Surface modification of titanium plate enhanced fibronectin-mediated adhesion and proliferation of MG-63 cells

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Abstract: An understanding of osteoblast adhesion and proliferation on biomaterials is crucial to optimizing the surfaces of artificial implants used in clinical practice. Polished, anodic oxidation (AO) and micro-arc oxidation (MAO) treated titanium (Ti) plates were used as model surfaces to study the adhesion of MG-63 cells. Cells were monitored for 0.5 and 4 h; faster adhesion and spreading of MG-63 cells were observed on the AO and MAO modified samples. Stimulated secretion of fibronectin (FN) influenced the adhesion rates. In addition, AO and MAO modified surfaces promoted cell proliferation through apparent up-regulation of FN and integrin α_5 transcription via outside-in signaling. This strongly suggests that FN secretion by osteoblasts plays an essential role in enhanced cell adhesion, spreading and proliferation on these modified Ti surfaces.
Key words: titanium; surface modification; Outside-in signaling; fibronectin; integrin

1 Introduction

Titanium is an ideal choice for the long-term replacement of hard tissue because of its excellent mechanical properties, corrosion resistance and biocompatibility. Enhancement of titanium bioactivity via surface modification has been reported for particular clinical applications [1,2]. Frequently used methods are anodic oxidation (AO) and micro-arc oxidation (MAO) [3,4]. Anodic oxidation produces vertically aligned TiO_2 nanotubes on a titanium substrate, and micro-arc oxidation can provide titanium with a microporous calcium phosphate coating. Both modifications have been well tested in vivo and in vitro [5−7], and have exhibited benefits for osteoblast growth and a potential for future clinical application.

For the successful clinical use of biomaterial implants, cell/surface interactions are considered to be a key determinant [8]. Research is focused on establishing the relevant surface characteristics of biomaterials, such as chemical composition [9,10] and topography [11,12], to unravel the processes whereby biomaterials affect cell behavior. However, because of the complex and varied surface properties of biomaterials, and the application of different cell culture conditions, it is difficult to arrive at a general conclusion on how biomaterials regulate cell behavior [13]. Our mechanistic understanding of cell alteration on biomaterial surfaces remains incomplete [14]. The relationship between osteoblast behavior and titanium surface modification is the focus of study here, in order to provide further insight into cell/biomaterial interface. Moreover, the system has clinical relevance for hard tissue replacement.

Outside-in signaling is one of the most important processes mediating the interaction of cells and biomaterials. The cell, typically, uses integrins to transduce information from the extracellular matrix (ECM) to the cell interior [13,15], but such outside-in signaling also mediates the response to biomaterial surfaces [16,17]. This signaling exerts significant influence on cell adhesion and proliferation, and if we can fully understand this signaling function, we can also better control cell behavior through improved design biomaterials. An outside-in signaling study of the interaction of osteoblasts with AO or MAO modified

titanium implants has not yet been reported.

The specific objective of this study is to understand the mechanism of outside-in signaling at osteoblasts cultured on AO or MAO modified titanium. Polished, AO and MAO treated Ti plates were used as the biomaterial models, and MG-63 cells, a human osteosarcoma cell line were used as the osteoblast model. Protein expression and gene transcription of fibronectin (FN) and collagen I (COLI), respectively, were observed by Western blot and RT-PCR assay. RT-PCR assay was also employed to examine the FN and COLI-related integrin transcription.

2 Experimental

2.1 Biomaterial preparation

Commercially available titanium plates (>99.9% purity, ASTM GR.1, Baoji INT Titanium Material Co., Ltd., China) of 2 mm×10 mm×10 mm were chemically polished and used for AO or MAO treatment. Scratches or blemishes on the plates were first removed using 400-grits SiC polishing paper, and chemical polishing was then carried out by using acid etch solution comprising HNO_3 (65%−68% in mass fraction) and HF (40%) in volume ratio of 1:1. The samples were then ultrasonically cleaned using double distilled water (ddH_2O) and ethanol (99.7%), respectively. Finally, the samples were dried at room temperature.

AO treatment of the titanium plates was performed in electrolyte solution containing NaF (0.138 mol/L) and H_3PO_4 (0.5 mol/L) according to a reported procedure [18]. A constant voltage of 10 V was also applied to the titanium plates for 20 min at 40 °C. All prepared samples were then ultrasonically cleaned with ddH_2O and dried at room temperature.

