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Anorganic bovine bone and a silicate-based synthetic bone activate different microRNAs

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Abstract: Bio-Oss (BO), composed of anorganic bovine bone, is widely used in several bone regeneration procedures in oral surgery. PerioGlas (PG) is an alloplastic material that has been used for grafting of periodontal osseous defects since the 1990s. However, how these biomaterials alter osteoblast activity to promote bone formation is poorly understood. We attempted to address this question by using microRNA microarray techniques to investigate differences in translational regulation in osteoblasts exposed to BO and PG. By using miRNA microarrays containing 329 probes designed from human miRNA sequences, we investigated miRNAs whose expression was significantly modified in an osteoblast-like cell line (MG-63) cultured with BO vs PG. Three up-regulated miRNAs (mir-337, mir-200b, mir-377) and 4 down-regulated miRNAs (mir-130a, mir-214, mir-27a, mir-93) were identified. Our results indicated that BO and PG act on different miRNAs. Globally, PG causes activation of bone-forming signaling, whereas BO also activates cartilage-related pathways. (J. Oral Sci. 50, 301-307, 2008)

Keywords: bone graft; miRNA; microarray; gene expression; gene profiling.

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Introduction

Bio-Oss (BO) is a deproteinized sterilized bovine bone material comprising calcium-deficient carbonate apatite, and is chemically and physically identical to human bone (1-8). BO has a compressive strength of 35 Mpa and its porous nature (75-80% of the total volume) serves to greatly increase the surface area of the material, thus providing a substratum for increased angiogenesis and a scaffold for bone formation (1-8).

PerioGlas (PG) is an alloplastic material that has been used for grafting of periodontal osseous defects since the 1990s. In animal models it has been shown to facilitate good histological repair of surgically created defects. In monkey (9-11) PG demonstrates biocompatibility and osteo-conductive activity. It is mostly resorbed and replaced by bone, and the remaining granules stay in close contact with bone. In a rabbit model, PG has been shown to be capable of improving bone healing at the interface between titanium dental implants and bone (12), whereas in ovariectomized rats it enhances de novo formation of bone trabeculae in extraction sockets (13).

In a clinical trial, bioactive glass was shown to be effective as an adjunct to conventional surgery in the treatment of intrabony defects (14) as well as dental extraction sites before implant placement, in order to implement bone regeneration and augment early fixation of the implant (15). However, PG has no regenerative properties for cementum and periodontal ligament (16).

Because the mechanism by which BO and PG stimulate osteoblast activity to promote bone formation is poorly

understood, we attempted to address this question by using microRNA microarray techniques.

MicroRNAs (miRNAs) represent a class of small, functional, noncoding RNAs of 19-23 nucleotides (nt) cleaved from 60- to 110-nt hairpin precursors (17,18). Hundreds of miRNAs have been identified in plants and animals, where they are involved in various biological processes, including cell proliferation and cell death during development, stress resistance, and fat metabolism, through the regulation of gene expression (19) via post-transcriptional RNA silencing pathways. The RNA interference (RNAi) and miRNA pathways regulate gene expression by inducing degradation and/or translational repression of target mRNAs. These pathways are generally initiated by various forms of double-stranded RNA (dsRNA), which are processed by Dicer, an RNase III family endonuclease, to 21-22-nt RNA molecules that serve as sequence-specific guides for silencing (20,21).

MicroRNAs are transcribed as long primary transcripts (pre-miRNAs), which are processed by a nuclear RNase III Drosha-containing complex into short hairpin intermediates (pre-miRNAs). Pre-miRNAs are transported to the cytoplasm, where they are further processed by a second Rnase III-family enzyme called Dicer to generate 22-bp RNA duplexes with 2-nt 3' overhangs (22-25).

MicroRNAs are loaded onto an argonaute containing an effector ribonucleoprotein (RNP) complex, referred to as miRNP or RISC (RNA-induced silencing complex), which is capable of recognizing cognate mRNAs and inhibiting protein expression.

