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Response of human bone marrow stromal cells to a novel ultra-fine-grained and dispersion-strengthened titanium-based material

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ABSTRACT

A novel titanium-based material, containing no toxic or expensive alloying elements, was compared to the established biomaterials: commercially pure titanium (c.p. Ti) and Ti6Al4V. This material (Ti/1.3HMDS) featured similar hardness, yield strength and better wear resistance than Ti6Al4V, as well as better electrochemical properties at physiological pH 7.4 than c.p. Ti grade 1 of our study. These excellent properties were obtained by utilizing an alternative mechanism to produce a microstructure of very fine titanium silicides and carbides (<100 nm) embedded in an ultra-fine-grained Ti matrix (365 nm). The grain refinement was achieved by high-energy ball milling of Ti powder with 1.3 wt.% of hexamethyldisilane (HMDS). The powder was consolidated by spark plasma sintering at moderate temperatures of 700 °C. The microstructure was investigated by optical and scanning electron microscopy (SEM) and correlated to the mechanical properties. Fluorescence microscopy revealed good adhesion of human mesenchymal stem cells on Ti/1.3HMDS comparable to that on c.p. Ti or Ti6Al4V. Biochemical analysis of lactate dehydrogenase and specific alkaline phosphatase activities of osteogenically induced hMSC exhibited equal proliferation and differentiation rates in all three cases. Thus the new material Ti/1.3HMDS represents a promising alternative to the comparatively weak c.p. Ti and toxic elements containing Ti6Al4V.

1. Introduction

Titanium (Ti) is the metal of choice for the fabrication of dental and orthopedic implants due to its excellent biocompatibility and high corrosion resistance in physiological environment [1,2]. However commercially pure (c.p.) titanium exhibits low strength and wear resistance, which is related to low hardness. Mechanical properties of the bulk material can be ameliorated by several hardening mechanisms, e.g. solid solution (alloying), grain refinement or dispersion strengthening.

Ti6Al4V represents one of the most common α–β alloys [3]. However, its drawback could arise from the alloying elements with toxic effects. Vanadium ions are classified as toxic [1,4–6], inhibit enzymes or can interact with intercellular components [7], especially mimicking the metabolic effect of insulin [6,8]. To overcome these concerns, the toxic V was substituted by the inert Nb to create the alloy Ti6Al7Nb [1,4] similar to or with even slightly better properties than Ti6Al4V [9,10]. The preference for the particular alloy varies throughout the world. Ti6Al7Nb is more prevalent in the western world; Ti6Al4V is more common in Asia. Here, Ti6Al4V was chosen as reference material because there are significantly more published studies available for comparison. Still, aluminum ions can be released, which are suspected to cause some forms of dementia or osteomalacia [11,12] especially after slow accumulation over the years [13].

Recent advances in the research of Ti-based biomaterials focused on β-Ti alloys [14,15]. The advantage is the high strength combined with low Young's modulus diminishing the risk of aseptic loosening due to stiffness missmatch (stress shielding). However, only few β alloys are found dealing with non-toxic elements [16]. One class is represented by the high alloy Ti system Ti–Nb–Ta–Zr [14]. Limited access and high prices of some alloying elements reduce their broad applicability. Therefore the driving force for the development of Ti-based materials was to exclude any toxic or expensive alloying elements.

High strength of metallic implants without using alloying elements can be achieved by grain refinement of c.p. Ti as the Hall–Petch relation predicts, and which was experimentally proven [17]. C.p. Ti with ultra-fine grains can be obtained by the massive forming technology of severe plastic deformation (SPD) [18]. However, such samples are limited in size [19]. Another technology used to produce materials of small grain size is spark plasma sintering (SPS) [20]. This powder metallurgy technique aims at near net-shape production, e.g. of c.p. Ti [21]. This method is attractive for the processing of amorphous, nano-crystalline or ultra-fine-

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grained powders because of the short processing time and low heat impact hindering grain growth. Initial powder with a nano-crystalline microstructure can be fabricated by high-energy milling – a process which is also called mechanical alloying [22]. A new approach for the generation of Ti materials with suitable mechanical properties is the distribution of finest Ti silicide and carbide dispersoides in an ultra-fine-grained Ti matrix. This is accomplished by high-energy milling of Ti with silicon powder or a liquid carbosilane to create fine grains, followed by SPS compaction keeping the ultra-fine-grained microstructure [23–25].

Besides the mechanical properties, the initial success of bone implants depends on rapid osteogenesis around the implant. Therefore, the attachment of osteoblast-like cells or osteoprogenitor cells is considered an important indicator for the suitability of an implant material. Mesenchymal stem cells (MSC) are capable of differentiation into various cell types and are therefore potential precursors of osteoblasts [26,27]. In the case of direct implantation, this cell population is in contact with the biomaterial in situ. Therefore, the reaction of MSC to potential implant materials is eminently relevant for the evaluation of initial osseointegration.

The aim of the present study was to investigate the suitability of novel ultra-fine-grained and dispersion-strengthened material Ti/1.3HMDS as implant material. The mechanical and electrochemical behavior is summarized and compared to the well established implant materials c.p. Ti and Ti6Al4V. Special care was taken to illustrate how the microstructure influences the mechanical behavior. The novel Ti-based material was tested in an in vitro model involving human MSC (hMSC). We characterized the attachment, proliferation and osteogenic differentiation of hMSC on the material in comparison to the classical titanium materials c.p. Ti and Ti6Al4V.