MAO treatment of plates (prepared by Shanghai Institute of Ceramics, Chinese Academy of Sciences) was carried out in electrolyte solution comprising $Ca(CH_3COO)_2$ (0.1 mol/L) and $Na_2C_3H_5(OH)_2PO_4$ (0.1 mol/L) at 10 °C [19,20]. During this treatment, an AC voltage was applied for 4 min at 800 Hz and current density of 0.15 A/cm^2. The MAO treated samples were cleaned by the same method as for AO treatment.

2.2 Surface analysis

The surface morphology of the specimens was observed using a field emission scanning electron microscope (FESEM, JSM6700F, JEOL, Japan).

2.3 Cell culture

A human osteoblast-like MG-63 cell line (purchased from the Center of Cell Resource, Shanghai Institutes for Biological Science, China) was used to evaluate the biological response to the samples. Cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic (100 U/mL penicillin, and 100 g/mL streptomycin) at 37 °C in a humidified incubator (BB15, Thermo Scientific, USA) with an atmosphere of 5% CO_2. All reagents were from Thermo Scientific, USA.

2.4 Cell morphology

Titanium samples were collected and washed twice with PBS prior to their use for MG-63 cell culture. Culture periods of 0.5 h and 4 h were used. For imaging, samples were pre-fixed with 3% glutaraldehyde for 30 min, and then dehydration gradients of ethanol and of hexamethyldisilazane were applied. The dehydrated specimens were observed by a field emission scanning electron microscope (FESEM, JSM6700F, JEOL, Japan).

2.5 AlamarBlue assay

AlamarBlue (Invitrogen, USA) assay of cell viability was carried out according to the protocol described previously [9].

2.6 Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from MG-63 cells which had been cultured on polished Ti, AO and MAO treated Ti for either 4 h or 5 d using TRIzol reagent (Invitrogen, USA). The RNA was precipitated with isopropanol, washed with 75% ethanol and dissolved in RNase-free water.

Following DNase I (Fermentas, USA) treatment, the extracted RNA was used to produce cDNA using a reverse transcription kit (Fermentas, USA). PCR reactions were undertaken using a thermal cycle system (2720 Thermal Cycler, Applied Biosystems, USA) with the following programs: a denaturation step for 2 min at 94 °C followed by a sequence of 25−30 cycles at 94 °C for 30 s, 55−57 °C for 30 s and 72 °C for 60 s. The PCR products were detected using a gel image analysis system (2600R GIS, Tanon, China). Typically, five repeats were performed for every sample. The primers used in this study are listed in Table 1.

2.7 Western blotting assay

MG-63 cells were collected after they had been incubated with samples for 4 h and 5 d, respectively, and were washed once with PBS and once with washing buffer (10 mmol/L tris (pH 8.0), 150 mmol/L NaCl, 1 mmol/L EDTA (pH 8.0)). The cells were then lysed in 200 μL of lysis buffer which contained 20 mmol/L tris-HCl (pH 8.0), 30 mmol/L $MgCl_2$, 2 mmol/L ethyleneglycol tetraacetic acid (EGTA), 10% glycerol, 1% CHAPS, 1.2 μL of 10% β-mercaptoethanol and 100 mmol/L phenylmethanesulfonyl fluoride (PMSF).

Table 1 Primer pairs used for RT-PCR

Gene symbol	Primers (F, forward; R, reverse)
FN	F 5'- GCCAACCTTTACAGACCTATCC -3' R 5'- ATCCAGGTACTGTGGCTCATCT -3'
COLI	F 5'- CACCCACCGACCAAGAAACCAC -3' R 5'- TTGGCACCATCCAAACCACTGA -3'
Integrin β_1	F 5'- GAAAGTCCCAAGTGTCATGAAGG -3' R 5'- TCTTTATGCTCAGCACAGACACC -3'
Integrin α_1	F 5'- AAGCAGACGTGCTGTTCATCTC -3' R 5'- GTTTCCTTTCCCTCCATATGGC -3'
Integrin α_2	F 5'- GGTTATTCAGGCTCACCGAGG -3' R 5'- CATCCAAGGACCTCCCAAAGT -3'
Integrin α_3	F 5'- GCCTTTGGTTTATCTGTGGCCAG-3' R 5'- GTGATGTTTCGCCTGTAGTTGGG -3'
Integrin α_4	F 5'- GATGGCTTTGAAGATGTTGC -3' R 5'- CTGCTGGACACCTGTATGCT -3'
Integrin α_5	F 5'- CTATGAGGCTGAGCTTCGGGTC -3' R 5'- GTGGTGCAGTTGAGTCCCGTAA -3'
Integrin α_v	F 5'- CCAGTTCACGCCTGCTAACAT -3' R 5'- TTGTGCTCCCAGTTTGGAATC -3'
β-actin	F 5'- GGAGCAATGATCTTGATCTTCATTG -3' R 5'- AGATCATGTTTGAGACCTTCAACAC-3'

