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Biocompatibility studies of human fetal osteoblast cells cultured on gamma titanium aluminide

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Abstract Ti-48Al-2Cr-2Nb (at. %) (γTiAl), a gamma titanium aluminide alloy originally designed for aerospace applications, appears to have excellent potential for bone repair and replacement. The biological response to γTiAl implant is expected to be similar to other titanium-based biomaterials. Human fetal osteoblast cells were cultured on the surface of γTiAl and Ti-6Al-4V disks with variable surface roughness for both SEM and immunofluorescent analysis to detect the presence of collagen type I and osteonectin, proteins of the bone extracellular matrix. Qualitative results show that cell growth and attachment on γTiAl was normal compared to that of Ti-6Al-4V, suggesting that γTiAl is not toxic to osteoblasts. The presence of collagen type I and osteonectin was observed on both γTiAl and Ti-6Al-4V. The results obtained suggest γTiAl is biocompatible with the osteoblast cells.

Introduction

Titanium and Ti-6Al-4V are popular biocompatible materials used in bone repair and replacement due to their high strength, low weight and excellent corrosion resistance. However, they suffer from low fracture toughness, poor wear properties and high coefficients of friction in both metal-metal and bone-metal interfaces [1, 2]. The quality and intensity of bone cell response to a Ti implant appears to depend on factors linked to the nature of the material and on the ability of bone cells to interact with the surface and their microenvironment [3].

Several studies have been conducted using titanium implants with different roughness in order to determine if surface properties affect expression of a differentiated cell phenotype and integration of the implant in the human body. Many investigators have concluded that the integrity of the bone-implant interface is correlated with an increasing roughness of implant surfaces [4–13]. These results are in contradiction with some reports in which osteoblast-like cells cultured on titanium plates with three different surface roughness showed that roughness of titanium surfaces affects neither the proliferation of the cells nor the bone growth [14–16]. Other studies have reported a significant correlation between surface roughness and cell growth with greater proliferation of osteoblasts on smoother surfaces resulting in better osteointegration [17–20].

Ti-48Al-2Cr-2Nb (at. %), a gamma titanium aluminide alloy (γTiAl) originally designed for aerospace applications, has excellent properties for possible biological implant applications. This titanium alloy has superior corrosion resistance, high specific strength and rigidity [21], and lower density than currently used Ti alloys. There has been a report that titanium oxide debris is formed in currently used Ti alloy implants causing an immunological reaction resulting in loosening of the implants at the metal-bone interface [22]. γTiAl can be oxidized to preferentially form a predominantly alumina surface layer which is expected to provide a much higher wear resistance because of its inherent hardness [23]. Studies have also shown that

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Ti-6Al-4V implants could possibly release vanadium, considered a toxic element [24], which may give rise to biocompatibility problems, altering the stability of this alloy and its viability as a biomaterial [21, 25]. The absence of vanadium in γTiAl will obviate its toxicity when used in biological functions.

In an effort to establish a baseline to evaluate the potential of γTiAl as a biocompatible material, the main objective of this research was to study in vitro cell attachment and bone tissue formation of human fetal osteoblast cells cultured on γTiAl with different surface roughness, and hence determine its biocompatibility in a qualitative manner by assaying collagen Type I and osteonectin, both proteins of the bone extracellular matrix, using Scanning Confocal Microscopy (SCM). The expressions of these two proteins are good indicators of the viability of the osteoblast cells which are in contact with γTiAl. Cell attachment was qualitatively checked using Scanning Electron Microscopy. Ti-6Al-4V was used for comparison with γTiAl.

