Short-period Effects of Zirconia and Titanium on Osteoblast MicroRNAs

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ABSTRACT

Background: MicroRNAs (miRNAs) are a class of small, functional, noncoding RNAs of 19 to 23 nucleotides which induce degradation of specific messenger RNAs (mRNAs), thus controlling the translational process (ie, synthesis of proteins from mRNAs). In addition, mRNAs regulate the promoter of specific miRNAs activating an autoregulatory feedback loop.

Purpose: Titanium and zirconium dioxide ceramics (ZDCs) are used to make dental implants. Because the molecular mechanism by which ZDC and Ti act on osteoblasts is incompletely understood, we attempted to get more information by comparing the effect of ZDC and Ti on osteoblast miRNAs.

Materials and Methods: By using miRNA microarray technique, we identified in osteoblast-like cell line (MG63) grown on grade 3 Ti and ZDC disks several miRNAs whose expression was modified. We collected mRNAs after 24 hours of cell culturing to better understand molecular events related to early bone healing around inserted implants. An mRNA microarray technique was then performed as a control.

Results: There were six up- and four down-regulated miRNAs. Because every miRNA regulates hundreds of genes, we focused only on those related to bone formation. Among them, the most notable are BMP4 and 7, which are both up-regulated in osteoblasts cultured on Ti disks.

Conclusion: The detected miRNAs differentially expressed in osteoblast-like cells grown on ZDC versus Ti act on a limited number of miRNAs and bone-related genes. The most notable are BMP4 and 7, which are more expressed in osteoblasts exposed to Ti surface. Consequently, we suggest that Ti surfaces could provide some advantages to immediate load implantology.

KEY WORDS: gene expression, gene profiling, miRNA, RNA interfering, ZrO_2

MicroRNAs (miRNAs) represent a class of small, functional, noncoding RNAs of 19 to 23 nucleotides (nt) cleaved from 60 to 110 nt hairpin precursors.[1,2] miRNAs are involved in various biological processes, including cell proliferation and cell death through the regulation of gene expression (ie, the amount of messenger RNAs [mRNAs]) in post-transcriptional RNA silencing pathways.[3] miRNA pathways regulate gene expression by inducing degradation and/or translational (ie, synthesis of proteins from mRNA) repression of target mRNAs. These pathways are generally initiated by various forms of double-stranded RNA, 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.[4,5]

miRNAs are transcribed as long primary transcripts, 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.[6–9]

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miRNAs are loaded onto an Argonaute containing effector ribonucleoprotein complex, referred to as miRNP or RNA-induced silencing complex, which is capable of recognizing cognate mRNAs and inhibiting protein expression.

In addition, mRNAs can bind the promoter of specific miRNAs activating an autoregulatory feedback loop; thus, when a specific mRNA is up-regulated, the related miRNA is also over-expressed.[10]

Titanium is the gold standard material in implantology and has been used since the 1960s;[11] zirconium dioxide (ZrO_2) is a bioinert nonresorbable metal oxide which is used to make dental implants.[12,13] Zirconium dioxide's ivory color, similar to the color of natural teeth, makes it useful in aesthetically important areas of the oral cavity, and its ability to transmit light renders it a suitable material in aesthetic restorations. The good biocompatibility of this material has been found with regard to direct bone apposition of implants.[12,13]

Because the mechanism by which Ti and ZrO_2 act on osteoblasts is incompletely understood, we therefore attempted to get more information by analyzing differences in miRNAs between osteoblast-like cells (ie, MG63) cultured on ZrO_2 versus grade 3 machined Ti disks.

To reach this target, we used a recently developed methodology for miRNA screening based on the hybridization of miRNAs extracted from cells on a microchip, a slide printed with hundreds of unique probes of miRNA sequences. 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 the cells cultured on Ti and zirconium dioxide ceramic [ZDC]), 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 cells cultured on ZDC and Ti. The overall result is the generation of a so-called genetic portrait. It corresponds to up- or down-regulated miRNA in the investigated cell systems. Thus, we compared hundreds of miRNAs expressed in osteoblast-like cells (ie, MG63) cultured on zirconium dioxide versus grade 3 machined Ti disks to detect those differentially expressed. These differentially expressed miRNAs act on a series of specific mRNAs, and we focused on those related to bone formation.