Proteins (~20 μg) were resolved by gel electrophoresis using 8% SDS-polyacrylamide gel and transferred to polyvinylidene (PVDF) membranes (Millipore, USA) at 100 V for 1.5 h. The membranes were blocked by overnight exposure to 5% non-fat milk in 1×PBS and 0.1% Tween 20 at 4 °C. Samples were incubated with primary and secondary antibody at room temperature for 2 h and 1 h respectively. After samples had been treated with enhanced chemiluminescence reagent (ECL) (Pierce, USA), they were exposed to photographic films (Kodak). Primary antibodies used in this study were rabbit anti-β-actin antibody (1:1000) (Cell Signaling Technology, USA), anti-Fibronectin (1:1000) (Sigma-Aldrich, USA) and anti-collagen I (1:200) (Millipore, USA). Secondary antibodies were HRP-conjugated anti-rabbit IgG (1:2000) (Cell Signaling Technology, USA).

2.8 Statistical analysis

All quantitative data were collected from triplicates, or more, and expressed as mean ± standard deviation (s.d.). Statistical comparison between groups was performed using a two-tailed unpaired t-test (two-group comparison). A value of p less than 0.05 was considered to be statistically significant.

3 Results

3.1 Morphology of titanium specimens

The surface morphology of Ti specimens was analyzed by FESEM (Fig. 1). The surface of the titanium plates was smooth as a result of initial polishing (Fig. 1(a)). The surface of AO treated Ti plates exhibited a similar appearance to that of polished Ti plates at low magnification (Fig. 1(b)), but it also showed clearly at high magnification (Fig. 1(c)) that nanotubes of around 50 nm diameter were evenly distributed on its surface.
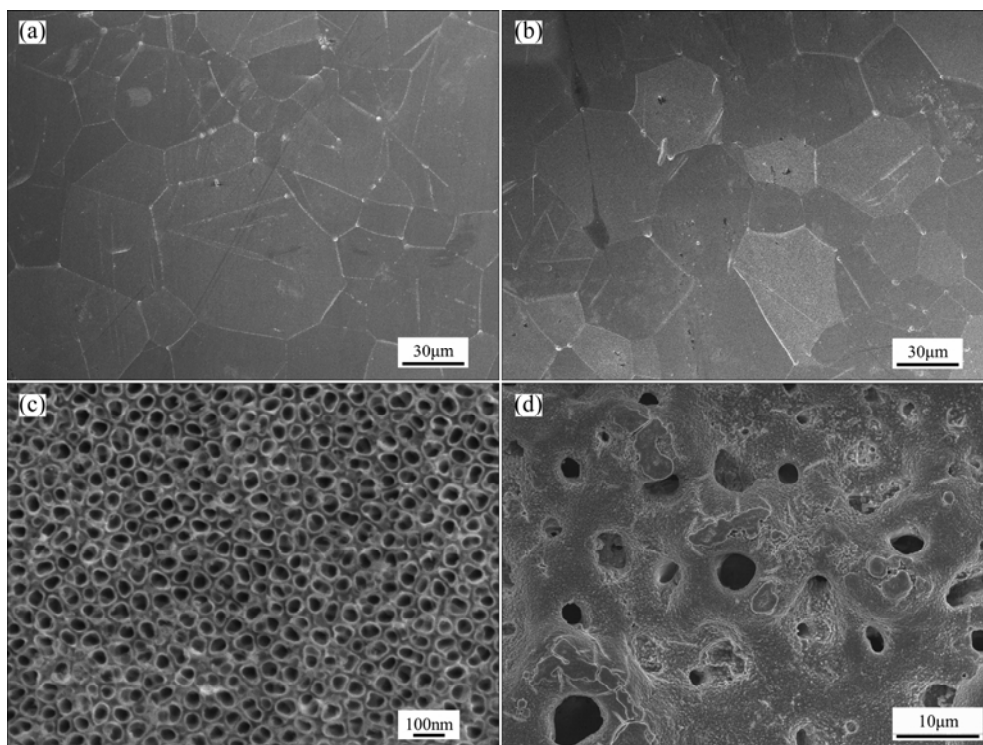