Materials and methods

Cell culture method

Human osteoblast cell line hFOB 1.19 (CRL-11372) (ATCC, Manassas, Virginia) was cultured in 90% Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 Ham (DMEM) (Sigma-Aldrich, St. Louis, Missouri) with 2.5 mM L-Glutamine and 15 mM Hepes, without phenol red, supplemented with 0.3 mg/mL geneticin antibiotic (G418, Calbiochem, San Diego, California) and 10% Fetal Bovine Serum (FBS) (Hyclone, Logan, Utah). The cells were grown in 25 cm^2 plastic culture flasks (Corning, Corning, New York) and incubated at 33 °C until confluence, washed three times with phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO4, 1.4 mM KH_2HPO_4) and harvested using trypsin-EDTA (Gibco, Gaithersburg, Maryland) at 37 °C for 5 min. The harvested cells were pelleted by low-speed centrifugation (3300 rpm) for 7 min, and subcultured in a 1:3 ratio and later stored in liquid nitrogen in 72% culture medium, 20% FBS and 8% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) for cell attachment experiments.

Titanium alloy sample preparation

γTiAl and Ti-6Al-4V disks were prepared with three different surface preparation media: 600 grit paper, 3 μm diamond paste and Mastermet® (Buehler, Lake Bluff, Illinois). The disks were 1 cm in diameter and 0.1 cm in thickness. The surface preparation was performed mechanically and manually to induce parallel orientation of residual grooves. The metal disks were then cleaned using deionized water, absolute alcohol and deionized water. The average roughness was measured using a Mitutoyo Surftest SJ-201 surface roughness tester on six samples (5 measurements on each sample) for each surface condition. The average roughness values obtained are given in Table 1. Figure 1 shows typical topography of these surfaces. The titanium disks were sterilized by autoclaving at 5 psi and 121 °C and placed in 35 mm tissue culture plates (Corning, Corning, New York).

Cell attachment

Cells were cultured on γTiAl and Ti-6Al-4V disks of the different surface roughness at a density of 4×10^5 cells per mL. For the SEM analysis, the samples were incubated for 21 days at 37 °C using a cell growth control (cells growing on a glass cover slip), negative control group (metal disks incubated with culture media without cells), a positive control group (cells cultured on Ti-6Al-4V), and the

Table 1 Roughness values for the Ti alloy surfaces for different surface preparations

Average surface roughness (nm)	Ti Alloy	Surface preparation		
		600 grit	3 μm diamond paste	Mastermet®
	γ-TiAl	600 ± 137	517 ± 13	474 ± 23
	Ti-6Al-4V	558 ± 52	500 ± 14	482 ± 13

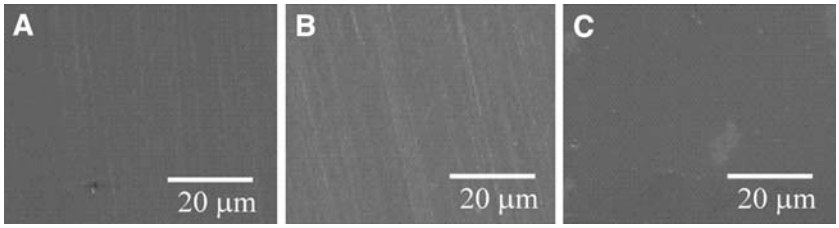

Fig. 1 Scanning electron micrographs of typical γTiAl and Ti-6Al-4V samples with three different average surface roughness: (**a**) 575 nm, (**b**) 500 nm and (**c**) 475 nm (see Table 1)

experimental group (cells cultured on γTiAl). For immunofluorescent labeling analysis, the samples were divided into four different groups: Group I was incubated for 7 days at 37 °C, Group II was incubated for 14 days at 37 °C, Group III was incubated for 21 days at 37 °C and Group IV was incubated for 28 days at 37 °C. For each experimental group a cell growth control, a negative control and a positive control were used.

Scanning electron microscopy

The titanium alloy disk samples were washed carefully with PBS and fixed overnight in 4% glutaraldehyde at 4 °C. After washing three times with PBS, the samples were dehydrated in graded alcohol ranging from 10% to 100% ethanol for 10 min each. The 100% ethanol was changed three times every 10 min. After critical point drying (EMS 850) (Electron Microscopic Science, Washington), the samples were sputter-coated with gold in EMS 550X and were examined in JEOL JSM-5410 LV SEM (JEOL, Japan) at 10 KV at various magnifications.

