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Involvement of N-cadherin/β-catenin interaction in the micro/nanotopography induced indirect mechanotransduction

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ABSTRACT

Topographical modification at micro- and nanoscale is widely applied to enhance the tissue integration properties of biomaterials, but the underlying molecular mechanism is poorly understood. The biomaterial topography modulates cell functions via mechanotransduction of direct and indirect. We propose that N-cadherin may play a role in the topographically induced indirect mechanotransduction by regulating the β-catenin signaling. For confirmation, the cell functions, N-cadherin expression and β-catenin signaling activation of osteoblasts on titanium (Ti) surfaces with micro- or/and nanotopography are systemically compared with naive and N-cadherin down-regulating MC3T3-E1 cells. We find that the N-cadherin expression is reversely related to the intracellular β-catenin signaling and the N-cadherin/β-catenin signaling is modulated differentially by the micro- and nanotopography. The nanotopography significantly up-regulates the N-cadherin expression leading to lower β-catenin signaling activity and consequently depressed differentiation, whereas the microtopography down-regulates the N-cadherin expression resulting in enhanced β-catenin signaling and thus osteoblast differentiation. Artificial down-regulation of the N-cadherin expression can significantly up-regulate the β-catenin signaling and consequently enhance the osteoblast differentiation on all the Ti surfaces. The study for the first time clarifies the involvement of the N-cadherin/β-catenin interaction in the micro/nanotopography induced indirect mechanotransduction and provides a potentially new approach for biomaterial modification and biofunctionalization by down-regulating the cell N-cadherin expression to achieve improved clinical performance.

1. Introduction

Titanium (Ti) implants are widely used in clinic due to their excellent compatibility with the bone tissue. Suitably topographical modification of Ti at micro- and nanoscale is reported to alter the cell functions [1], but the underlying molecular mechanism remains largely elusive. A deeper understanding of the involved molecular events might direct the implant surface design thereby accelerating the biomaterial research. Mechanotransduction is the process by which exogenic force exerted on cells and the modulation of endogenic force in cells by the surface properties of the adhering substrate (topography, stiffness, chemistry, etc.) are transmitted into the nucleus to induce adaptive cellular functional changes [2]. According to how the signals are transported into cell nucleus, mechanotransduction can be divided into direct and indirect mechanotransduction [2]. The direct mechanotransduction indicates the process of transferring of the force via the conformational change of cytoskeleton to induce cell nucleus distortion and shape change accompanied with alteration of gene expression. The indirect mechanotransduction refers to the process of transducing the mechanical cues into chemical signal pathways and

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relaying the message by them to the nucleus. There are increasing reports on the signal pathways induced by biomaterial micro/nanotopography, but mainly focusing on the events related to the cell–substrate interaction, such as focal adhesion kinase [3] and integrin linked kinase (ILK) [4] that both locate in the focal adhesions. As to the further downstream events, the β-catenin signaling, which plays a crucial role in osteoblast differentiation and maturation [5], is demonstrated to be involved in the topography induced indirect mechanotransduction [6,7]. Yet the role of the cell-to-cell interaction in the biomaterial induced indirect mechanotransduction has rarely been described.

Cadherins are transmembrane structures responsible for direct cell-to-cell adhesion via homotypic interactions. The osteoblasts express a repertoire of cadherins, including N-cadherin [8]. The β-catenin functions in both transcription and cell-to-cell adhesion [9], which links N-cadherin to the β-catenin signaling [10–14]. Besides, there are some other mechanisms proposed for the cadherins to modulate the β-catenin signaling [15,16]. A manifest effect for the topographical cues on cells is the alteration in cell morphology [4,17], which shall be accompanied with the changes in cell-to-cell interaction and cadherin expression. Our previous study demonstrates an increased ILK expression in osteoblasts by micro/nanotopography [4], which is reported to down-regulate the cadherin expression and lead to β-catenin nuclear accumulation [18,19]. Based on these aspects, a reasonable postulation is that N-cadherin and its relation to the β-catenin signaling may be involved in the effect of biomaterial topography on osteoblast functions. This hypothesis is to great extent supported by the reports that the mechanical stimuli from fluid flow can induce disassembly of the cadherin-β-catenin complexes and a consequent increase in cytoplasmic β-catenin [11,12], because the mechanical stimuli and the biomaterial topographical cues are believed to influence cell functions by the same mechanism of mechanotransduction.

The present study is designed to confirm the above-mentioned hypothesis. Four different surface topographies on Ti, including the nanotubular topography, the micropitted topography, the hybrid micro- and nanoscale topography and the control topography absent of definite micro- or nanoscale cue are included. The cell functions, N-cadherin expression, and β-catenin signaling activation of MC3T3-E1 osteoblasts on these surfaces are systemically compared. Afterwards, the N-cadherin expression is down-regulated by RNA interference (RNAi) and then the supposed downstream events including the β-catenin protein levels as well as the cell functional changes are monitored again. Our study is hoped to uncover the role of the N-cadherin/β-catenin interaction in the biomaterial micro/nanotopography evoked indirect mechanotransduction.