Fig. 1 FESEM morphologies of polished titanium surface (a), AO treated titanium surface (b, c) and MAO treated titanium surface (d)

The surface of MAO treated Ti plates showed micropores of varying size (Fig. 1(d)). Whilst both AO and MAO treatment led to increased surface roughness, and the surface morphologies were quite different.

3.2 Cell morphology on titanium

The morphologies of MG-63 cells incubated on three different treated titanium plates for 0.5 h are shown in Figs. 2(a)−(c). In contrast to MG-63 cells on polished Ti, those seeded on AO treated and MAO treated Ti plates formed filopodia and lamellipodia, consistent with these rougher surfaces stimulating cellular adhesion and ECM formation. At 4 h, cells on the AO and MAO treated surfaces exhibited a regular, polygonal morphology, whereas those on polished Ti remained round, with no indication of stimulated adhesion (Figs. 2(d)−(f)).

3.3 Fibronectin production on titanium by MG-63 cells

The extracellular matrix proteins, FN and COLI, play a key role in cell adhesion, but the surface properties that induce this ECM production are less clear. To help determining biological mechanisms for such stimulated ECM protein production by MG-63 cells, an enhanced adhesion, combined use, was made of Western blotting and RT-PCR (Fig. 3). On this basis, total measured COLI levels were stable across all surfaces.
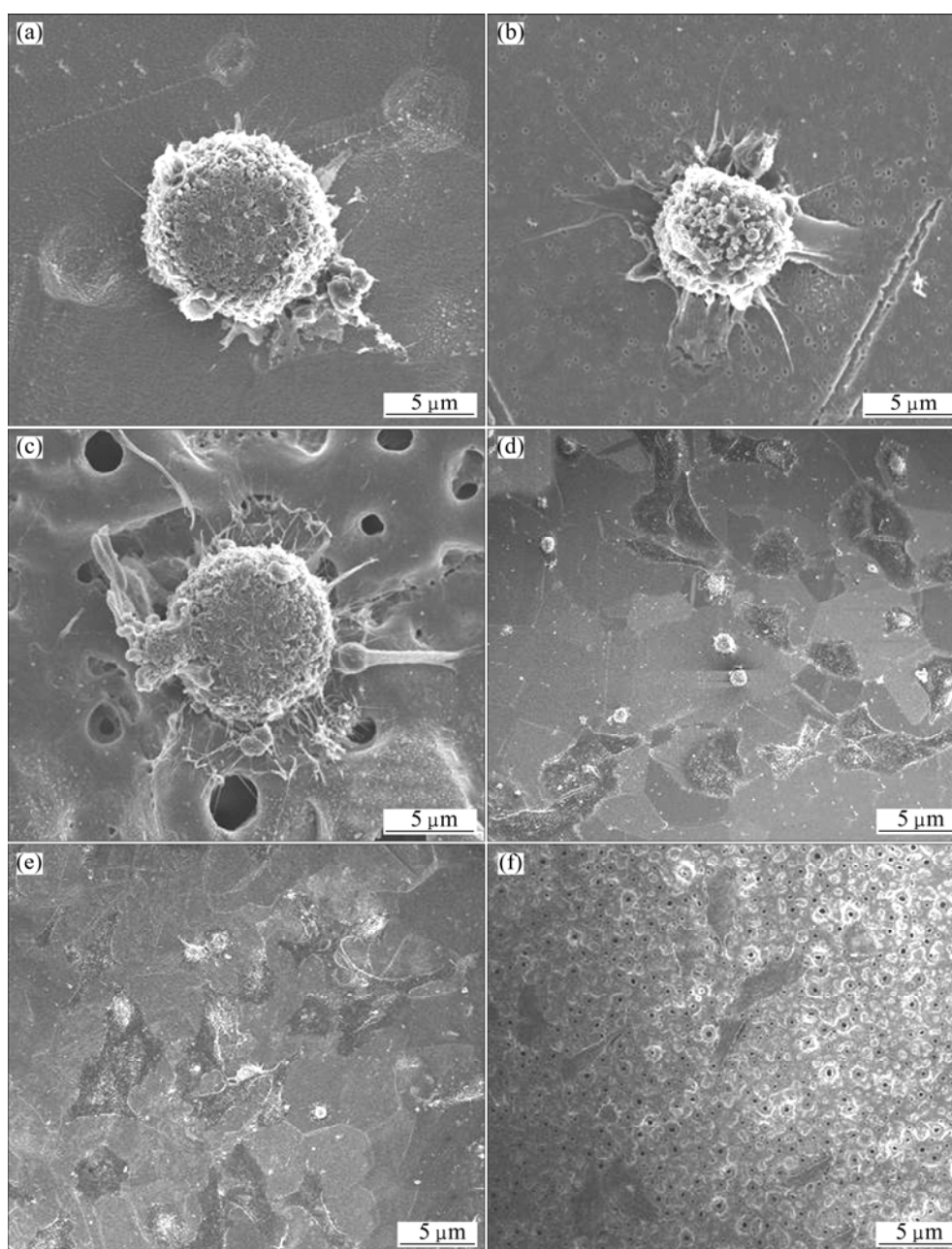

