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Ti-6Al-7Nb promotes cell spreading and fibronectin and osteopontin synthesis in osteoblast-like cells

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Abstract The purpose of this study was to compare the early response of human osteoblast-like cells (SaOS-2) on commercially pure titanium (cpTi) and titanium-6-aluminium-7-niobium (Ti-6Al-7Nb) using glass slide as a control. In terms of cell attachment, no significance was observed when cells were seeded on the materials. However, morphological analysis by scanning electron microscope revealed that cells on Ti-6Al-7Nb showed better spreading after 4 hrs. After 48 hrs, both Western analysis and reverse transcription polymerase chain reaction analyses showed that cells cultured on Ti-6Al-7Nb synthesized a higher amount of fibronectin and osteopontin as compared to cells seeded on cpTi or on glass slide. These results suggest that Ti-6Al-7Nb possess a good potential to support SaOS-2 cells on spreading and fibronectin and osteopontin synthesis, therefore, this material may be one of a candidate material used in implant dentistry.

1. Introduction

Titanium and titanium alloys are widely used in implant dentistry because of their excellent biocompatibility. Titanium is highly inert and highly corrosive resistance. Moreover, the spontaneous protective oxide layer is formed on the surface of titanium, which affects the corrosion behavior of the material. This oxide layer is not breakdown under the physiological condition [1]. The commercially pure titanium (cpTi), which is commonly used in medical and dental implant, has been reported in clinical failure due to the fracture caused by the corrosion-fatigue mechanism [2]. It also releases the metal debris into the surrounding tissue when it is placed in the wear and/or fretting conditions [3]. Therefore, the development of titanium alloys, such as titanium-6aluminium-4vanadium (Ti-6Al-4V) and titanium-6aluminium-7niobium (Ti-6Al-7Nb), has been made to improve the mechanical properties.

However, the use of Ti-6Al-4V was restricted because of the relatively cytotoxicity of vanadium. Vanadium causes cytotoxic effect in osteoblast-like cells due to the inhibition of cell growth and oxidative inductive stress [4]. Recently, Ti-6Al-7Nb has been introduced as a new biomedical material. Ti-6Al-7Nb is more ductile than Ti-6Al-4V and also has superior corrosive resistance compared to Ti-6Al-4V through the immersion test [5].

Osseointegration is the requirement for the success in implant treatment. This biological process is associated with the osteoblast/material interaction including attachment, proliferation and differentiation, and mineralization of matrix at the material interface [6]. Thus, to obtain these processes, the material must not have any deteriorate effects on osteoblast phenotype. In addition, the materials should also favor the attachment, adhesion and spreading of cells onto the surface [7].

The ability of materials to promote protein synthesis may reflect better biocompatibility. The association between the certain types of protein in the extracellular matrix and the differentiation state of osteoblast has been reported [8].

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Fibronectin, a glycoprotein found in the extracellular matrix, can promote cell adhesion and reorganization of the actin microfilaments, thus influencing the cytoskeletal network, cell morphology, and migration [9]. In addition, the presence of fibronectin also involves in differentiation process of osteoblast [10]. Osteopontin, one of the major non-collagenous protein in bone, plays an important role in cell attachment and regulation of crystal formation and growth [11]. It has been reported that the deposition of osteopontin on bone surface can promote attachment of osteoblast as occurs in the reversal line in which osteoclast deposits this molecule on the bone surface after the process of bone resorption [12]. In addition, the ability of osteoblast in osteopontin synthesis is also used as one of the marker of differentiation [8]. Thus, the ability of materials in promoting the synthesis of these proteins at the early stage should be beneficial in the osseointegration process of the implant.

Therefore the aim of this study was to investigate the attachment behavior, morphology and the expression of fibronectin and osteopontin of human osteoblast-like cells (SaOS-2) at the early stage on cpTi and Ti-6Al-7Nb in a cell culture model.

