chemical Co., St Louis, Mo, USA). The cells will be maintained at 37°C in a fully humidified atmosphere at 5% CO2 in air. Medium was changed after 24 hours. PB-hMSC were selected for adhesivity and characterized for staminality by immunofluorescence.

B) IMMUNOFLUORESCENCE

Cells were washed with PBS for three times and fixed with cold methanol for 5 min at room temperature. After washing with PBS, cells were blocked with bovine albumin 3% (Sigma Aldrich, Inc., St Louis, Mo, USA) for 30 min at room temperature. The cells were incubated overnight sequentially at 4 °C with primary antibodies raised against CD105 1:200, mouse (BD Biosciences, San Jose, CA, USA), CD73 1:200, mouse (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), CD90 1:200, mouse (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), CD34 1:200, mouse (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). They were washed with PBS and incubated for 1 h at room temperature with secondary antibody conjugated-Rodamine goat anti-mouse 1:200 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Subsequently, cells were mounted with the Vectashield Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) and observed under a fluorescence microscope (Eclipse TE 2000-E, Nikon Instruments S.p.a., Florence, Italy).

C) CELL CULTURE

PB-hMSCs at second passage were grown in Alphamem medium (Sigma Aldrich, Inc., St Louis, Mo, USA)

supplemented with 10% fetal calf serum, antibiotics (Penicillin 100 U/ml and Streptomycin 100 micrograms/ml) Sigma Aldrich, Inc., St Louis, Mo, USA) and amminoacids (L-Glutamine) (Sigma Aldrich, Inc., St Louis, Mo, USA). The cells were maintained at 37°C in a fully humidified atmosphere at 5% CO2 in air.

For the assay, cells were collected and seeded at a density of $1x10_5$ cells/ml into 9 cm_2 (3ml) wells by using 0.1% trypsin, 0.02% EDTA in Ca++ - and Mg – free Eagle's buffer for cell release.

One set of 6 wells were added with Allogro® (Ceramed, Lakewood, CO) at the concentration of 10 mg/ml. Another set of wells containing untreated cells were used as control. The medium was changed every 3 days.

After seven days, when cultures were sub-confluent, cells were processed for RNA extraction.

D) RNA PROCESSING

Reverse transcription to cDNA was performed directly from cultured cell lysate using the TaqMAn Gene Expressio Cells-to-Ct Kit (Ambion Inc., Austin, TX, USA), following manufacturer's instructions. Briefly, cultured cells were lysed with lysis buffer and RNA released in this solution. Cell lysate were reverse transcribed to cDNA using the RT Enzyme Mix and appropriate RT buffer (Ambion Inc., Austin, TX, USA).

Finally the cDNA was amplified by real-time PCR using the included TaqMan Gene Expression Master Mix and the specific assay designed for the investigated genes.

E) REAL TIME PCR

Expression was quantified using real time RT-PCR. The gene expression levels were normalized to the expression of the housekeeping gene RPL13A and were expressed as fold changes relative to the expression of the untreated PB-hMSCs. Quantification was done with the delta/ delta calculation method.. [9]

Forward and reverse primers and probes for the selected genes were designed using primer express software (Applied Biosystems, Foster City, CA, USA) and are listed in Table 1.