Fig. 2 FESEM morphologies of MG-63 cells cultured for 0.5 h on polished Ti (a), AO treated Ti plate (b), MAO treated Ti plate (c) and cultured for 4 h on polished Ti (d), AO treated Ti plate (e) and MAO treated Ti plate (f)

However, FN levels were actually reduced at MG-63 cells grown on AO and MAO treated Ti plates (Fig. 3(a)) compared with those on polished Ti plates. The results suggest that while MG-63 cells may well secrete increased FN, leading to filopodia and lamellipodia development. This is a distinct feature from total cellular FN production. To test reduced transcription of FN of cells cultured on AO and MAO treated Ti, the transcription of COLI and FN was determined by RT-PCR (Fig. 4(b)). However, the same mRNA levels for FN and COLI were observed for the three types of Ti samples. This indicates not only that the Ti surfaces had no differential effect on FN and COLI transcription, but also that there was no suppression. It appears that cells were not able to vary the transcription pattern of their integrin genes, within the resolution of the assay at this early phase regardless of measured change in FN. It is possible that the relevant variable was FN export rather than synthesis.
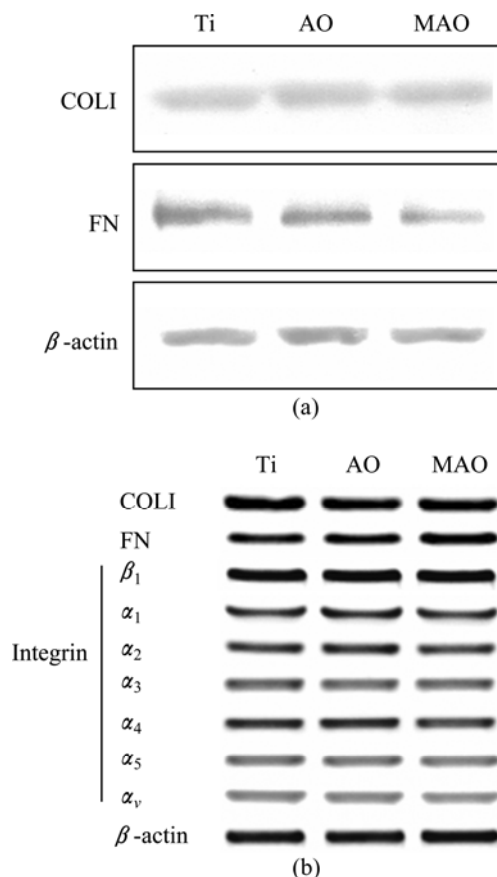
and 5 and normalized to day 1 to indicate proliferation rates. Compared with the polished Ti, the proliferation rates on the AO and MAO modified Ti at 3 and 5 d were significantly greater, with the highest value on MAO treated Ti (Fig. 4(a)).
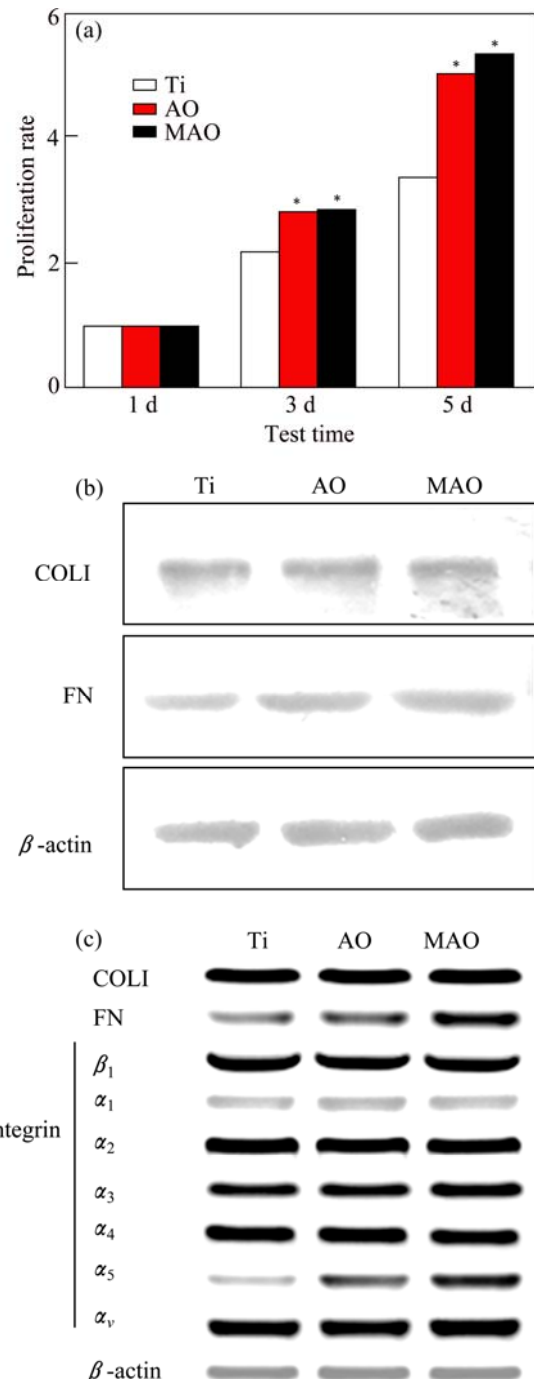