2. Materials and methods

2.1. Titanium disk preparation

Titanium disks (cpTi grade 2, Morita Company, Japan) and Ti-6Al-7Nb (Tough alloy, GC, Tokyo, Japan) were cast in disk shape, 12 mm in diameter and 2 mm thick. The specimens were polished using 1200-grit SiC paper in a polishing machine (DPS 3200, IMPTECH, South Africa) and consequently sandblasted with aluminous oxide particle, 50 μm in diameter, blown under 7 bars of pressure at the distance of 5 cm between nozzle and disk for 5 sec. Glass slides were used as the control. All specimens after sandblasted were ultrasonically cleaned with deionized water for 1 hr then consequently rinsed with 70% ethanol. Prior to cell culture experiment, each specimen was washed again with 70% ethanol followed by three times rinsing with deionized water and then autoclaved.

2.2. Surface characterization

Surface roughness was measured by contact profilometry using a profilometer (Talyscan 150, Taylor Hobson, UK). Average surface roughness (R_a) measurements were randomly taken at five different locations on each disk under the following condition: measurement speed at 2.0 mm/s, one way direction measurement. Surface morphology of the specimens was also examined using a scanning electron microscope.

2.3. Cell culture

Human osteoblast-like cells, SaOS-2, kindly provided by Professor Erik Thompson, St Vincent's Institute of Medical Research, were seeded on the specimens in 24 well plates (Nunc, Napervile, IL, USA) at a density of 80,000 cells per well in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 unit ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 5 μg ml^{-1} amphotericin B under standard condition (at 37°C in 100% humidity and 5% CO_2). Medium and the supplement were from Gibco BRL (Carlsbad, CA, USA).

2.4. Reverse-transcription polymerase chain reaction (RT-PCR)

Cells were seeded on the materials for 48 hrs. Total RNA of each sample was extracted with Trizol (Gibco BRL, Carlsbad, CA, USA) according to manufacturer's instruction. Then, the first-strand DNA was synthesized using AMV (Avain myeloblastosis virus, Promega, Medison, WI, USA) for 1.5 h at 42°C. For polymerase chain reaction, aliquots of synthesized cDNA were added to PCR mixtures containing Taq polymerase (Qiagen, Hilden, Germany) and performed on a DNA thermal cycler (Tpersonal, Whatman Biometra, Germany). Primers for PCR were as follows:

Fibronectin	sense 5′ GGATCACTTACGGAGAAACAG 3′
	antisense 5′ GGATTGCATGCATTGTGTCCT 3′
Osteopontin	sense 5′ AGTACCCTGATGCTACAGACG 3′
	antisense 5′ CAACCAGCATATCTTCATGGCTG 3′
GAPDH	sense 5′ TGAAGGTCGGAGTCAACGGAT 3′
	antisense 5′ TCACACCCATGACGAACATGG 3′

PCR products were then electrophoresed on a 1.8% agarose gel and visualized by ethidium bromide fluorostaining. The density of band was determined using Scion Image Software (Scion Corporation, Maryland, USA).

2.5. Western Blot analysis

After cells were cultured on the materials for 48 hrs, the condition medium was collected and centrifuged to remove all debris and then concentrated 5-fold by lyophilization for fibronectin and osteopontin analyses. In addition, fibronectin was also analyzed from cell layer. Cell layer was extracted using RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing a cocktail of protease inhibitors (Sigma, St Louis, MO, USA). The amount of protein was determined by BCA protein assay kit (Pierce, Rockford, IL, USA). All samples, 25 μg of total protein per lane, were separated by 7.5% SDS/PAGE and then transferred to nitrocellulose membranes (BioTrace®NT, Life

Science, Pensacola, FL, USA). Membranes were incubated with 1:1,000 dilutions of either a mouse monoclonal antibody immunoreactive against cell-attachment domain of human fibronectin (clone 568, ascites fluid mouse IgG1, Sigma, St Louis, MO, USA), or a rabbit polyclonal antibody immunoreactive against amino acids 75–90 of human osteopontin (AB1870, Chemicon International, Tamecula, CA, USA). Horseradish peroxidase-conjugated either a biotin goat anti-mouse IgG (Zymed, South San Francisco, CA, USA) or biotin goat anti-rabbit IgG (Zymed, South San Francisco, CA, USA) were used at a 1:5,000 dilution, and blots were developed by using an enhanced chemiluminescence kit (Pierce, Rockford, IL, USA) and the signal was captured with CL-Xposture film (Pierce, Rockford, IL, USA).