Figure 3

Table 1. Primer and probes used in real time PCR

Gene symbol	Gene name	Primer sequence (5'>3')	Probe sequence (5'>3')
SPP1	osteopontin	F-GCCAGTTGCAGCCTTCTCA R-AAAAGCAAATCACTGCAATTCTCA	CCAAACGCCGACCAAGGAAAACTCAC
COL1A1	collagen type I alpha1	F-TAGGGTCTAGACATGTTCAGCTTTGT R-GTGATTGGTGGGATGTCTTCGT	CCTCTTAGCGGCCACCGCCCT
RUNX2	runt-related transcription factor 2	F-TCTACCACCCCGCTGTCTTC R-TGGCAGTGTCATCATCTGAAATG	ACTGGGCTTCCTGCCATCACCGA
ALPL	alkaline phosphatase	F-CCGTGGCAACTCTATCTTTGG R-CAGGCCCATTGCCATACAG	CCATGCTGAGTGACACAGACAAGAAGCC
COL3A1	collagen, type III, alpha 1	F-CCCACTATTATTTTGGCACAACAG R-AACGGATCCTGAGTCACAGACA	ATGTTCCCATCTTGGTCAGTCCTATGCG
BGLAP	osteocalcin	F-CCCTCCTGCTTGGACACAAA R-CACACTCCTCGCCCTATTGG	CCTTTGCTGGACTCTGCACCGCTG
CD105	endoglin	F-TCATCACCACAGCGGAAAAA R-GGTAGAGGCCCAGCTGGAA	TGCACTGCCTCAACATGGACAGCCT
FOSL1	FOS-like antigen 1	F-CGCGAGCGGAACAAGCT R-GCAGCCCAGATTTCTCATCTTC	ACTTCCTGCAGGCGGAGACTGACAAAC
RPL13A	ribosomal protein L13	F-AAAGCGGATGGTGGTTCCT R-GCCCCAGATAGGCAAACTTTC	CTGCCCTCAAGGTCGTGCGTCTG

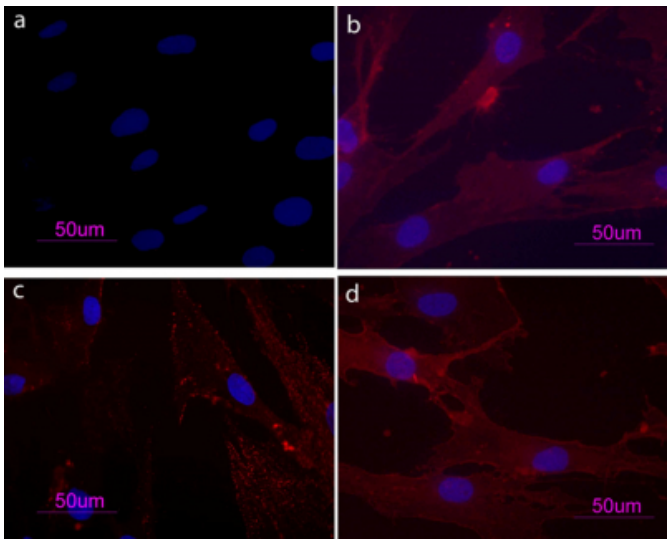
All PCR reactions were performed in a 20 µl volume using the ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA). Each reaction contained 10 µl 2X TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA, USA), 400 nM concentration of each primer and 200 nM of the probe, and cDNA. The amplification profile was initiated by 10-minute incubation at 95°C, followed by two-step amplification of 15 seconds at 95°C and 60 seconds at 60°C for 40 cycles. All experiments were performed including non-template controls to exclude reagents contamination. PCRs were performed with two biological replicates.

RESULTS

PB-hMSCs were characterized by immunofluorescence. The cell surfaces were positive for mesenchymal stem cell marker, CD105, CD90 and CD73 and negative for markers of hematopoietic origin, CD34 (Figure 3).

Figure 4

Figure 3. PB-hMSCs by indirect immunofluorescence (Rodamine). Cultured cells were positive for the mesenchymal stem cell marker CD73 (b), CD90 (c), CD105 (d) and negative for the hematopoietic markers CD34 (a). Nucleuses were stained with DAPI. Original magnification x40



Transcriptional expressions of several osteoblast-related genes (RUNX2, SPP1, COLIA1, COL3A1, BGLAP, ALPL and FOSL1) and mesenchymal stem cells marker (ENG) were examined after 7 days of treatment with Allogro®.