In the present study, we used a recently developed methodology for miRNA gene expression profiling based on the hybridization of a microchip, the Ncode Multi-Species miRNA Microarray (Invitrogen, Carlsbad, CA, USA), a slide printed with approximately 900 unique miRNA sequence probes for *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Danio rerio*. By analysis of the 329 human miRNAs sequences spotted on the array, we compared miRNA expression and consequently gene regulation in human MG63 cells treated with BO and PG.

Materials and Methods

Cell culture

Osteoblast-like cells (MG63) were cultured in sterile Falcon wells (Becton Dickinson, New Jersey, USA) containing Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) (Sigma, Chemical Co., St Louis, MO, USA) and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml; Sigma Chemical Co.). Cultures were maintained in a 5% CO_2 humidified atmosphere at 37°C.

MG63 cells were collected and seeded at a density of 1×10^5 cells/ml into 9-cm^2 (3-ml) wells by using 0.1% trypsin, 0.02% EDTA in Ca^{++}, Mg-free Eagle buffer for cell release. One set of wells was treated with Bio-Oss® (Geistlich, Wolhusen, Switzerland) at 10 mg/ml. Another set of wells was treated with PerioGlas® (US Biomaterials Corp., Alachua, FL) at 0.04 g/ml. After 24 h, when cultures were sub-confluent, the cells were processed for RNA extraction. This time point and doses were chosen in order to ensure that the results were comparable to previous studies performed by our group (26,27).

miRNA Microarray

MicroRNAs were extracted from the cells using a PureLink™ miRNA Isolation Kit (Invitrogen, Milan, Italy). Four hundred nanograms of miRNA from each sample (treated and control) was used for hybridization of NCode™ Multi-Species miRNA Microarray, a slide containing 329 Human miRNAs sequences in duplicate.

The Ncode™ miRNA Labeling System (Invitrogen, Milan, Italy) was used for labeling and hybridization of miRNA to the microarray, in accordance with the manufacturer's instructions. Briefly, a poly(A) tail was added to each miRNA using a poly A polymerase and an optimized reaction buffer. Then a capture sequence was ligated to the miRNA using a bridging oligo(dT). Following a purification step, the tagged miRNAs were hybridized to the microarray and incubated overnight.

After incubation for 18-20 h, the array was washed and hybridized with Alexa Fluor® 3 (for PG) and Alexa Fluor® 5 (for BO) capture reagents in the first experiment, and then switched. After another wash the array was scanned using a standard microarray scanner (Axon Instruments, Sunnyvale, CA, USA).

After scanning, each spot was identified by means of a GAL (GenePixR Array List) file downloaded from www.invitrogen.com/ncode, that lists the identities and locations of all the probes printed on the array.

Images were quantified by GenePix 6.0 software (Axon Instruments, Sunnyvale, CA, USA). Signal intensities for each spot were calculated by subtracting local background from total intensities. The data were normalized using DNMAD and Preprocessing (http://gepas.bioinfo.cipf.es/cgi-bin/tools) (28,29). This generates an average value of the two spot replicates of each miRNA.

To select the differentially expressed miRNA, the data obtained were analysed using the *SAM* (significance analysis of microarray) package (30).

For target predictions and validations, miRNAs were processed using miRBase Target, a web resource

(http://microrna.sanger.ac.uk/targets/v4/) developed by the Enright Lab at the Wellcome Trust Sanger Institute. This source uses an algorithm called miRanda to identify potential binding sites for a given miRNA in genomic sequences.

The gene target list was then processed by FatiGO (http://fatigo.bioinfo.cnio.es), a web interface that carries out simple data mining using Gene Ontology. The data mining involves assignation of the most characteristic Gene Ontology term to each cluster of regulated genes.

