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Titanium nanotubes activate genes related to bone formation *in vitro*

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ABSTRACT

Background: Titanium is used worldwide to make osseointegrable devices, thanks to its favorable characteristics as mechanical proprieties and biocompatibility, demonstrated by *in vivo* studies with animal models and clinical trials over a forty-year period. However, the exact genetic effect of the titanium layer on cells is still not well characterized.

Materials and Methods: To investigate how titanium nanotubes stimulate osteoblasts differentiation and proliferation, some osteoblast genes (SP7, RUNX2, COL3A1, COL1A1, ALPL, SPP1 and FOSL1) were analyzed by quantitative Real Time RT- PCR.

Results: After 15 days, osteoblasts cultivated on titanium naotube showed the up-regulation of bone related genes SP7, ENG, FOSL1 and SPP1 and the down-regulation of RUNX2, COL3A1, COL1A1, and ALPL. After 30 days of treatment, the bone related genes SP7, ENG, FOSL1 and RUNX2 were up-regulated while COL3A1, COL1A1, ALPL and SPP1 were down-regulated.

Conclusions: Our results, demonstrates that titanium nanotubes can lead to osteoblast differentiation and extracellular matrix deposition and mineralization in dental pulp stem cells by the activation of osteoblast related genes SPP1, FOSL1 and RUNX2.

Key Words: Gene expression, nanotubes, osteoblasts, titanium disks

INTRODUCTION

Titanium and its alloys are employed as implant materials because of their desirable mechanical properties and "biocompatibility".[1-3] For decades, oral, maxillofacial and orthopedic surgeons have utilized dental implants, screws and plates, and prostheses to substitute lost teeth, to fix bone fragments and to replace joints, respectively. Also, many surgical instruments, such as drills and saws, are made with titanium alloys. The "biocompatibility" of titanium has been demonstrated by *in vivo* studies with both animal models and clinical trials, for more than forty years.[1,4] Several authors studied other different aspect of implant morphology in order to value their impact on implant stability.[5-11] Macro-, mini-, micro- and nano-design characterizes prostheses. Macro-design is the shape of the prosthesis: some examples are offered by the cylindrical or root form of dental implants. Mini-design is the dimension of the threads or the shape of the neck of the dental fixture. The dimensions range from 1 to 0.1 mm. Micro-design is the shape of the implant surface: An example of which is provided by the "grooves and holes" resulting from surface treatments like machination, acid etching and sand blasting procedures. These treatments determine the roughness of the surface and the "holes" have a cellular dimension. Nano-design is determined by the molecular composition of the surface, such as those composed of: hydroxyapatite, zirconium and titanium. Usually, mechanical proprieties are related to macro- and mini-design, whereas biological properties are related to micro- and nano-design. Mechanical properties are responsible for primary

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implant stability, whereas biological properties act on the osseointegration process.[12]

When titanium is exposed to oxygen a stochastic distribution of three titanium-oxide isoforms (i.e., rutile, brookite and anatase) are available on the surface of the prosthesis. These isoforms are responsible for its material biological properties.[12]

Although previous studies have demonstrated that titanium surfacing is capable of modulating osteoblast gene expression,[13] they failed to separate the effect of roughness (i.e., micro-dimension) from that of composition (i.e., nano-dimension).

Effect of the titanium nanotubes layer on osteoblast gene expression has been studied, comparing the expression profiling of osteoblasts (HOb) cultivated on two type of surface: pure titanium disk (TD) and nanotubes titanium disk (NTD) in order to detect if NTD surface stimulates osteoblast proliferation.

The quantitative expression of the mRNA of specific genes, like transcriptional factors (RUNX2), bone related genes (SPP1, COL1A1, COL3A1, ALPL, and FOSL1) was examined by means of Real Time Reverse Transcription-Polymerase Chain Reaction (Real Time RT-PCR).