MATERIALS AND METHODS

Cell Culture

Osteoblast-like cells (MG63) were cultured in sterile Falcon wells (Becton Dickinson, Franklin Lakes, NJ, 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% ethylenediaminetetraacetic acid in Ca^{2+}- and Mg^{2+}-free Eagle's buffer for cell release. One set of wells contained sterile metal disks of machined grade 3 Ti (diameter 3 cm) (control), whereas another set of wells contained sterile disks of ZDC with a diameter of 3 cm covering the same area (35 cm^2) as the reference wells. Ti and zirconia disks were the same as those of previously reported experiments (where production, composition, and surface characteristic of the test materials – Ti and ZrO_2 – are available).[14,15] After 24 hours, the medium (3 mL of MEM with 10% FCS) was changed. Finally, after 24 hours, when the cultures were sub-confluent, cells were processed for RNA extraction. We performed the experiment after 24 hours of treatment as early genetic effects are of paramount importance to better understand bone healing mechanisms around inserted implants, especially if one focuses on variables potentially related to immediate loading.

miRNA Microarray

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

The NCode™ miRNA Labeling System (Invitrogen) was used for labeling and hybridizing miRNA to 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

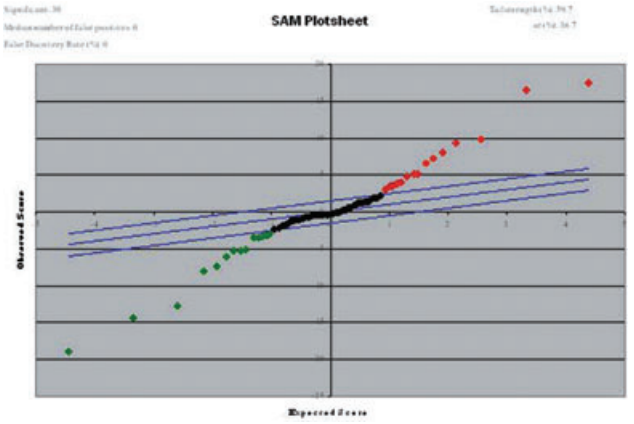

Figure 1 Significance analysis of microarray (SAM) plot of MG63 cultured for 24 hours on titanium and zirconium disks.

purification step, the tagged miRNAs were hybridized to the microarray and incubated overnight.

After an incubation of 18 to 20 hours, the array was washed and hybridized with Alexa Fluor® 3, (Invitrogen), capture reagents (for the control = Ti), and Alexa Fluor® 5 capture reagents (for cells grown on ZDC disks) 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 the GenePixR Array List, file downloaded from http://www.invitrogen.com/ncode, that lists the identities and locations of all the probes printed on the array.

The images were quantified by GenePix 6.0 software (Axon Instruments). Signal intensities for each spot were calculated by subtracting the local background from the total intensities. The data were normalized by using the DNMAD and preprocessing (http://gepas.bioinfo.cipf.es/cgi-bin/tools).[16,17] This generates an average value of the two spot replicates of each miRNA.

To select the differentially expressed miRNA, the data obtained were analyzed using the significance analysis of microarray package (Figure 1).[18]

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 which carries out simple data mining using Gene Ontology (http://www.geneontology.org). The data mining consists in assigning the most characteristic Gene Ontology term to each cluster of regulated genes.

To verify the mRNAs, a messenger microarray experiment was performed, as previously described.[14,15]

RESULTS

The hybridization of miRNA (derived from MG63 cultured on Ti and ZDC disks) to the sequences spotted on the slide allowed us to perform a systemic analysis of miRNAs and to provide primary information as regards the regulation of the translation process induced by ZrO_2 compared to Ti.

There were six up-regulated miRNAs in ZDC compared to Ti (ie, mir-214, mir-337, mir-423, mir-339, mir-377, and mir-193b), and four down-regulated miRNA (ie, mir-143, mir-17-5p, mir-24, and mir-22) for false discovery rate (FDR) = 0 and score > 7.

Score (d) is a T-statistic value. The local FDR for a gene is the FDR for genes having a similar score d as that gene. It is estimated by taking a symmetric window of 0.5% of the genes on each side of the target gene and estimating the FDR in that window. If 1.0% times the total number of genes in the data set is less than 50, then the percentage is increased so that the number of genes is 50. The FDR is computed as [the median (or 90th percentile) of the number of falsely called genes] divided by [the number of genes called significant].

Because miRNA potentially regulates thousands of genes, in this study we selected only genes related to osteogenesis and bone remodeling, and regulated in the same way by almost two independent miRNAs (Table 1). Genes with the opposite type of regulation were excluded. Then, we compared the theoretically regulated mRNAs with those obtained with the messenger microarray technique.

DISCUSSION

ZDCs have outstanding mechanical properties, a high biocompatibility, and a high resistance to scratching;

TABLE 1 List of Messenger RNAs (mRNAs) Regulated by Detected microRNAs (miRNAs)

MiRNA	Biological function	Target mRNAs
Up	Skeletal development	FGFR1 COMP MSX1
	Cartilage development	NOG
Down	Cartilage development	BMP7

thus, dental implants have been made with ZDC[4,5] for some time. Zirconium dioxide seems to have a high affinity with bone tissue.[4] Bone was in close contact with the surface of the zirconium dioxide implants, and the bone-implant interface was similar to that seen around Ti implants.[5] Ti is the gold standard material in implantology and it has been used since the 1960s.[11]

Because the mechanism by which ZDC and Ti implants act on osteoblasts is incompletely understood, we attempted to get more information by analyzing miRNAs in osteoblast-like cells exposed to ZDC compared to Ti.