2.6. Analysis of cell attachment and spreading by scanning electron microscopy

Cells on different materials were fixed after 20 min, 4 h, 8 h and 16 h of culture using 2.5% glutaraldehyde (Sigma, St Louis, MO, USA) in phosphate buffer saline (PBS) for 30 min and rinsed three times in 0.1 M PBS. They were dehydrated through a series of alcohols and then critical point dried with CO_2 (CPD 7501, FISONS Instrument, UK). A thin layer of gold was sputter-coated on the surface and they were examined in a scanning electron microscope (JSM 5410LV, JEOL, Japan). Ten fields of cells on the materials were randomly photographed at 500X magnification. The number of cell/area was assessed in each field and averaged.

Table 1 Surface roughness determination

Material	Ra (μm)
Glass	0.003 ± 0.001
cp Ti	0.041 ± 0.007*
Ti-6Al-7Nb	0.046 ± 0.013*

*$p < 0.05$, the mean Ra values were statistically different between control group (Glass) and experimental group (cp Ti and Ti-6Al-7Nb).

2.7. Statistical analysis

The experiments were performed in triplicate. Data were expressed as mean ± standard deviation. Statistical analyses were performed using a one way analysis of variance (ANOVA), follow by Dunnett test at $p < 0.05$.

3. Results

3.1. Surface topographical analysis

The value of R_a measurement is presented in Table 1. The results showed in the mean of fifteen measurements of three types of materials. The R_a parameter of cpTi and Ti-6Al-7Nb are 0.041 ± 0.007 μm and 0.046 ± 0.013 μm, respectively compared to 0.003 ± 0.001 μm for glass slide. The R_a values of the glass slides were significantly lower than those of cp Ti and Ti-6Al-7Nb. However, no significant difference is indicated between these two types of materials. The computer-generated images from profilometer and

