THE EFFECT OF TITANIUM ALLOY MODIFIED WITH a-C:N:H AND a-SiC_XN_Y(H) COATINGS ON ADHESION AND IMMUNE RESPONSE OF HUMAN OSTEOBLAST-LIKE MG-63 CELLS

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Abstract

The study was conducted in order to determine the effects of modified titanium alloy (Ti-6Al-4V) surfaces on the biological response of a human osteoblast-like cell line. MG-63 cells were cultured on disk-shaped Ti-alloys: unmodified, and covered with a-C:N:H or a-SiC_xN_y(H) layers. Interactions between materials and cells were examined through determination of cells adhesion and secretion of cytokines involved in the development of immune response.

Keywords: *titanium alloy, a-C:N:H and a-SiC_xN_y(H) coatings, osteoblast-like MG-63 cells, cell adhesion, cytokines*

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Introduction

Titanium and its alloys are widely used as a material for implants because of their mechanical properties, high strength-to-weight ratio, biocompatibility, and high corrosion resistance [1-3]. Despite numerous advantages, titanium and its alloys can not be used without limitations. This is connected with a possibility of metallose and other diseases caused by a movement of metal ions to the surrounding tissues. Many efforts have been made to improve the biocompatibility of titanium and its alloys, mainly by modifying the topography and physicochemical properties to promote cell activity at the surface of implants. The surface treatment may be achieved with one of the following techniques: nitriding, oxygenating, carbonating, nitro-carbonating or deposition of carefully tailored layers [4]. There is a growing interest in search of new layer materials of complex chemical composition, containing elements of various groups of periodic table. Most of the studies are focused on four elements: carbon, hydrogen, silicon and nitrogen [5]. The objective of our study was to estimate the effect of Ti-alloy surface modification achieved by the application of a-C:N:H and a-SiC_xN_y(H) layers on adherence and activity of MG-63 osteoblast-like cells.

Materials and methods

Surface modyfication

Titanium alloy (Ti-6Al-4V) disks, 13mm in diameter and 3mm in thickness, were used in the study. The disks were mechanically polished, cleaned in isopropyl alcohol and next covered with two types of layers: a-C:N:H and a-SiC_xN_y(H). The layers were grown with application of plasma assisted chemical vapour deposition: RFCVD (13,56MHz, 60W, and cathode autopotential about -300mV) and MWCVD (2,45GHz, 800W). The mixtures of gaseous CH_4, N_2, H_2 or CH_4, N_2, SiH_4, H_2 respectively, provided at various proportions were used in the syntheses. Disks with non-modified surface were used as a reference material. Before the *in vitro* experiment examined disks were sterilized in autoclave and afterwards placed in 24-well plates (Nunclon, Denmark).

Cell culture

The ability to promote cell adhesion and cytokine production of titanium alloy disks with modified surfaces was compared using MG-63 osteoblast-like cells.

The cells were routinely grown in 75 mL flask in Dulbecco's Modified Eagle Medium with glucose and L-Glutamine (PAA, Austria), 10% foetal bovine serum (PAA, Austria), 10 U/mL penicillin, and 10U/mL streptomycin (Sigma, Germany) in a 5% CO_2 and 95% air atmosphere at 37°C. A flask of cells was brought into suspension after incubating for 5min in 0.5% trypsin plus EDTA (PAA, Austria). Following trypsinization, cells were washed by centrifugation at 400g for 5 min to give a pellet that was resuspended in fresh supplemented medium to a concentration of $3x10^4$ cells/mL. Next, 1ml of cell suspension was added to each well of 24-well plates (Nunclon, Denmark) containing sterile Ti-alloy samples. Tissue culture polystyrene (TCPS) bottom of wells served as a positive control. Cultures were performed for 3 or 7 days at 37°C in a 5% CO_2 and 95% air atmosphere.

Adhesion of cells and cytokine production

At the selected time points (3 or 7 days), supernatants from above cells cultured on biomaterials were collected and frozen for cytokines evaluation. Next, the colorimetric assay measuring cell adherence was performed. Estimation of adherent cell mass was achieved by crystal violet staining/extraction (CV test) [6]. Cells cultured on studied materials were washed twice with PBS, fixed in 2% paraformaldehyde for 1h, stained with 0.5% crystal violet in 20% methanol for 5min, washed with tap water, and dried. The dye was extracted from the cells with 100% methanol and optical density was measured on an Expert Plus spectrophotometer (Asys Hitech, Austria) at 570nm.