Fig. 3 COLI, FN expression in MG-63 cells cultured with polished, AO and MAO treated Ti plates respectively for 4 h (a), mRNA level of genes listed in MG-63 cells cultured with polished AO and MAO treated Ti plates respectively for 4 h (b)

Fig. 4 Proliferation rates of MG-63 cells cultured on three tested samples (All results were measured by the AlamarBlue assay (a), COLI, FN expression in MG-63 cells cultured with polished, AO and MAO treated Ti plates respectively for 5 d (b), and mRNA level of genes listed in MG-63 cells cultured with polished, AO and MAO treated Ti plates respectively for 5 d (c) (*$p < 0.05$ compared with "Ti" of each group)

3.4 Proliferation of MG-63 cells

To determine the proliferation of MG-63 cells on the three surfaces, an AlamarBlue assay was carried out. The AlamarBlue reduction was determined at day 1, 3

3.5 Influence of surface modification on gene transcription at MG-63 cells

As well as conditioning cell adhesion, COLI and FN are also regulatory proteins for cell proliferation [21,22]. To understand the molecular correlates of enhanced cell proliferation on AO and MAO modified surfaces, levels of COLI and FN in MG-63 cells cultured for 5 d with the three samples were measured (Fig. 4(b)). Also, mRNA levels of genes coding integrin β_1, α_1, α_2, α_3, α_4, α_5, and α_v that interact with COLI and FN, were examined (Fig. 4(c)).

Figure 4(b) shows similar COLI levels for the three samples, indicating that the expression of COLI was not influenced by surface modification. FN production, however, was raised at day 5 (Fig. 4(b)) and contrasts with a lack of early stage response (Fig. 3(a)). In agreement with this, the transcription of FN by RT-PCR increased in these cells, both on AO and MAO treated Ti plates (Fig. 4(c)).