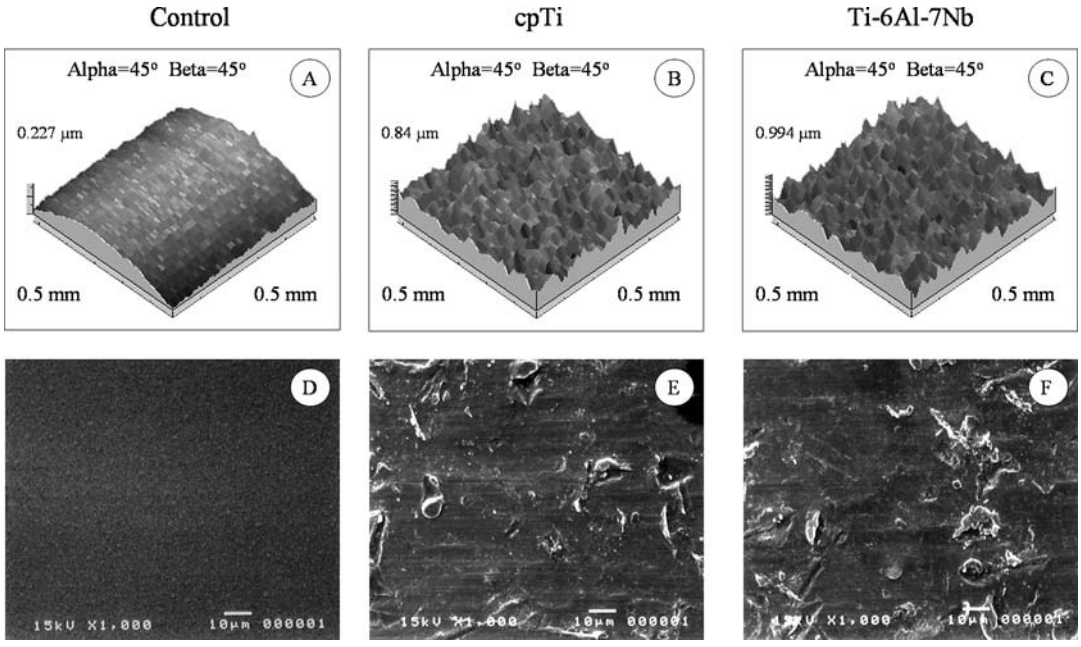

Fig. 1 A–C are images generated by the profilometer showing the roughness of the materials. D–E are micrographs showing the surface of the materials by scanning electron microscope.

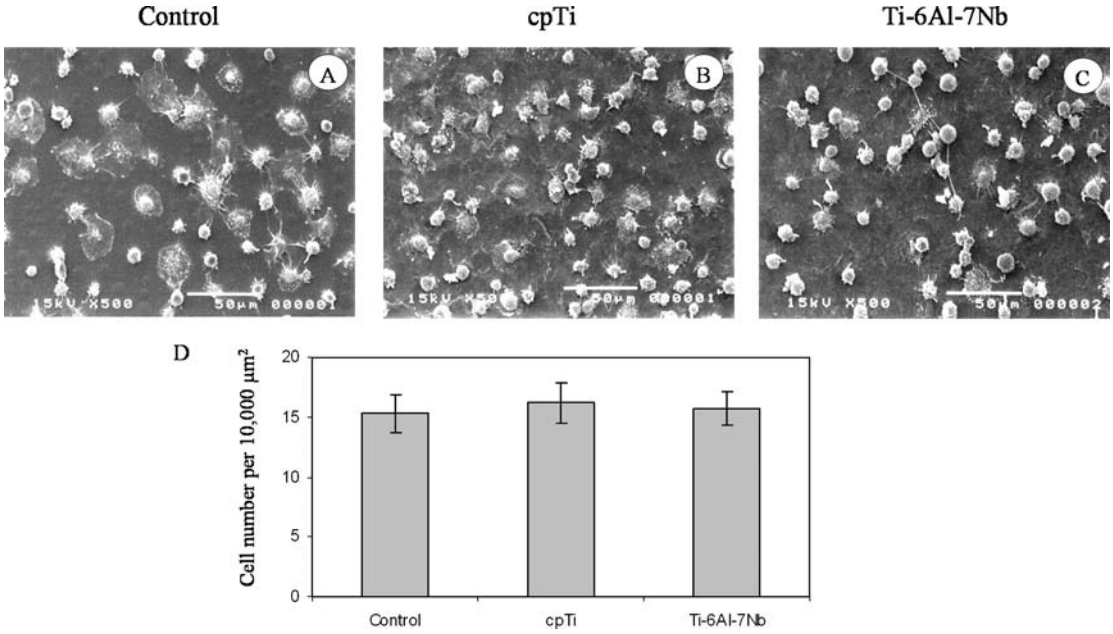

Fig. 2 Photographs taken by scanning electron microscope of SaOS-2 cultured on different types of materials for 20 min. (A) cells on glass slide, (B) cells on cpTi, (C) cells on Ti-6Al-7Nb. (D) Histogram shows the number of attached cells on various types of materials for 20 min.

micrograph from SEM observation show the difference of surface topographies (Fig. 1). The smooth and flat surfaces are clearly detected on the glass slides (Fig. 1A, 1D). On the contrary, the surface of cp Ti and Ti-6Al-7Nb shows similar appearance and homogeneous surface structure (Fig. 1B, 1C). The longitudinal rounded edge groove resulting from machining process and some scratches are also detected (Fig. 1E, 1F).

3.2. Cell morphology in scanning electron microscopy (SEM)

No significant difference on the number of cells of each condition was observed within 20 min after layering as shown in Fig. 2.

