TITANIUM ACTS ON OSTEOBLAST TRANSLATIONAL PROCESS

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Titanium is a highly biocompatible material and very osteogenic in vivo. However, how titanium regulates osteoblast activity to promote bone formation is incompletely characterized. We, therefore, attempted to get more information by using microRNA (miRNA) microarray techniques to investigate translation regulation in osteoblasts grown on titanium disks. The miRNA oligonucleotide microarray provides a novel method to carry out genome-wide miRNA profiling in human samples. By using miRNA microarrays containing 329 probes designed from the human miRNA sequence, several miRNA were identified in osteoblast-like cell line (MG 63) grown on titanium disks. There were 13 up-regulated miRNAs (ie, mir-23a, mir-222, mir-523, mir-22, mir-23b, mir-143, mir-377, mir-24, mir-422b, mir-26a, mir-29a, mir-17–5p, mir-182) and 2 down-regulated miRNAs (ie, mir-187, mir-339). The data reported are, to our knowledge, the first study on translation regulation in osteoblasts exposed to titanium. The data can be relevant to understand better the molecular mechanism of osteoblast activation and as a model for comparing other materials with similar clinical effects.

Key Words: titanium, miRNA, microarray, gene expression, gene profiling

INTRODUCTION

Several features of the implant surface including composition, topography, roughness, and energy play a relevant role in implant integration with bone.[1–21] Little is known about the structural and chemical surface properties that may influence biological responses, especially from a genetic point of view.[1] Titanium has been widely used in the biomedical field, but the factors and mechanisms underlying the biological response to titanium are not yet well understood,[1] and it is necessary to look for correlations between surface characteristics and response of biological tissues at different levels of resolution and sophistication.[1] Because the mechanism by which titanium stimulates osteoblast activity to promote bone formation is incompletely characterized, we therefore attempted to add new information by using microRNA microarray techniques.

MicroRNAs (miRNAs) represent a class of small, functional, noncoding RNAs of 19 to 23 nucleotides (nt) cleaved from 60- to 110-nt hairpin precursors.[22,23] Hundreds of miRNAs have been identified in plants and animals. The miRNAs 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 in posttranscriptional RNA silencing pathways.[24] The RNA interference (RNAi) and the microRNA (miRNA) pathway, regulate gene expression by inducing degradation and/or translational repression of target messenger RNAs (mRNAs). These pathways are generally initiated by various forms of double-stranded RNA

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(dsRNA), which are processed by Dicer, an RNase III family endonuclease, to 21 to 22 nt long RNA molecules that serve as sequence-specific guides for silencing.[25,26]

MicroRNAs are transcribed as long primary transcripts (pri-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 base pair (bp) RNA duplexes with 2-nt 3′ overhangs.[27–30]

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

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, Calif), a slide printed with approximately 900 unique probe of miRNA sequences for *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Danio rerio*.

By the analysis of the 329 human miRNA sequences spotted on the array, we compared miRNA expression and consequently gene regulation in human MG63 cells grown on machined titanium disks with untreated MG63 cells.

Materials and Methods

Cell culture

Osteoblast-like cells (MG63) were cultured in sterile Falcon tissue colture wells (Becton Dickinson, Franklin Lakes, NJ) containing Eagle's minimum essential medium supplemented with 10% fetal calf serum (Sigma Chemical Co, St Louis, Mo) and antibiotics (penicillin 100 U/mL and streptomycin 100 μg/mL, Sigma). 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++, and Mg++—free Eagle's buffer for cell release. One set of wells contained sterile metal disks of machined grade 3 titanium (diameter 3 cm; test). After 24 hours, when cultures were subconfluent, cells were processed for RNA extraction. Cell cultures were repeated twice.

MicroRNA microarray

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

NCode miRNA Labeling System (Invitrogen) was used for labeling and hybridizing miRNA to the microarray, according to 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 an incubation of 18–20 hours, the array was washed and hybridized with Alexa Fluor 3 capture reagents (for the control) and Alexa Fluor 5 capture reagents (for the treated cells) in the first experiments, and then the dyes were switched. After another wash, the array was scanned using a standard microarray scanner (Axon Instruments, Sunnyvale, Calif).

After scanning, each spot was identified by means of a GAL (GenePixR Array List) file[31] that lists the identities and locations of all probes printed on the array.

Images were quantified by GenePix 6.0 software (Axon Instruments). Signal intensities for each spot were calculated by subtracting local background from total intensities. The data were normalized by using the DNMAD and preprocessing software packages.[32–34] This generates an average value of the two spot replicates of each miRNA.