Immunofluorescent labeling

Immunofluorescent labeling analysis was performed in order to detect the presence of collagen Type I and osteonectin in samples cultured at 37 °C for 7, 14, 21 and 28 days. The cells were fixed using 3.7% formaldehyde in PBS at RT for 10 min, washed twice with PBS and permeabilized. Non-specific staining was blocked by incubation in blocking solution for 30 min at room temperature. Blocking solution was removed and 40 μL per sample of primary antibody monoclonal anti-human collagen Type I (mouse) (Calbiochem, San Diego, California) diluted in blocking solution was added. After 1.5 to 2 h incubation at room temperature, the samples were washed three times with blocking solution for 15 min each followed by the addition of 40 μL of secondary antibody anti-mouse IgG (goat) with Rhodamine (Calbiochem) diluted 1:100 in blocking solution. These samples were then incubated in the dark for 1 hr at RT and washed three times with blocking solution in the dark for 15 min each. A second primary antibody, monoclonal anti-osteonectin (mouse) (Zymed Laboratories, San Francisco, California) was diluted and 40 μL of dilution was added to each sample. The samples were incubated 1.5 to 2 h in the dark. The samples were washed in the dark three times with blocking solution for 15 min each and then 40 μL of secondary antibody anti-mouse IgG with Cy5 conjugate (Zymed Laboratories) were added. After 1 h incubation, the samples were washed three times with PBS, mounted in 20 × 40 mm cover slips with Fluorescent Mounting Media (Oncogene, San Diego, California) and stored at 4 °C in the dark. Samples were observed with a Confocal Laser Scanning Microscope (Fluo View™ 300 Confocal Microscope) (Olympus, USA).

Results

Scanning electron microscopy analysis showed that hFOB 1.19 cells grew on the surface of both γTiAl and Ti-6Al-4V disks. The controls (cells cultured on glass coverslips) are shown in Fig. 2a for reference. Cell attachment was similar for both metals (see Fig. 2b). Osteoblast cells appeared to spread and anchor on both metal surfaces, independent of

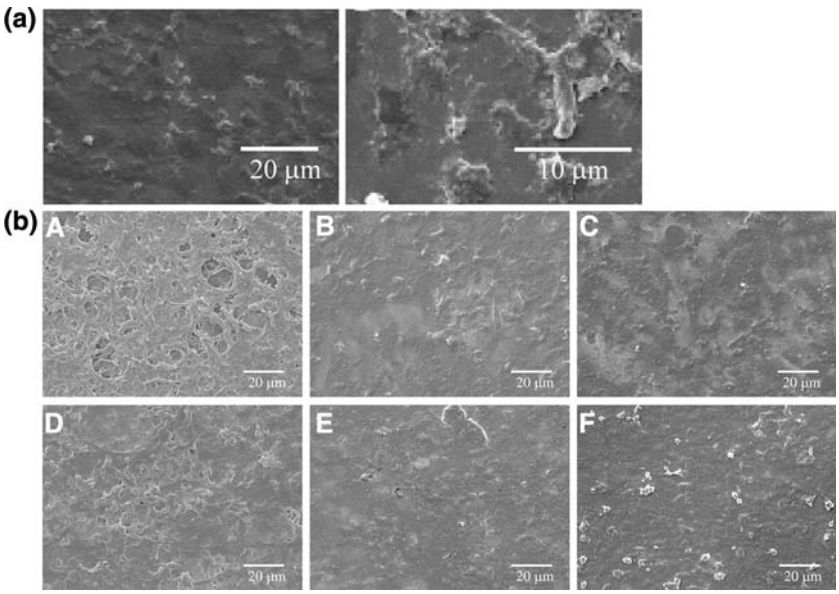

Fig. 2 Scanning electron micrographs of hFOB 1.19 cells cultured for 21 days at 37 °C on (**a**) glass coverslips as control and (**b**) γTiAl (A-C) and Ti-6Al-4V (D-F) with different surface roughness (see Table 1)

surface roughness. On the smoother surfaces, polished with 3 μm diamond paste and Mastermet®, the cells had a flattened appearance, were well spread and appeared to form a continuous layer, while on the grit polished surface cell topography exhibited ruffling and possibly formed multiple layers. This shows that these cells are indeed capable of distinguishing between changes in surface topography and respond differently to varying surface stimuli. For both γTiAl and Ti-6Al-4V, cells appeared to be in direct contact with the metal surface, and, at higher magnifications the cells appeared to have cellular extensions, microspikes and even indicated mitosis in agreement with Desagne [6] and Lauer [16], as shown in Fig. 3.