The level of cytokine production was estimated in cell-culture supernatants by flow cytometry. Human Inflammation Kit (Cytometric Bead Array, BD Biosciences, USA) was used to quantitatively measure 6 cytokines important for the process of inflammation. The method which allows to detect the presence of 6 cytokines in a single sample is based on fluorescence of beads coated with capture antibodies specific for: Interleukin-1 (IL-1), Interleukin-6 (IL-6), Interleukin-8 (IL-8), Interleukin-10 (IL-10), Interleukin-12 (IL-12), and Tumor Necrosis Factor (TNF) proteins. The six bead populations were mixed together and resolved in the F3 channel of a flow cytometer BD FACSCalibur™ (BD Biosciences, USA). The capture beads, phycoerythrin-conjugated detection antibodies, and recombinant standards or test samples were incubated together to form sandwich complexes. The presence of specific cytokine in the sample was detected owing to double signal for FL-3 and FL-2 lasers.

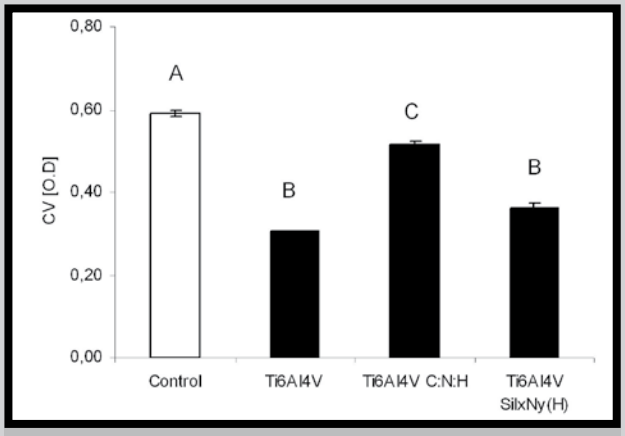

FIG. 1. The adherence of osteoblast-like MG-63 cells to TCPS (Control), Ti-alloy (Ti-6AL-4V) and modified Ti-alloy (Ti-6Al-4V C:N:H, Ti-6Al-4V-SiC_xN_y(H)) surfaces at 7 day of culture. O.D.- the optical density measured at 570nm. Letters (A, B, C) indicate values significant when tested against the control by means of the T-Tukey's test (p<0.05).

Statistical analysis

At each time point four replicates were tested for the experimental and control samples, and four measurements on each sample were performed. The results were reported as mean values plus or minus the standard error (SEM). Statistical analyses were performed using the T-Tukey's test. The statistical significance of differences was set at $p<0.05$.

Results and discussion

Cell adhesion to synthetic surfaces is involved in a variety of phenomena, such as immune response, wound healing, and tissue integration of biomaterials. Cellular attachment, adhesion and spreading belong to the first phase of cell/material interactions, and the success of this phase will influence proliferation and differentiation of cells on biomaterial surfaces. In the case of materials for bone tissue regeneration, they are expected to promote osteoblasts adherention and proliferation. The efficiency of orthopedic or dental implants is achieved if a solid interface is established with complete attachment between the material's surface and the bone tissue [7].