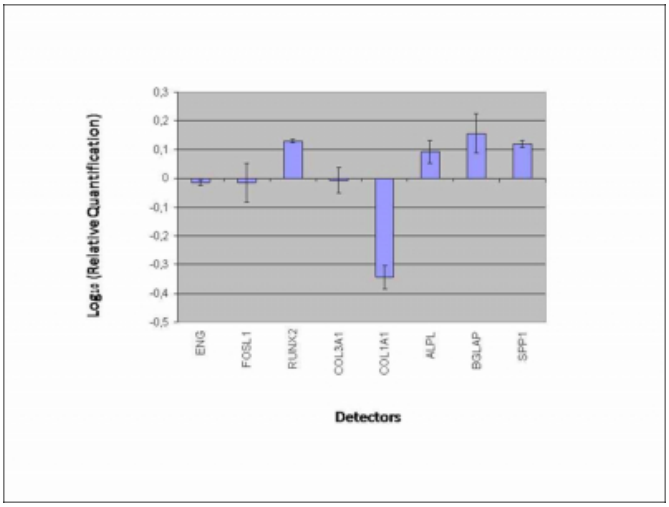
Quantitative real-time RT–PCR of RUNX2, ALPL, BGLAP and SPP1 showed a significant induction after treatment.

COL1A1, ENG and FOSL1 were decreased in the presence of Allogro® at day 7.

However, Allogro® treatment did not affect the mRNA expression of COL3A1 that were similarly in both treated and untreated PB-hMSCs. (Figure 4).

Figure 5

Figure 4. Gene expression analysis of PB-hMSCs after 7 days of treatment with Allogro®.



DISCUSSION

Although autogenous bone grafting continues to be considered the gold standard for sinus grafting and spinal fusion, [10] it is associated with an unacceptably high incidence of morbidity. [11] Chronic iliac crest bone graft harvest site pain and discomfort is reported by a significant percentage of patients undergoing this procedure more than three years following surgery, and these complications are associated with worse patient-reported disability. [2] Furthermore, operative time, blood loss, and length of hospitalization are often increased. [11] In order for a graft substitute to replicate the optimal bone healing properties of autogenous graft, 3 essential elements must be present: scaffolding for osteoconduction, growth factors for osteoinduction, and progenitor cells for osteogenesis. A composite graft that combines a synthetic scaffold with osteoprogenitor cells from bone marrow aspirate (BMA) may potentially deliver the advantages of autogenous bone grafts without the procurement morbidity [1] . Tissue engineering for bone grafting may emerge as an alternative to autogenous bone grafts. [12]

Research in regenerative medicine is developing at a significantly quick pace. Cell-based bone and cartilage replacement is an evolving therapy aiming at the treatment of patients who suffer from limb amputation, damaged tissues and various bone and cartilage-related disorders and dental and maxillofacial reconstructive surgery. Stem cells are undifferentiated cells with the capability to regenerate into one or more committed cell lineages. Stem cells isolated from multiple sources have been finding widespread use to

advance the field of tissue repair. [13] Bone regeneration for the defects in revision surgery of joint replacement is an increasingly important issue. To repair bone defects, bone cell activation by growth factors using synthetic resorbable scaffold is a useful and safe option. [14] Synthetic and biological materials are increasingly used to provide temporary or permanent scaffolds for bone regeneration. [15] Bone regeneration by autologous cell transplantation in combination with a biodegradable scaffold is one of the most promising techniques being developed in craniofacial and orthopedic surgery. [16] Tissue engineering approaches attempts to create tissue replacement by culturing autologous cells onto three-dimensional matrixes that facilitate cell progenitor migration, proliferation and differentiation. [17]

Maxillary sinus augmentation is frequently necessary before placement of dental implants in the posterior maxilla. Besides autogenous bone graft, various bone substitutes have been used, with favorable results. [18] In this study we focused our interest on a promising material for bone grafting (Allogro®) which is a demineralized freeze-dried bone allograft. Allogro® is useful as a scaffold to fill bone defects and to restore bone loss in orthopedic and maxillofacial surgery. [6]

In order to get more inside how Allogro® acts on PB-hMSCs, changes in expression of bone related marker genes (RUNX2, SPP1, COLIA1, COL3A1, BGLAP, ALPL and FOSL1) and mesenchymal stem cells marker (ENG) were investigated by real-time RT–PCR.