Immunofluorescent analysis of hFOB 1.19 cells cultured on γTiAl and Ti-6Al-4V with different surface roughness, and labeled with Cy5 and Rhodamine clearly revealed the presence of both fluorescent labels, corresponding to collagen Type I and osteonectin as seen in Fig. 4. However, the concentration of collagen Type I appeared to be higher. The formation of an extracellular matrix was evident and both collagen and osteonectin appeared to be restricted to specific parts of the cells, with the former appearing to form circles around the nucleus of the cells. A difference in the organization of the extracellular matrix was not apparent for cells grown neither on rough nor smooth surfaces. This is in contradiction with previous studies [12, 19] in which the extracellular matrix was organized and oriented differently when cells were grown on smooth surfaces compared to rough surfaces. There was no difference observed in the level of protein expression between cells cultured on rough or smooth surfaces in either γTiAl or Ti-6Al-4V, although there appears to be a slightly greater degree of osteonectin expression on γTiAl compared to Ti-6Al-4V.

Discussion

Cell growth, attachment and proliferation are major events that need to occur in order to maintain or repair a tissue. Since cells are sensitive to the physical properties of the materials with which they interact [3], the effects of the material on the surrounding tissue will determine its ability to be used as an implant in the human body. The fact that normal cell growth on γTiAl and Ti-6Al-4V was observed for different surface roughness with single cells having a typically flat appearance and a prominent nucleus area similar to an earlier study [19], indicates that γTiAl is not

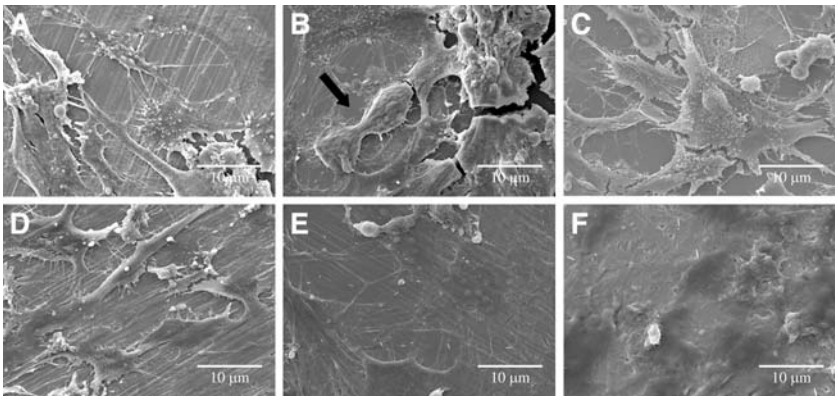

Fig. 3 Scanning electron micrographs of hFOB 1.19 cells cultured for 21 days at 37 °C on γTiAl (A–C) and Ti-6Al-4V (D–F) with different surface roughness (see Table 1). Arrow points to a mitosis-like structure in a γTiAl sample