Figure 3 shows the morphology of the cells seeded on the material at 4, 8, and 16 hrs. The cells seeded on Ti-6Al-7Nb appeared with extended process or pseudopodia within the first 4 hrs (Fig. 3C) and started to form contact with the adjacent cells within 8 hrs (Fig. 3F). Meanwhile, the pseudopodia appearance was observed in the cells seeded on the glass slide and cpTi at 8 hrs (Fig. 3D, E). At 16 hrs, the monolayer of cells was observed on Ti-6Al-7Nb (Fig. 3I) but not on glass slide and cpTi (Fig. 3G, H).

3.3. Measurement of fibronectin and osteopontin synthesis

The synthesis of fibronectin and osteopontin was evaluated using RT-PCR and Western blot analysis. Figure 4 revealed that SaOS-2 cells on cpTi and Ti-6Al-7Nb displayed increased fibronectin expression and synthesis as compared to cells on glass slide. However, no difference in the level of mRNA expression was observed on cells seeded on cpTi and Ti-6Al-7Nb (Figs. 4A and 4B). Different cycles of PCR were performed to assure that the PCR products were in the exponential phase. Interestingly, the results from Western blot indicated the different distribution of fibronectin in cells seeded on Ti-6Al-7Nb as compared to cpTi (Figs. 4C and 4D). While the amount of fibronectin in the culture medium was similar on cpTi and Ti-6Al-7Nb, the amount of fibronectin in the cell extract was slightly higher in Ti-6Al-7Nb than those cultured on cpTi.

Fig. 5 showed the level of osteopontin secreted from cell culture. Similar to the pattern of fibronectin, the cells cultured on cpTi and Ti-6Al-7Nb expressed higher level of osteopontin as compared to the cells on the glass slide. Although the level of osteopontin expression in the cells cultured on Ti-6Al-7Nb was slightly higher than those on cpTi but insignificant difference.

4. Discussion

SaOS-2 cells, which are human osteosarcoma cell line, were used in the present study. This cell line is well-characterized showing osteoblast-like features [13] and has been employed in many implant studies [14–17].

Response of osteoblasts to titanium implant is related to surface topography and composition of material. In this study, we prepared the surface of both cp Ti and Ti-6Al-7Nb