Extracellular FN interacts with specific integrin heterodimer complexes, which consist of one integrin β_1 and an integrin α subunit, notably integrin α_3, α_4, α_5 or α_v [23]. As shown in Fig. 4(c), the transcription of FN markedly increased in MG-63 cells cultured on MAO treated Ti plates. According to the quantified data, both AO and MAO modified surfaces significantly enhanced the FN transcription. Figure 4(c) also shows that mRNA levels of integrin β_1, α_1, α_2, α_3, α_4, α_5, and α_v, the transcription of integrin β_1, α_1, α_2, α_3, α_4 and α_v are not affected by the different surfaces modifications, with identical mRNA levels found in all samples. Only the transcript of integrin α_5 is up-regulated, suggesting this to be the integrin mediating the outside-in signaling.

4 Discussion

The initial cell interaction with a biomaterial, i.e. its adhesion, is regarded as the fundamental event which determines later events, including proliferation [23]. Previous works [9−12] have shown that both topography and chemistry influence cell adhesion on a surface, but how these elements affect the biological process is unclear. Here, the facilitated adhesion on AO and MAO modified Ti plates (Fig. 2), is consistent with the previous reports [7,11]. Moreover, the formation of filopodia and lamellipodia is also stimulated (Figs. 2(a)−(c)). Since faster formation of filopodia and lamellipodia supports earlier cell spreading, the majority of MG-63 cells are seen to be spread over the AO and MAO modified surfaces within 4 h (Figs. 2(d)−(f)). So, the promotion of attachment and spreading, at least of MG-63 cells, is an early process.

Combining Western blotting and RT-PCR assay employed in this study has shed some light on the involvement of COLI and FN. While no early gene transcription effects were seen for either FN or COLI (Fig. 3(b)). The drop in expressed FN (Fig. 3(a)) suggests that a more subtle process of FN regulation and partitioning is in play. The cell morphological changes on the modified surfaces point to higher extracellular FN, and therefore higher secretion, regardless of total measured amount, and the early translation of this into filopodia and lamellipodia formation. The higher proliferation rates of MG-63 cells on AO and MAO modified Ti plates (Fig. 3(a)) are consistent with the known effects of cell adhesion on proliferation enhancement [23].

The COLI level and the transcription of COLI remain stable in MG-63 cells regardless of the chemistry or morphology of the materials on which they are cultured. However, the protein level of FN increases (Fig. 4(b)), with, as expected, significant elevated transcription of FN (Fig. 4(c)), so at a later stage there is a definite transcriptional effect.

Outside-in signaling is the well established route to cell/biomaterial interaction in which integrins are the key cell surface proteins. Integrins can pass information about the extracellular matrix to the cell interior, driving a range of cellular responses [13]. In particular, integrins play a role in cellular proliferation [24,25]. Our data demonstrates clearly that it is specifically integrin α_5 that is responsible for stimulated growth on the modified titanium. MILNER et al [26] also found that FN and the α_5 integrin subunit are strongly up-regulated in capillary endothelial cells where vigorous cell proliferation is required.

5 Conclusions

1) Compared with the smooth surface of polished Ti plates, the AO and MAO treated Ti surfaces were decorated with nanotubes and micropores, respectively.

2) AO and MAO treated Ti plates significantly promoted cell adhesion and the formation of extracellular matrix by stimulating the secretion of FN from MG-63 cells.

3) AO and MAO treated Ti plates enhanced the cellular proliferation by stimulating the transcription of FN and α_5 integrin.

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表面改性处理钛增强纤维连接蛋白介导的
成骨细胞 MG-63 黏附和增殖

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摘 要：采用化学抛光处理钛、阳极氧化和微弧氧化处理钛作为生物材料模型，研究成骨细胞 MG-63 在其表面的黏附和增殖机理。结果表明，阳极氧化和微弧氧化处理的钛表面通过促进 MG-63 细胞分泌纤维连接蛋白形成细胞外基质从而使其快速附着和伸展。另外，阳极氧化和微弧氧化处理的钛表面通过 Outside-in 信号传导通路，上调纤维连接蛋白及与其相关的整合素 α_5 的转录水平，促进成骨细胞 MG-63 在其表面的增殖。

关键词：钛；表面改性；Outside-in 信号传导；纤维连接蛋白；整合素

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