MATERIALS AND METHODS

Titanium nanotubes disks preparation

Disks of commercially pure grade-1 titanium (Titania, Italy) have been used as substrate for the nanotube growth. The disks have diameter of 30 mm with a thickness of 0.5 mm, and were arranged to show an active area of 3.8 cm^2. After 3 minutes pickling in a HF (Carlo Erba)/HNO_3 (Carlo Erba) solution, made by a volumetric ratio of 1:3 and diluted in deionized water until to 100 ml, all the titanium sheets have been set in three-electrode cell, containing a KOH 1 M solution (Carlo Erba) and subjected to a prefixed and optimized density current (1 mA/cm^2), which is generated by a potentiostat/galvanostat Solartron 1286 for 3 minutes. The counter-electrode is a Platinum sheet, while the reference is a standard calomel electrode (SCE). The growth of the nanotube arrays has been made using a Glycol Ethylene solution with 1 %wt. H_2O and 0, 2% wt. NH_4F for 3 hours at 60 V. After the anodization treatment, all the samples are washed in glycole ethylene, left overnight in the dry room, in order to dry them. So to crystallize the TiO_2 nanotubes, obtained in amorphous form by anodic growth, after a pre-heat treatment at 80°C in vacuum for 3 hours, all the samples have been placed in a tubular furnace (Lenton) for 1 hour at 580°C, with a slope of 1°C/minute in air, so to be transformed into the anatase phase.

Primary osteoblasts (HOb) cell culture

Fragments of bone derived from skull of an healthy volunteer were sampled during operation and transferred in 75 cm^2 culture flasks containing DMEM medium supplemented with 20% 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). Cells were grown in a humidified atmosphere of 5% CO2 at 37°C. The medium was changed the next day and every 3 days thereafter. After 15 days, the pieces of bone tissue were removed from the culture flask. Cells were harvested after 30 days of incubation.

Cell culture

For the assay, HOb were trypsinized upon subconfluence and seeded on NTD and TD.

The medium was changed every 3 days. The cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. Cells were collected, for RNA extraction at 15 and 30 days.

RNA processing

Reverse transcription to cDNA was performed directly from cultured cell lysate using the TaqMAN Gene Expression 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.

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 cells. Quantification was done with the delta/delta calculation method.[14]

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.

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.

RESULTS

HObs were cultivated on two type of surface, NTD and TD. Gene expression in HOb cultivated on NTD was compared with that cultivated on TD (control) in order to check whether NTD is more osteoinducent respect TD.

After 15 days, HOb cultivated on NTD showed the up-regulation of bone related genes SP7, ENG, FOSL1 and SPP1 and the down-regulation of RUNX2, COL3A1, COL1A1, and ALPL [Figure 1a].

After 30 days of treatment, the bone related genes SP7, ENG, FOSL1 and RUNX2 were up-regulated in HOb cultivated on NTD, while COL3A1, COL1A1, ALPL and SPP1 were down-regulated [Figure 1b].

DISCUSSION

Titanium is well tolerated by the bone and is able to induce osseointegration of the implant. Osteointegration is primary element when considering implant stability.[15-17] Recently titania film have been used to cover prosthetic implants in medical applications such as orthopedic or dental surgery.[18] Titania film

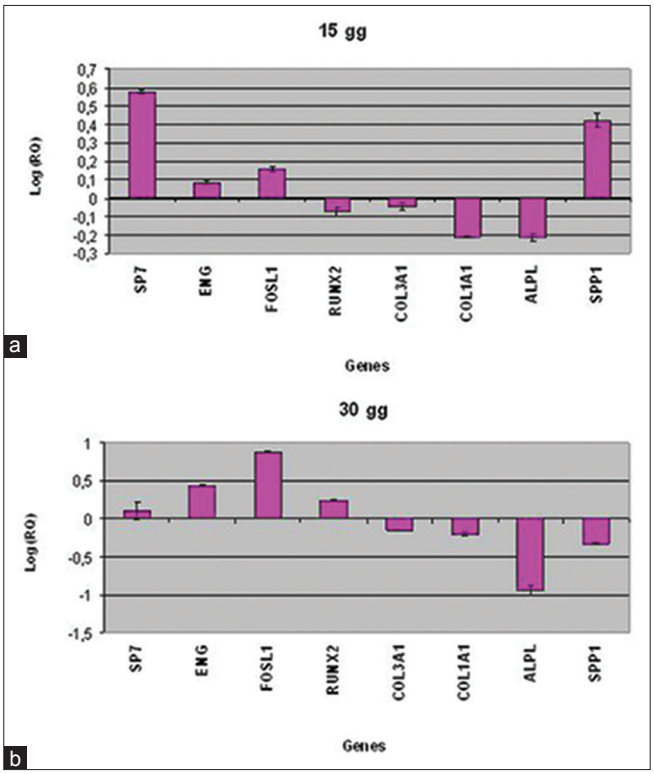

Figure 1: Relative gene expression of HOb cultivated on titanium disks for 15 days (a) and 30 days (b)