2. Materials and methods

2.1. Sample preparation

In this work, a novel ultra-fine-grained and dispersion-strengthened Ti material, Ti/1.3HMDS, was manufactured by powder metallurgy where an initial powder is densified by sintering. The reference material Ti6Al4V (Robert Zapp Werkstofftechnik/Stahlwerk Ergste Westig, Schwerte, Germany) was conventionally produced by metallurgy via melting and casting. For cell culture experiments, casted c.p. Ti 99.6 + % (Goodfellow, Bad Nauheim, Germany) was used, being a commercially available and technically relevant product. Detailed preparation descriptions of the novel material Ti/1.3HMDS were already published elsewhere [23,25].

Briefly, nano-crystalline powder was achieved by high-energy milling for 64 h with 150 rpm of a mixture of gas atomized Ti powder grade 1 (TLS Technik, Bitterfeld, Germany) blended with 1.3 wt.% hexamethyldisilane (HMDS) (ABCR, Karlsruhe, Germany) in a RETSCH planetary ball mill PM400 with balls of 10 mm in diameter and a powder-to-ball mass ratio of 1:10. The whole process was carried out under argon atmosphere. A subsequent heat treatment under high vacuum conditions at 400 °C for 30 min was executed to remove hydrogen which had been introduced by the silane. The granules were consolidated by SPS in a Dr. Sinter SPS 5155 laboratory facility (Sumitomo Coal Mining Company, Tokyo, Japan). Prior experiments found optimized parameters for consolidation [23]. Densities of more than 99.5% were achieved at a heating rate of 100 K min^{-1} to 700 °C, a holding time of 6 min and with a pressure of 80 MPa [23]. SPS-consolidated c.p. Ti without mechanical alloying and free of alloying elements was used for testing the mechanical, tribological and electrochemical properties to investigate the effect of grain refinement, alloying elements and dispersoides.

Precursors of all materials were trimmed to a diameter of 8 mm and 3.2 mm height. The top surface of the disks was ground and polished to 1 μm diamond followed by final polishing with colloidal silica "Final" (Buehler, Düsseldort, Germany) with intermediated etching in aqueous 2% HF + 2% HNO_3. For three-point bending experiments, beams with the geometry of 18.5 × 3.5 × 2.3 mm (length, width, height) were ground with silicon carbide paper until to a grade of P4000.

2.2. Materials characterization

The microstructure was visualized by optical microscopy with an Epiphot (Nikon, Tokyo, Japan) in differential interference contrast (DIC) and by scanning electron microscopy with a Zeiss DSM982 Gemini (Zeiss, Oberkochen, Germany) after etching of the samples with a mixture of 2% HF, 2% HNO_3, 96% H_2O. Surface roughness of the polished specimens was diagnosed by atomic force microscopy, using a Bioscope machine (Digital Instruments, Santa Barbara, USA) in tapping mode with a scan area of 2 × 2 μm^2.

Vickers hardness HV0.5 was measured with a HMV2000 (Shimadzu, Tokyo, Japan), Youngs modulus was determined by ultrasound (Tektronix Inc., Bracknell, UK) and three-point bending tests with a bearing distance of 13.5 mm were performed at a Zwicki Z005 (Zwick, Ulm, Germany).

2.3. Investigations of biocompatibility

2.3.1. Human mesenchymal stem cells

The hMSC, isolated from bone marrow aspirate of a healthy donor (33 years old), were kindly provided by Prof. Bornhäuser and co-workers, Medical Clinic I, Dresden University Hospital. Expansion of the cells was performed in Dulbecco's Modified Eagle's Medium low glucose (Biochrom, Berlin, Germany), containing 10% fetal calf serum (Biochrom), 10 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Biochrom) at 37 °C in a humidified, 7% CO_2/93% air incubator.

2.3.2. Cell seeding and cultivation on titanium disks

Prior to cell culture experiments, sample disks were autoclaved. For cell seeding, 5×10^3 cells were given to the surface of each sample disk within 18 μl of cell culture medium. After 1 h of initial adhesion, 1 ml cell culture medium was added to each sample. After 24 h of adhesion, the cell culture medium was supplemented with 10^{-7} M dexamethasone (Sigma–Aldrich GmbH, Taufkirchen Germany), 3.5 mM β-glycerophosphate (Sigma) and 0.05 mM ascorbic acid 2-phosphate (Sigma). The medium was changed twice weekly. Samples for biochemical measurements (n = 3) were taken on day 1, 9 and 18 of culture. After removing the medium, cell-seeded scaffolds were washed twice with PBS and frozen at −80 °C until further analysis. Cell experiments on proliferation and osteogenic differentiation were performed twice independently. Samples for microscopic analyses were taken at 1, 4 and 24 h of initial adhesion. These samples were fixed with 3.7% formaldehyde in PBS.

2.3.3. Biochemical analysis of cytosolic lactate dehydrogenase (LDH) activity and specific alkaline phosphatase (ALP) activity

Frozen cell-seeded sample disks were thawed for 20 min on ice, followed by cell lysis with PBS containing 1% Triton X-100 for 50 min on ice. During cell lysis, samples were sonicated for 10 min in an ultrasonic bath (Bandelin, Sonorex TK 52, Berlin, Germany).

One aliquot of the resulting cell lysate was added to ALP reaction buffer, containing 1 mg ml^{-1} p-nitrophenyl phosphate (Sigma), 0.1 M diethanolamine, 1% Triton X-100 (pH 9.8) and the mixture was incubated at 37 °C for 30 min. 1 M NaOH was added