In our study, mesenchymal stem cells from peripheral blood were isolated by gradient and characterized by morphology and immunophenotype. Isolated PB-hMSCs showed fibroblast-like morphology and were positive for MSCs surface molecules (CD90, CD105, CD73) and negative for markers of hematopoietic progenitors (CD34) (i.e. CD34 positive cells differentiate in hematopoietic line).

After 7 day of treatment with Allogro®, the expression levels of osteo-differentiation genes were measured by relative quantification methods using real-time RT–PCR.

The transcriptional factors RUNX2 was up-regulated in treated PB-hMSCs with respect to control. RUNX2 is a prerequisite for osteoblast differentiation and consequently mineralization. It is expressed in the earlier stages and induces the expression of two bone related genes BGLAP and SPP1 that were up- regulated in PB-hMSCs after treatment.

BGLAP, the most abundant protein in bone, is a mature osteoblast marker and its expression correlates with bone formation. [19] SPP1 encodes osteopontin, which is a phosphoglycoprotein of bone matrix and it is the most representative non collagenic component of extracellular bone matrix. [15] Osteopontin is actively involved in bone resorbitive processes directly by ostoclasts. [16] Osteopontin produced by osteoblasts, show high affinity to the molecules of hydroxylapatite in extracellular matrix and it is chemo-attractant to osteoclasts. [20]

Allogro® induced the expression of ALPL in treated stem cells with respect to control. ALPL is widely used as a marker of osteoblasts differentiation, an increase in ALPL expression should be associated with osteoblast differentiation.

ENG (CD105), a surface marker used to define a bone marrow stromal cell population capable of multilineage differentiation, [21] was weakly down expressed in treated PB-hMSCs with respect to control at 7 days, indicating the differentiation effect of this biomaterial on stem cells. The disappearance of the CD105 antigen during osteogenesis suggests that this protein, like others in the Transforming Growth Factor TGF-β superfamily, is involved in the regulation of osteogenesis. [22]

Allogro® also modulates the expression of genes encoding for collagenic extracellular matrix proteins like collagen type 1α1 (COL1A1). COL1A1 was significantly down expressed as compared to the control when exposed to Allogro®, probably because this gene is activated in the late stage of differentiation and is related to extracellular matrix synthesis. COL3A1 expression didn't have significant change in treated cells respect to control after 7 day of treatment.

FOSL1, another weakly down-regulated gene in treated stem cells, encodes for Fra-1, a component of the dimeric transcription factor activator protein-1 (Ap-1), which is composed mainly of Fos (c-Fos, FosB, Fra-1 and Fra-2) and Jun proteins (c-Jun, JunB and JunD). AP-1 sites are present in the promoters of many developmentally regulated osteoblast genes, including alkaline phosphatase, collagen I, and osteocalcin. McCabe et al. (1996) [23] demonstrated that differential expression of Fos and Jun family members could play a role in the developmental regulation of bone-specific gene expression and, as a result, may be functionally significant for osteoblast differentiation. Kim et al. (2008) [24] studied the effect of a new anabolic agents that stimulate

bone formation. They found that this gene is activated in the late stage of differentiation, during the calcium deposition.

The present study shows the effect of Allogro® on PB-hMSCs in the early differentiation stages, as indicated by the activation of bone related markers RUNX1, BGLAP, SPP1 and ALPL. The down regulation of genes like COL1A1 demonstrated that 1 week of treatment was not enough for osteoblast differentiation. Moreover, we have chosen to perform the experiment after 7 days in order to get information on the early stages of stimulation. It is our understanding, therefore, that more investigations with different time points are needed in order to get a global comprehension of the molecular events related to Allogro®. The reported model is useful to investigate the effects of different substances on stem cells.

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