Results

Hybridization of miRNA to the sequences spotted on the slide allowed us to perform systematic analysis of microRNAs and to provide primary information on the regulation of translation processes induced by BO and PG. There were 3 up-regulated miRNAs (mir-337, mir-200b, mir-377) and 4 down-regulated miRNAs (mir-130a, mir-214, mir-27a, mir-93) for FDR (false discovery rate) = 0 and score > 4. Figure 1 shows the graphical output of SAM (Statistical Analysis for Microarray) for differentially expressed miRNAs. Because any given miRNA can potentially regulate thousands of genes, in this study we select only genes related to osteogenesis and bone remodeling (Table 1). Genes presenting conflicting regulation by miRNA were excluded.

We performed an indirect PCR validation rather than a direct one by comparing our results with those published previously by our group using the microarray technique (26,27). Lect1 was detected among the messenger RNAs up-regulated by down-regulated miRNAs, whereas CALCR was detected among the messenger RNAs down-regulated by up-regulated miRNAs, as reported previously (26,27).

Discussion

Previous histological studies have shown that most BO particles are surrounded by newly formed mature, compact bone with well-organized osteons (2,5,7,8). In some fields, osteoblasts are observed in the process of apposing bone directly on the particle surface. No gaps are present at the bone-particle interface, and the bone is always in close contact with the particles (31-35). No inflammatory cell infiltrate has been observed around the particles or at the interface with bone.

PG is a silicate-based synthetic bone augmentation material that has been used to fill periodontal defects, showing bonding and integration to both soft tissue and bone. Previous studies using animal models have shown that PG facilitates good histological repair of surgically created defects (9-13). In clinical trials, PG has been effective as an adjunct to conventional surgery in the treatment of intrabony defects (14) as well as dental extraction sites (15).

As it is still unclear how these biomaterials alter osteoblast activity to promote bone formation, we attempted to address this question by using miRNA microarray to identify genes that are differentially translated in osteoblasts exposed to BO and PG.

MicroRNAs (miRNAs) are a recently discovered class of small, ~19-23-nucleotide non-coding RNA molecules. They are cleaved from 70-110-nucleotide hairpin precursors and play an important role in post-transcriptional regulation.

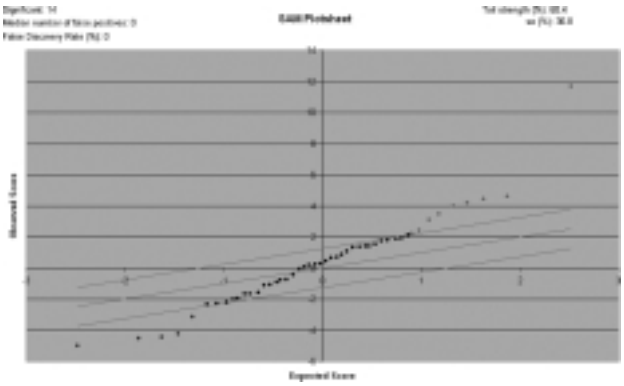

Fig. 1 SAM (significance analysis of microarray) plot of MG63 cells treated for 24 h with Bio-Oss® at a concentration of 100 mg/10 ml vs MG63 cells treated for 24 h with PerioGlas at a concentration of 0.04 g/ml.

Table 1 Down- and up- regulated genes

miRNA	Biological Function	Target Genes
Up-	skeletal development cartilage development	CALCR, CASR, COL11A2, TNFRSF11B, PAPSS1, EBP, EN1, INBH, CMKLR1, COMP NOG
Down	skeletal development cartilage development bone remodeling	MATN3, DLL3, CDH11, DLX5, PTH, IGF2, FGFR1, HOXA13, ADAMTS4 LECT1, BMP1, MATN1 SPP1, AHSG, PMF1, ALPL

MicroRNAs are not translated into proteins: instead, they regulate the expression of other genes by either cleaving or repressing the translation of their messenger RNA (mRNA) targets.