2. Materials and methods

2.1. Sample preparation

Pure Ti foils (99.9%, 10 × 10 × 1 mm^3, Northwest Institute for Nonferrous Metal Research, China) were used in this study. The Ti foils were polished using SiC sandpaper from 400 to 1500 grits to form polished Ti (PT) sample that is absent of obvious micro- or nanoscale cue. After ultrasonic cleaning, the PT sample was etched in 0.5 wt% hydrofluoric acid for 30 min to generate an etched Ti surface with micropitted cue (MT), which was immediately rinsed with distilled water, and then dried. The PT and MT samples were anodized for 1 h in an electrolyte containing 0.5 wt% hydrofluoric acid and 1 M phosphoric acid using a DC power supply with a platinum electrode as the cathode at 5, 10 and 20 V to generate TiO_2 nanotubular surfaces (NT) and hierarchical hybrid micropitted/nanotubular surfaces (MNT) of different tube size, respectively. The morphology of the Ti samples was inspected by field-emission scanning electron microscopy (FE-SEM, S-4800, Hitachi). After ultrasonic cleaning, all the samples, including PT, NT, MT and MNT were sterilized by cobalt 60 irradiation before cell plating.

2.2. Antibodies and reagents

The mouse monoclonal antibodies against N-cadherin and β-catenin were acquired from BD Transduction Laboratories. Those against α-tubulin and cavelion-1 were purchased from Cell Signaling Technology. That for β-actin was obtained from Abcam. The rhodamine- or FITC-conjugated secondary antibodies were obtained from Jackson Immuno Research Laboratories, and the horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signaling Technology.

2.3. Cell culture

Mouse osteoblastic MC3T3-E1 cells were cultured in α-modified essential medium (α-MEM, Hyclone) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% penicillin/streptomycin, and 2 mM L-glutamine and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. For osteoblastic differentiation, the cells were cultured in the presence of 100 μg/ml ascorbic acid, 10 nM dexamethasone and 10 mM β-glycerophosphate.

2.4. RNA interference

Small interfering RNA (siRNA) specific to N-cadherin or ILK as well as the control siRNA were synthesized by RiboBio. Three clones of siRNA sequences target at each of N-cadherin and ILK were evaluated firstly to choose those possessing the highest knockdown efficiencies. The siRNA were transiently transfected into the MC3T3-E1 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Before transfection, the siRNA were incubated with Lipofectamine 2000 in OPTI-MEM™ reduced serum medium (Gibco) for 20 min at room temperature. The siRNA-Lipofectamine 2000 complex was added to individual wells containing the cells and growth medium without antibiotic and incubated for 4 h at 37 °C. The knockdown efficiencies were determined by western blot.

2.5. Cell viability assay

The cells were seeded at a density of 2×10^4 cells/well on the samples mounted in 24 well plates. After culturing for 3 and 7 days, the cell vitality was assessed with the MTT assay. In brief, at the prescribed time points, the Ti samples with cells were rinsed gently with phosphate buffered saline (PBS) and transferred to a new 24-well culture plate. The MTT solution was added and the specimens were incubated at 37 °C to allow the formazen formation. Afterwards, the formazen was dissolved using dimethyl sulfoxide and the optical density (OD) was measured at 490 nm on a spectrophotometer (Bio-tek, Germany).

2.6. SEM observation of cell morphology

After 3 days of culturing on the samples, the cells were introduced to SEM observation. After washing with PBS, fixing with 2.5% w/v glutaraldehyde (Sigma) at 4 °C overnight and washing with PBS again, the cells on the substrates were then dehydrated in a graded series of ethanol (50, 70, 90 and 2 × 100 vol %) for 30 min each and dried in a critical point dryer (E-1045, Hitachi). The dried samples were sputter-coated with gold (ES-2030, Hitachi) to inspect the cell morphology by the FE-SEM.

2.7. Fluorescent staining of actin cytoskeleton, N-cadherin and β-catenin

After 3 days of culture on the samples, the cells were fixed in 4% paraformaldehyde and then permeabilized with 0.1% Triton-X100. To show the actin cytoskeleton, the cells were stained with Rhodamine-conjugated Phalloidin solution (50 μg/ml in PBS) for 40 min at room temperature and then washed thoroughly with PBS to remove the unbound dye. For immunofluorescent staining of N-cadherin and β-catenin, after blocking in goat serum blocking solution at 37 °C for 30 min, the cells were incubated with mouse monoclonal anti-N-cadherin and anti-β-catenin overnight, respectively. Afterwards, the cells were incubated with the goat anti-mouse antibody conjugated to Rhodamine for 2 h at room temperature and washed with PBS thrice. Finally, the samples were observed using a confocal laser scanning microscope (CLSM, Olimpus).

2.8. RNA isolation and real time reverse transduction polymerase chain reaction

Real time reverse transduction polymerase chain reaction (real-time RT-PCR) analysis was used to determine the expression of N-cadherin, β-catenin and other osteogenesis related genes by cells cultured on the Ti samples. The MC3T3-E1 cells were seeded on the samples at a density of 2×10^4/well and cultured for 3 and 7 days in the osteogenic medium. Total RNA was extracted from cells using the E.Z.N.A.™ Total RNA Kit I (OMEGA) following the manufacturer's recommendation. One μg of total RNA was converted to cDNA using the PrimeScript™ RT reagent kit (TaKaRa). The real-time PCR analysis was performed using SYBR Premix ExTaq II (TaKaRa) on the Applied Biosystems 7500. The names of the genes and the sets of primers were listed in Table 1. The expression levels of the target genes were normalized to that of the housekeeping gene GAPDH.

2.9. ALP product

The cells were seeded on the substrates at a density of 2×10^4 cells/well and cultured in the osteogenic medium. After culturing of 7 days, the cells were stained with the BCIP/NBT ALP color development kit (Beyotime) for 15 min. Then the samples were washed with PBS to take the images.

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