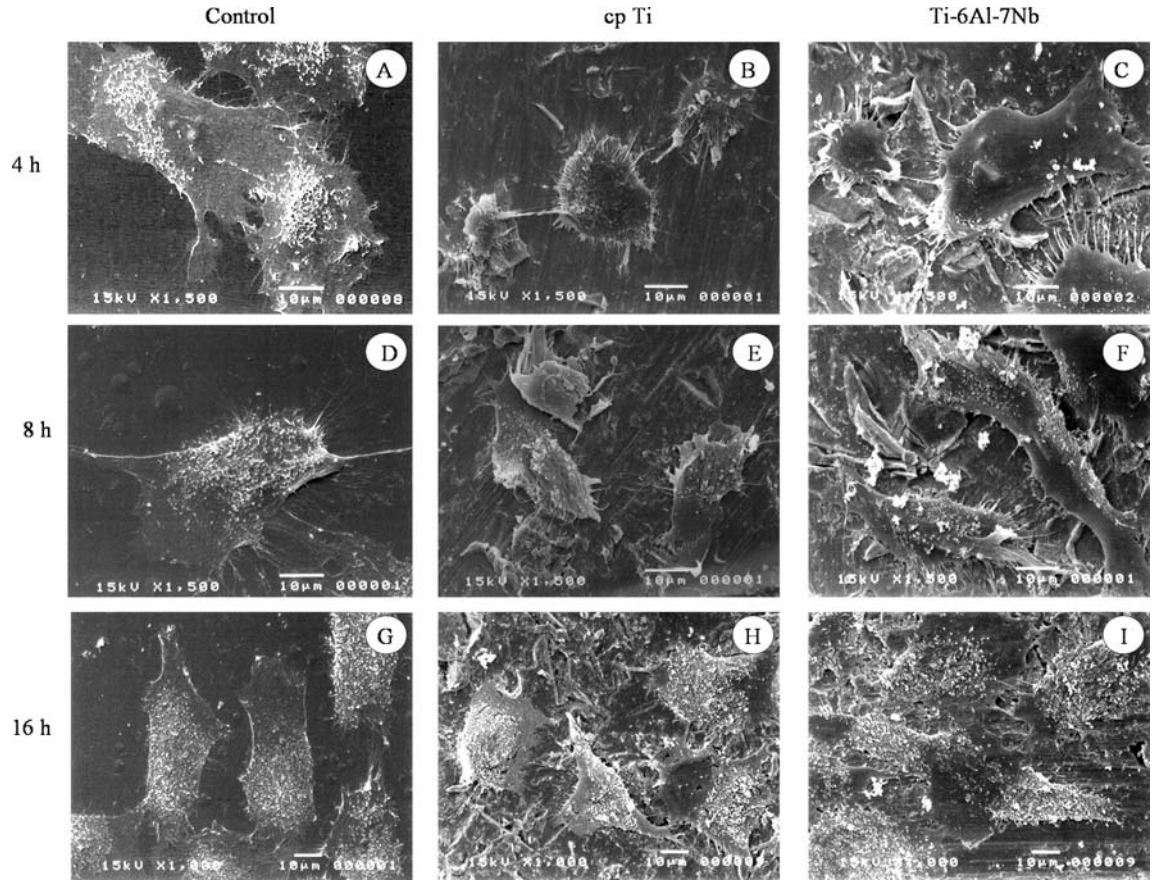

Fig. 3 Scanning electron microscopy demonstrated the morphology of SaOS-2 after 4, 8 and 16 hrs of the incubation on the materials. (A, D, G) cells on glass slide, (B, E, H) cells on cpTi, (C, F, I) cells on Ti-6Al-7Nb.

in a similar fashion. The mean of R_a values is not significantly different between two types of titanium implant material. Several studies have demonstrated that surface roughness and texture can affect the osteoblast response. Cells decrease in proliferation but increase in alkaline phosphatase production [18] and Cbfa1 and BSP II gene expression [19] on rough surface. In addition, cells on smooth surface can proliferate more than those on rough surface [20]. However, it has been report that the type of titanium surface does not influence *in vitro* adhesion and spreading of osteoblasts on titanium [16, 21]. The percentage of human mandibular alveolar bone cells attachment does not differ significantly between the specimens blasted with 180–300 micro particles and the smooth specimens [22]. This may imply that cell adhesion on titanium in early hours of culture is influenced by other factors rather than surface roughness, which shows significant difference in this study.

The response of cells, when contact with the material surface, will attach, adhere and spread respectively. The attachment phase occurs rapidly and involves a physiochemical linkage between cells and material such as ionic and van der Walls forces**.** [23] Subsequently, the adhesion phase occurs and involves various biological molecules such as extracellular matrix, cytoskeletal and membrane receptors. Thereafter, the interaction of these molecules initiate signaling pathway in the cells and will influence their morphology as well as their capacity for proliferation and differentiation [23].

From scanning electron microscope observation, we found that the total numbers of cells attached on the glass slide, cpTi and Ti-6Al-7Nb were not obviously different within the first 20 min. This indicates that both cpTi and Ti-6Al-7Nb can support the attachment of the cells. However, at 4 hrs, cells cultured on the Ti-6Al-7Nb appeared obviously extended filopodia which were not observed on the other cultured condition. The appearance of extended filopodia was later found on cpTi and glass slide at 8 hrs which indicates the delay cell spreading as compared to the cells on Ti-6Al-7Nb. Furthermore, forming of monolayer at 16 hrs in Ti-6Al-7Nb may also indicate the ability of Ti-6Al-7Nb on supporting early attachment of the cells. This characteristic of early promotion of cell attachment may attribute to the compositional parameter on the surface of the metal rather than the surface roughness [6]. This is because the Ra of both cpTi and Ti-6Al-7Nb used in this study is not significantly different as shown in Table 1.