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 phospatasi	F-CCGTGGCAACTCTATCTTTGG R-CAGGCCCATTGCCATACAG	CCATGCTGAGTGACACAGACAAGAAGCC
COL3A1	Collagen, type III, alpha 1	F-CCCACTATTATTTTGGCACAACAG R-AACGGATCCTGAGTCACAGACA	ATGTTCCCATCTTGGTCAGTCCTATGCG
CD105	Endoglin	F-TCATCACCACAGCGGAAAAA R-GGTAGAGGCCCAGCTGGAA	TGCACTGCCTCAACATGGACAGCCT
FOSL1	FOS-like antigen 1	F-CGCGAGCGGAACAAGCT R-GCAGCCCAGATTTCTCATCTTC	ACTTCCTGCAGGCGGAGACTGACAAAC
SP7	Osterix	F-ACTCACACCCGGGAGAAGAA R-GGTGGTCGCTTCGGGTAAA	TCACCTGCCTGCTCTTGCTCCAAGC
RPL13A	Ribosomal protein L13	F-AAAGCGGATGGTGGTTCCT R-GCCCCAGATAGGCAAACTTTC	CTGCCCTCAAGGTCGTGCGTCTG

creates an active surface that is able to improve the biocompatibility of titanium implants.[19] In prostheses made of titanium, the titania film surface is mainly composed of two polymorphs of titanium, rutile and anatase. Rutile is considered the stable form of titania while anatase is metastable and converts to rutile at high temperatures.[19] Anatase titania is more advantageous for medical applications than rutile. Compared to rutile, anatase exhibits stronger interactions between metal and support, and the surface of anatase titania can absorb more OH- and PO43- than that of rutile titania in body fluid, which favors the depositing of bone-like apatite.[20]

Effect of the titanium nanotubes on HOb gene expression has been studied, comparing the expression profiling of osteoblasts cultivated on two type of surface: Pure titanium disk (TD) and nanotubes titanium disk (NTD) in order to detect if NTD surface stimulates HOb proliferation.

The quantitative expression of the mRNA of specific genes, like transcriptional factors (RUNX2 and SP7) and bone related genes (SPP1, COL1A1, COL3A1, ALPL, ENG and FOSL1) was examined by means of real time Reverse Transcription-Polymerase Chain Reaction (real time RT-PCR).

After 15 days of treatment the bone related genes SP7, ENG, FOSL1 and SPP1 were up-regulated in HOb cultivated on NTD respect to HOb cultivated on TD. These results indicated that NTD enhance osseointegration.

SP7 is a zinc finger transcription factor that regulates bone formation and osteoblast differentiation *in vitro* and *in vivo* and that is expressed in the early stage of osteogenic differentiation.

ENG is a receptor for TGF-β1 and -β3[21] and modulates TGF-β signaling by interacting with related molecules, such as TGF-β1, -β3, BMP-2, -7, and activin A. It is speculated that these members of the TFG-β super family are mediators of cell proliferation and differentiation and play regulatory roles in cartilage and bone formation.[22]

NTD also modulate the expression of FOSL1 that 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, osteocalcin (OC).

Maccabe *et al*.[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.

SPP1 encodes osteopontin, which is a phosphoglycoprotein of bone matrix and it is the most representative non collagenic component of extracellular bone matrix.[24] Osteopontin is actively involved in bone resorbitive processes directly by ostoclasts.[25] Osteopontin produced by osteoblasts, show high affinity to the molecules of hydroxylapatite in extracellular matrix and it is chemo-attractant to osteoclasts.[26]

After 30 days of treatment these genes, SP7, ENG and FOSL1, continue to be up-regulated in HOb cultivated on NTD, indicating the osteodifferentiative properties of titanium nanotubes.

In addition another bone related genes, RUNX2, was over-expressed in HOb cultivated on NTD respect to control. RUNX2 is a transcriptional factor activated in the first stage of differentiation and plays a fundamental role in osteoblast maturation and homeostasis. RUNX2-null mice have no osteoblasts and consequently bone tissue.[27]

CONCLUSION

Our results, demonstrates that NTD can lead to osteoblast differentiation and extracellular matrix deposition and mineralization in dental pulp stem cells by the activation of osteoblast related genes SPP1, FOSL1 and RUNX2.

These results showed that NTD is more osteoinducent surface compared to TD, promoting the osseointegration.

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