To select for differentially expressed miRNA, the data obtained were analyzed using the SAM package (statistical analysis of microarray).[35]

For target predictions and validations, miRNA were processed using miRBase Target,[36] a web resource 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,[37] a web interface which carries out simple data mining using Gene Ontology.[38] The data mining consists on the assignation of the most characteristic gene ontology term to each cluster of regulated genes.

Results

Hybridization of miRNA (derived from MG63 cultured on disks of machined grade 3 titanium) to the sequences spotted on the slide allowed us to perform a systemic analysis of miRNAs and to provide primary

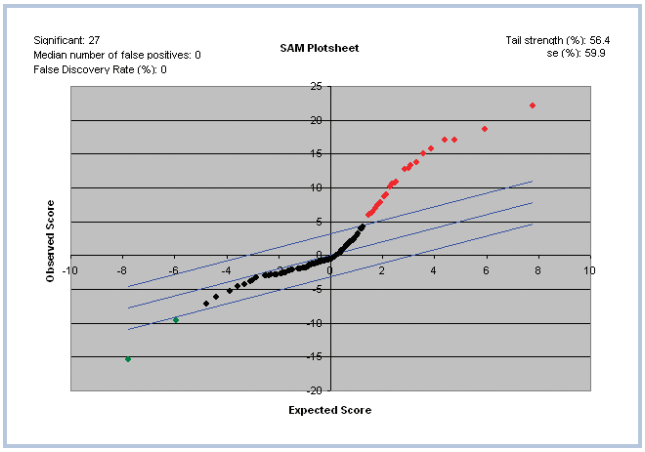

FIGURE. SAM (significance analysis of microarray) plot of MG63 cultured for 24 hours on titanium disks.

information with regard to the regulation of the translation process induced by titanium. There were 13 up-regulated miRNAs (ie, mir-23a, mir-222, mir-523, mir-22, mir-23b, mir-143, mir-377, mir-24, mir-422b, mir-26a, mir-29a, mir-17–5p, mir-182) and 2 down-regulated miRNAs (ie, mir-187, mir-339) for FDR (false discovery rate) = 0 and score >9. The Figure is the graphical output of SAM and it shows differentially expressed miRNA. Because miRNA potentially regulates thousands of genes, in this study we selected only the genes related to osteogenesis and bone remodeling (Table). Because we detected some mRNAs regulated in an opposite manner by different miRNAs, these mRNAs with not univocal regulation were excluded from the subsequent analysis since the global effect of microRNAs on these messenger RNAs was not determined, yet.

DISCUSSION

Recent studies in animal models have demonstrated that the titanium surface is of paramount importance in influencing the timing of bone healing, and rougher surfaces have been demonstrated to present a higher quantity of bone-implant contact and a higher removal torque value.[6,7,10,11,21] Moreover, the surface roughness has demonstrated to be able to alter the responsiveness of different types of cells.[8,13,14] In addition, previous reports on gene expression have given primary information with regard to the genetic effects of titanium on osteoblast-like cells.[39–41]

However, because it is poorly understood how the titanium surface alters osteoblast activity to promote bone formation, we therefore attempted to address this question by using a new method, miRNA microarray, to identified genes that are differently translated in osteoblasts grown on machined grade 3 titanium surfaces.

MicroRNAs are a recently discovered class of small, ~19 to 23-nucleotide noncoding RNA molecules. They are cleaved from 70 to 110-nucleotide hairpin precursors and play an important role in the posttranscriptional regulatory process. MicroRNAs are not translated into proteins: instead, they regulate the expression of other genes by either cleaving or repressing the translation of their mRNA targets.[25,26]

Recent advances in spotted oligonucleotide microarray labeling and detection have enabled the use of this high-throughput technology for miRNA screening.