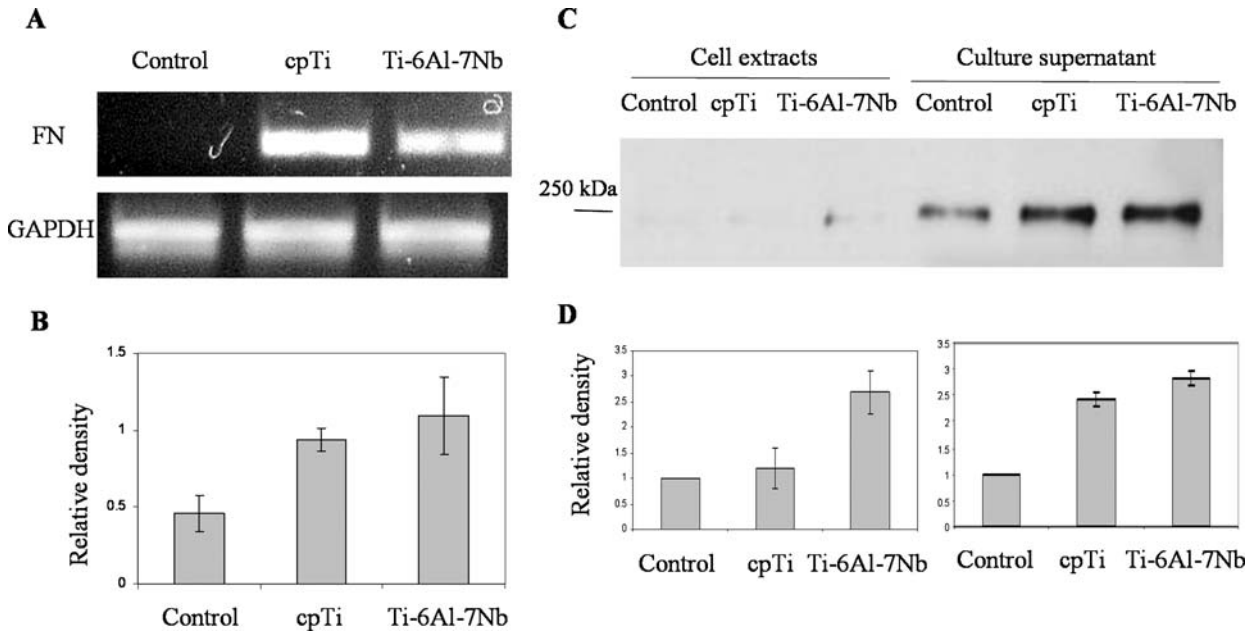

Fig. 4 Figure shows fibronectin (FN) expression of the cells grown on glass slide, cpTi, and Ti-6Al-7Nb analyzed by reverse-transcription polymerase chain reaction (A). (B) The density of bands was quantitated and the relative density was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level. A photograph of Western blot analysis of fibronectin. The cells were cultured on two types of the titanium alloys (cpTi and Ti-6Al-7Nb) and on glass slide (Control). Total cellular protein extraction and culture supernatant were collected at 48 h and consequent analyzed with Western blot analysis. The number on the left indicates the position of molecular weight marker. (D) Graphs showed the relative density of the bands in (C) normalized to the control (band density of control = 1).

Attachment on the titanium can also influence matrix synthesis of osteoblast-like cells. The results here indicated that cells seeding on both cpTi and Ti-6Al-7Nb increase the synthesis of both fibronectin and osteopontin as compared to the cells cultured on glass surface. As indicated by osteopontin synthesis, no obvious difference was observed between the cells on cpTi or Ti-6Al-7Nb. For fibronectin expression, Western analysis shows an increased amount of fibronectin in the cell extract as compared to the cells on cpTi or glass surface. However, similar amount of fibronectin in the medium