miRNAs are a recently discovered class of small, ~19 to 23 nt noncoding RNA molecules. They are cleaved from 70 to 110 nt hairpin precursors and play an important role in the post-transcriptional regulatory process. miRNAs are not translated into proteins; instead, they regulate the expression of other genes by either cleaving or repressing the translation of their mRNA targets. In addition, mRNAs can bind the promoter of specific miRNAs activating an autoregulatory feedback loop; thus, when a specific mRNA is up-regulated, the related miRNA is also over-expressed.[10]

Recent advances in spotted oligonucleotide microarray labeling and detection have enabled the use of this high-throughput technology for miRNA screening. Hybridization of miRNAs derived from MG63 cultured on Ti and ZDC disks to the sequences spotted on the slides allowed us to perform a systemic analysis of miRNAs and to provide information as regards the regulation of the translation induced by ZDC compared to Ti. These differentially expressed miRNAs act on a series of specific genes, and we focused on those related to bone formation (see Table 1). The concordant regulation of mRNAs and related miRNAs was detected by using messenger microarray technology (ie, some mRNAs were up-regulated together with related miRNAs). However, fold changes of up-/down-regulation were different (additional data are available on requests).

Up-regulated Genes

The most relevant up-regulated bone-related detected gene in MG63 cultured on Ti is BMP7, a member of the transforming growth factor-beta (TGF-β) superfamily. It is a secreted signaling molecule that can induce not only ectopic bone growth and formation but also has a role in early development.[19]

Down-regulated Genes

Among the down-regulated genes, MG63 cultured on Ti is noggin, encoded by the NOG gene. It binds and inactivates members of the TGF-β superfamily signaling proteins, such as BMP4. By diffusing through extracellular matrices more efficiently than members of the TGF-β superfamily, noggin has a principal role in creating morphogenic gradients. Noggin appears to have a pleiotropic effect, both early in development as well as in later stages. It is involved in numerous developmental processes, such as neural tube fusion and joint formation.[20]

MSX1 is a member of the muscle segment homeobox gene family. It has roles in limb-pattern formation, craniofacial development, and odontogenesis.[21] FGFR1 is a member of the fibroblast growth factor receptor (FGFR) family, where members differ from one another in their ligand affinities and tissue distribution. A full-length representative protein consists of an extracellular region, composed of three immunoglobulin-like domains, a single hydrophobic membrane-spanning segment, and a cytoplasmic tyrosine kinase domain. The extracellular portion of the protein interacts with fibroblast growth factors, setting in motion a cascade of downstream signals, ultimately influencing mitogenesis and differentiation. This particular family member binds both acidic and basic fibroblast growth factors and is involved in limb induction.[22]

The few genes discussed are only a limited number among those differentially regulated by miRNAs. We briefly analyzed some of those with better known functions and directly related to bone formation. Notable over-translated mRNAs are those coding for BMP4 and 7 in Ti versus ZDC. Previously, reports have demonstrated that Ti is able to induce BMP overexpression.[15,23–25] Here, the datum is confirmed and in addition an autoregulatory feedback loop between mRNAs and miRNAs is shown.

The increased expression of BMP4 and 7 could provide some advantages to the early phase of the osseointegration process (ie, 24 hours after implant insertion) for Ti with respect to ZDC implants.

Immediate implant loading means fitting the final or provisional prosthetic restoration immediately or within 24 hours from the surgical procedure, whereas early loading means prosthetic rehabilitation is delivered from the second day within a week after surgery.[26–29] Starting with a couple of implants placed in the

mandible and immediately loaded after surgical placement with an overdenture,[30,31] this concept evolved to immediately loading multiple implants in both the maxilla and the mandible.[26–29]

The identification of guidelines for high success rates in immediate loading implantology is the main goal of the recent literature. Several variables can influence the final result, but in general they are grouped as: (1) surgery-, (2) host-, (3) implant-, and (4) occlusion-related factors.[32] The surgery-related factors comprise several variables such as an excess of surgical trauma like thermal injury,[33] bone preparation, drill sharpness, and design.[34] Bone quality and quantity are the most important host-related factors,[35,36] while design,[37–39] surface coating,[37–40] diameter,[41] and length[42] are the strongest implant-related factors. Finally, quality and quantity of force[43] and prosthetic design[44] are the variables of interest among the occlusion-related factors. All these variables are a matter of scientific investigation because they may affect the clinical outcome. Here, we have focused on implant surface composition and its effects on miRNA regulation; a potentially higher osseointegration capacity has been detected for Ti versus ZDC.

The reported data are derived from a study performed on MG63 which are cell lines and not normal osteoblasts. Notwithstanding this, the advantages of using a cell line are related to the fact that the reproducibility of the data is higher because there is not the variability in 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 differentiation states.

In conclusion, the effect of Ti on bone formation has been detected (see BMP7 and the action of nogging on BMP4) and could be relevant for clinical applications.

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