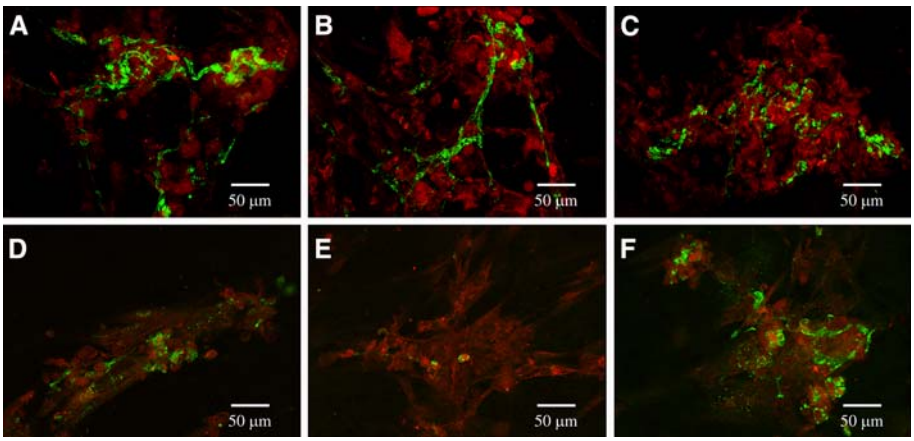

Fig. 4 Confocal Laser Scanning Microscopy analysis of the expression of human collagen type I (Rhodamine labeled, red) and osteonectin (Cy5 labeled, green) in hFOB 1.19 cells cultured for 21 days at 37 °C on γTiAl (A–C) and Ti-6Al-4V (D–F) with different surface roughness (see Table 1)

toxic to osteoblasts. The observation of a mitosis-like structure (Fig. 3, arrow), with cells in the process of separating, is a clear indication that cells can grow and proliferate on γTiAl.

Various investigators have found that grooved surfaces were found to stimulate a linearly oriented, cellular topography known as "contact guidance", where cells align themselves in an end-to-end fashion parallel to the direction of the grooves [11, 26]. The data in this study indicates that although cell appearance was different on rough and smooth surfaces concurring with previous studies [27–29], cells were able to attach and grow on both surfaces with no significant difference in cell number, also in accordance with an earlier report [22]. Clearly, osteoblast cells could be successfully cultivated on γTiAl with no observable difference irrespective of the different surface roughness.

It is demonstrated that surface roughness plays an important role in the success or failure of a metal implant [30, 31] and while roughness of the metal is believed to be critical to at bone-implant interface, the subsequent steps in bone healing around an implant may depend more on cells at the surface than on the surface per se [26, 32]. Bone formation and regulation involves hormones, cytokines and growth factors which are produced by the osteoblasts, along with the production of the bone extracellular matrix (90% collagen, and 10% glucosaminoglycans, small glycoproteins (osteocalcin, osteonectin and osteopontin), and sialoproteins [33]. The cell line hFOB 1.19 in synthesizing the proteins of the extracellular matrix indicates normal physiological activities on the γTiAl surface.

An earlier study [34] has demonstrated that synthesis and mineralization of the bone extracellular matrix were both enhanced when osteoblasts were cultured on rough-textured and porous-coated (but not on smooth) titanium surfaces, yet the current data does not agree with these results, since similar organization of the extracellular matrix was observed for hFOB 1.19 osteoblast cells grown on both rough and smooth surfaces. Expression of collagen Type I and osteonectin was found to qualitatively increase as a function of time for cells cultured on both γTiAl and Ti-6Al-4V, independent of surface topography. The increase observed is in accord with previous studies of Anselme et al [19]. At the present time, there is no acceptable explanation as to why the expression of osteonectin on γTiAl appears to be slightly greater than on Ti-6Al-4V.

Conclusions

Based on the results of this study, γTiAl appears to be a biocompatible implant material for possible medical applications. It does not appear to have toxic effects on hFOB 1.19 cells since cell attachment and growth were similar to that obtained for Ti-6Al-4V, and osteoblast cells grew and expressed their normal phenotype when cultured on γTiAl. The synthesis of collagen Type I and osteonectin was a clear indication that the presence of this metal does not interfere with normal cell function, allowing cells to produce the extracellular matrix. A significant difference in cell attachment and growth could not be observed for samples cultured on γTiAl and Ti-6Al-4V with varying surface roughness. The use of γTiAl does not appear to affect the biological activity of hFOB 1.19 osteoblast cells, suggesting that this titanium alloy has the potential for being used as implant material.

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