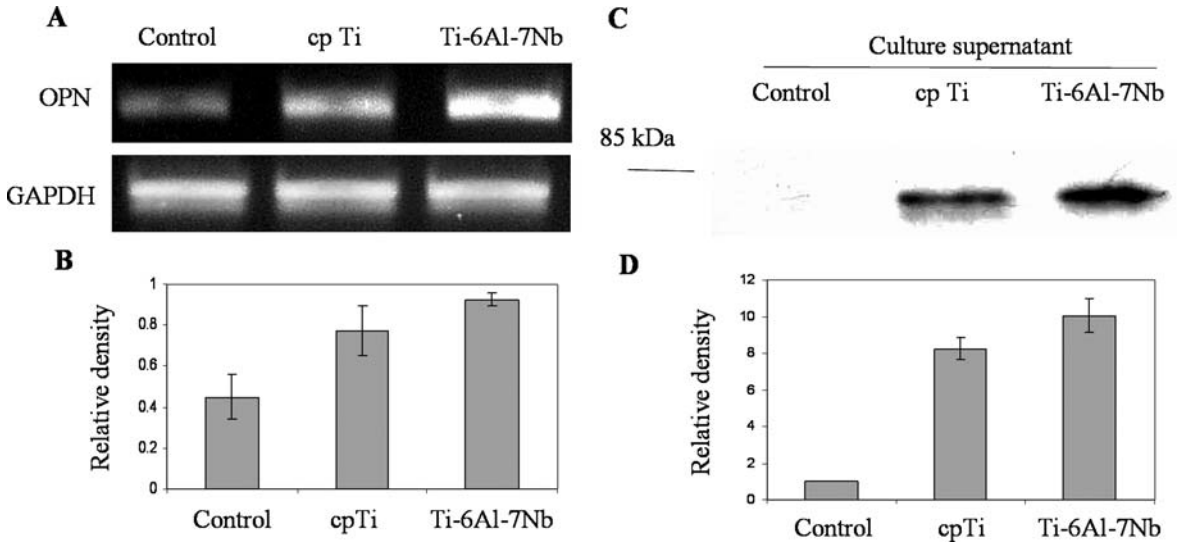

Fig. 5 Shows osteopontin (OPN) expression of the cells on glass slide, cpTi, Ti-6Al-7Nb quantitated by reverse-transcription polymerase chain reaction (A). (B) The density of bands was quantitated and the relative density was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level. A photograph of Western blot analysis showing osteopontin (OPN) in the supernatant collected at 48 h. The cells were cultured on two types of the titanium alloys (cpTi and Ti-6Al-7Nb) and on glass slide (Control). The number on the left indicates the position of molecular weight marker. (D) Graph showed the relative density of the bands in (C) normalized to the control. (band density of control = 1).

was detected in cells cultured on cpTi and Ti-6Al-7Nb. This result suggested that the surface of Ti-6Al-7Nb might facilitate the synthesis of fibronectin around the cell surface. However, further investigation is required to clarify this issue.

Fibronectin has been reported to promote both cell adhesion and proliferation in many cell types [24, 25]. Since this molecule also involves in the differentiation process of osteoblast. [10], the inductive effect of Ti-6Al-7Nb on fibronectin synthesis may facilitate both adhesion and differentiation of osteoblast. Fibronectin can also influence the behavior of other cells types rather than osteoblast. It has been shown that fibronectin involved in the migration of endothelial cells and help in the process of angiogenesis, and probably increase the number of mesenchymal cells that usually associated with the newly form blood vessels [26].

Regarding to osteopontin, it plays an important role in cell attachment [27] and calcification of mineralized tissue [11]. It has been reported that the increasing level of osteopontin synthesis may associate with the level of osteoblast differentiation [28]. In this study, the level of osteopontin protein in cells cultured on Ti-6Al-7Nb is higher than that of cpTi. In this regard, Ti-6Al-7Nb may provide better environment for osteoblast differentiation. However, the implication of differentiation resulted from this study could not be conclusively drawn. Further study on differentiation stage is now undergoing in our laboratory.

In conclusion, our data demonstrate that the different types of titanium affect adhesion, morphology, the synthesis of fibronectin and osteopontin of the human osteoblast-like cells. The result suggests that Ti-6Al-7Nb provides the proper environment in the early stage for the interaction with osteoblast and may favor a better biological outcome of dental implants. Further studies are needed to support this hypothesis.

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