Microarray is a molecular technology that enables the analysis in parallel on a very large number of DNA or RNA fragments, spanning a significant fraction of the human genome. Gene expression is performed by a process of (1) miRNA extraction, (2) labeling (different dyes are used for reference untreated cells and investigated cells, ie, cultured on titanium disks), and (3) hybridization on slides containing miRNA probes. Then, the slides are scanned with a laser system, and two false color images are generated for each hybridization with miRNA from the investigated and reference cells. The overall result is the generation of a so-called genetic portrait. It corresponds to up- or down-regulated miRNA in the investigated cell system. Hybridization of miRNA derived from MG63 cultured on titanium disks to the sequences spotted on the slide allowed us to perform systemic analysis of

TABLE

Down-regulated genes

miRNA*	Biological Function	Genes
UP-	Skeletal development Cartilage development Bone remodeling	FGFR3 IGF1 KLF10 GUSL2_HUMAN GDF10 ADAMTS4 FLG EN1 CHRD CALCA GHRHR MSX1 ANXA2 MATN1 BMPR1B BMP1 BMP7 COL1A1 COL11A1 CASR PHEX KAZALD1

*miRNA indicates microRNA; UP, up-regulated.

miRNAs and to provide primary information with regard to the regulation of translation induced by titanium (Table).

Notable is that miRNAs are up-regulated, and thus, there is a down-regulation of several genes, most of them related to osteogenesis. Down-regulation of genes is caused by the silencing process determined by microRNAs (ie, miRNAs) on messenger RNAs (ie, mRNAs). Several BMPs have a negative transcriptional control like BMP1 (or procollagen C proteinase), BMPR1B (a member of the BMP receptor family), BMP7, and GDF10 (another a member of the BMP family). Another group of mRNAs down-regulated are collagen like COL1A1 (the major component of type I collagen) and COL11A1 (one of the two alpha chains of type XI collagen).

Additional down-regulated mRNAs encode for receptors like CASR (ie, calcium-sensing receptor that functions as a sensor for parathyroid and kidney to determine the extracellular calcium concentration and thus helps to maintain a stable calcium concentration), FGFR3 (a member of the fibroblast growth factor receptor family that binds acidic and basic fibroblast growth hormone and plays a role in bone development and maintenance), and GHRHR (a receptor for growth hormone-releasing hormone).

Other repressed mRNAs encode for hormones and morphogenetic proteins. Insulin growth factor 1 (IGF1 or somatomedin) mediates many of the growth-promoting effects of growth hormone whereas MSX1 encodes a member of the muscle segment homeobox gene family. MSX1 functions as a transcriptional repressor during embryogenesis through interactions with components of the core transcription complex and other homeoproteins. It has roles in limb-pattern formation and craniofacial development, particularly odontogenesis.

The fact that several genes related to bone formation have a translational negative control can be related to the time point analyzed (eg, MG63 cultured for 24 hours until they are subconfluent). This phase is characterized by an elevated cellular kinetics and to a low extracellular matrix production and differentiation.

In the present study a comparison between MG63 cultured on titanium disks vs MG63 cultured on plastic wells was performed. It is well known that titanium is biocompatible, and it is osseointegrated when inserted in bone. This is not the case for plastic. Thus, the comparison produced the maximum contrast with regard to osseointegration associated genetic variables. In addition, the early period of titanium-cell contact was analyzed because immediate loading is the actual target in implantology. Immediate loading means placing the final or provisional prosthetic restoration immediately or within 24 hours of the surgical procedure; early loading means that implants are loaded in a period ranging from 24 hours to 7 days after fixtures are inserted.[42] Thus, the choice to study titanium-cell interaction at 24 hours derived from the definition of immediate loading. The detection of increased cell proliferation after 24 hours of titanium-cell contact is relevant because osseointegration depends on 2 phases: cell proliferation and then differentiation. The increase of cell kinetics explains why not sterile conditions can interfere with bone healing process. Proliferation is an additional variable to be considered in immediate loading.

We expected that in a subsequent period (ie, later time points) a differentiation process will start and a different panel of miRNAs will be activated. Additional experiments are needed to get information to subsequent titanium-cell contact periods.

The genes discussed are only a limited number among those differentially regulated by miRNA reported in the Table. We briefly analyzed some of those with a better known function and those that are directly related to bone formation, skeletal development, cartilage remodeling, and bone production.

It is worth noting that MG63 is a cell line and not primary osteoblast cell culture. The advantage of using a cell line is related to the fact that the reproducibility of the data is higher because there is not the variability of the patient studied. Primary cell cultures provide a source of normal cells, but they also contain contaminating cells of different types and cells in variable states of differentiation. Moreover, we have chosen to perform the experiment after 24 hours in order to get information on the early stages of stimulation. It is our knowledge, therefore, that more investigations with other osteoblast-like cell lines, primary cultures, and different time points are needed to get a global comprehension of the molecular events related to titanium action. However, a global effect on bone formation is detected, and the reported data can be a model to compare different substances with similar effects.

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