Recent advances in spotted oligonucleotide microarray labeling and detection have enabled the use of this high-throughout technology for miRNA screening. Microarray is a molecular technology that enables parallel analysis of a very large number of DNA or RNA fragments, spanning a significant fraction of the human genome. Gene expression is facilitated by a process of (i) miRNA extraction, (ii) labeling (different dyes are used to stain reference untreated cells and investigated cells), (iii) and hybridization on slides containing miRNA probes. The slides are then scanned with a laser system, and two false color images are generated for each hybridization with miRNA from the investigated and the reference cells. The overall result is the generation of a so-called genetic portrait, corresponding to up- or down-regulated miRNAs in the cell system investigated.

Hybridization of miRNA derived from MG63 cells cultured with 100 mg/10 ml B vs those cultured with 0.04 g/ml PG to the sequences spotted on the slide allowed us to perform systematic analysis of miRNAs, and to obtain primary information about differences in translational regulation (Table 1).

Some genes up-regulated by BO (due to down-regulated miRNA) are homeoboxes, i.e. genes spatially and temporally regulated during embryonic development for control of morphogenesis. Noggin (NOG) inactivates members of the TGF-beta superfamily signaling proteins, such as BMP4. By diffusing through extracellular matrices more efficiently than members of the TGF-beta superfamily, noggin has a principal role in creating morphogenic gradients (36). The human engrailed homolog 1 (i.e. EN1) is implicated in the control of pattern formation during limb development (37). Other enhanced mRNAs encode extracellular matrix (ECM) components and enzymes such as COLL1A2 and COMP (a non-collagenous ECM protein that binds to other ECM proteins such as collagen).

Hormonal receptors such as calcitonin receptor (CALCR) and calcium-sensing receptor (CASR) may also be up-regulated. CASR functions as a sensor for the parathyroid and kidney, determining the extracellular calcium concentration and thus helping to maintain a stable calcium concentration. Mutations that inactivate CASR cause familial hypocalciuric hypercalcemia, whereas mutations that activate CASR are the cause of autosomal dominant hypocalcemia (38). Osteoprotegerin (TNFRSF11B) is a member of the TNF-receptor superfamily. This protein is an osteoblast-secreted decoy receptor that functions as a negative regulator of bone resorption, binding specifically to its ligand, osteoprotegerin ligand (TNFSF11/OPGL), thus playing a key role in osteoclast development (39).

Some of the genes up-regulated by PG are homeoboxes, like DLX5 and HOXA13. DLX5 has a role in bone development and fracture healing. Mutation in this gene is associated with split-hand/split-foot malformation (40).

Another group of genes are hormones such as PTH and IGF2. IGF2 is member of the insulin family of polypeptide growth factors that are involved in development and growth (41). Additional proteins act in the extracellular matrix (ECM) compartment. BMP1 (or procollagen C proteinase) is a secreted metalloprotease that cleaves the C-terminal propeptides of procollagens I, II, and III and requires calcium for cartilage and bone formation (42). ALPL is a non-specific alkaline phosphatase that acts on matrix mineralization (43). MATN1 and 3 are enzymes involved in the formation of filamentous networks in the ECM of various tissues, playing a role in the development and homeostasis of cartilage and bone (44). Bone sialoprotein or osteopontin (SPP1) is a protein produced by osteoblasts upon stimulation with calcitriol and binds tightly to hydroxyapatite, thus anchoring osteoclasts to the mineral of bone matrix (45).

The genes discussed above represent only a limited number of those differentially regulated by the miRNAs listed in Table 1. We have briefly analyzed some of those whose functions are better known and directly related to bone formation, skeletal development, cartilage remodeling and bone production.

It should be noted that MG63 is a cell line and not normal osteoblasts. However, the use of a cell line allows higher data reproducibility, and avoids the variability of responses seen among individual patients. Primary cell cultures provide a source of normal cells, but they also contain contaminating cells of different types and cells in various states of differentiation. Moreover, we elected to perform the experiment after 24 h of culture in order to obtain information on the early genetic events that might occur in the early phase of grafting. In fact the postoperative period is of paramount importance for any successful grafting procedure.

In conclusion, BO and PG act on different miRNAs. Globally, PG causes activation of bone-forming signaling, whereas BO also activates cartilage-related pathways.

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