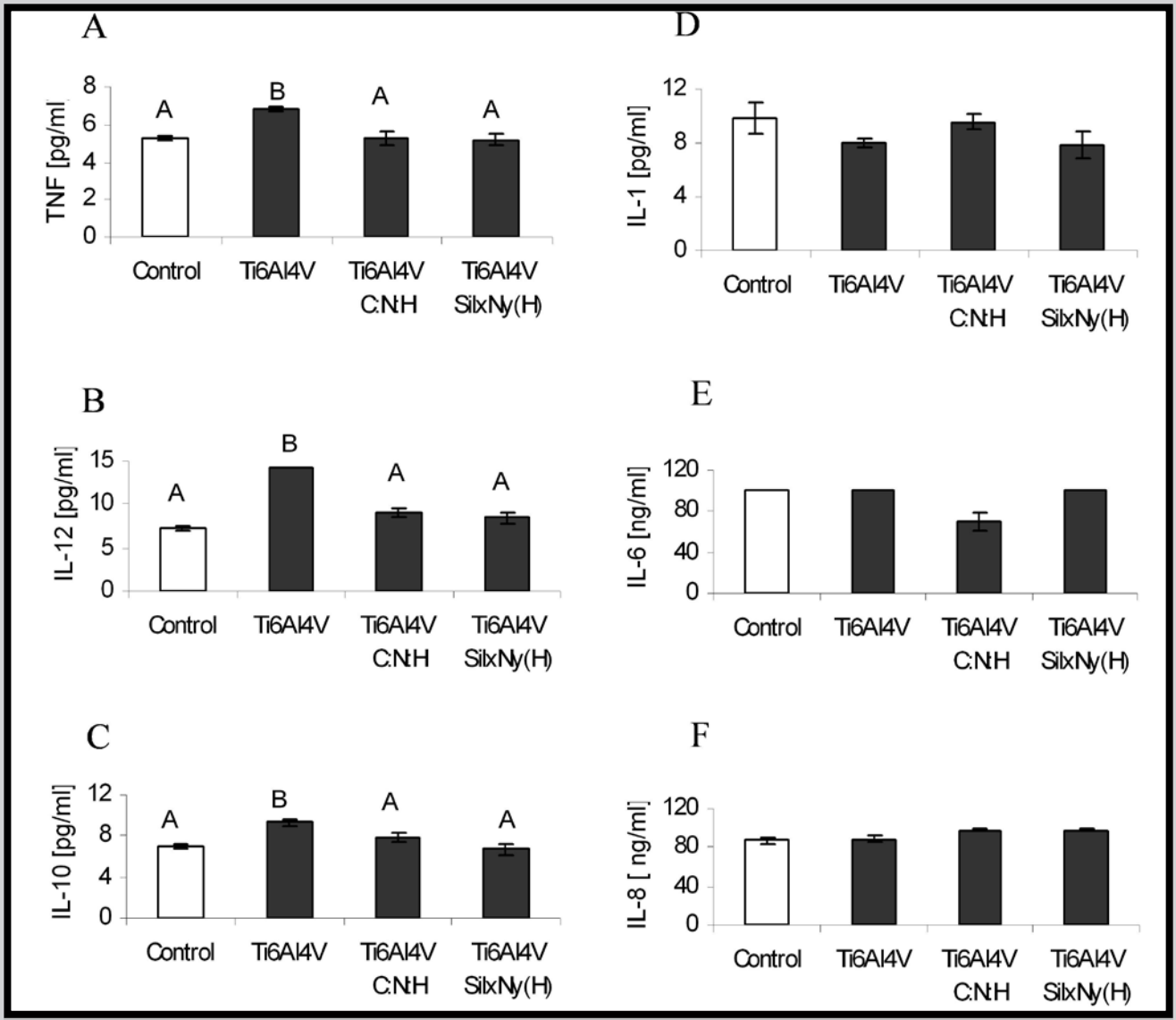

FIG. 2. The cytokine levels measured in supernatants from above cells cultured in contact with TCPS (Control), Ti-alloy, and Ti-alloy covered with a-C:N:H or a-SiC_xN_y(H) layers. Letters (A, B) indicate values significant when tested against the control by means of the T-Tukey's test (p<0.05).

Results of our study showed better ability to promote cell adhesion of titanium alloy modified with a-C:N:H or a-SiC_xN_y(H) layers compared to unmodified titanium alloy. Though all the three materials evoked lower adherence of osteoblast-like cells compared to the control, it should be stressed that the coverings improved biological features of Ti-alloy. After 3 days of culture only the tendency was observed (data not shown) but after 7 days the a-C:N:H covering gave much better results than unmodified Ti-alloy surface (FIG. 1).

Interactions between materials and cells may influence the secretory response of the cells. The secretion of the pro-inflammatory and down regulating cytokines was examined in the supernatants collected from MG-63 cells cultures. Cytokines, used extensively in cellular communication, are critical to the development of the immune response. They are often secreted by cells that have encountered a pathogen, thereby activating and recruiting immune cells to increase the system's response [8].

In our experiment the level of cytokines secreted by MG-63 cells cultured on Ti-alloy disks for 3 days didn't show statistically significant differences (results not shown).

However, after 7 days the statistically significant increase in the level of pro-inflammatory TNF (FIG. 2A) and IL-12 (FIG. 2B), and anty-inflammatory IL-10 (FIG. 2C) produced by cells cultured on unmodified surface of titanium alloy was observed. On the contrary, the level of these cytokines secreted by cells cultured on a-C:N:H and a-SiC_xN_y(H) coatings didn't differ significantly compared to the control (FIG. 2). TNF is one of the most important cytokines initiating inflammation [9]. This cytokine initiates, among others, cascade reaction causing migration of inflammatory cells which function is destruction of a foreign body penetrating to the system. Another cytokine induced during inflammation is IL-12, which increases activity and proliferation of lymphocytes T, population of cells very effectively fighting pathogens [10]. The increase in secretion of above mentioned pro-inflammatory cytokines means that titanium alloy Ti-6AL-4V can as well evoke inflammatory reaction in *in vivo* conditions. Such reaction in the case of lack of infection would be harmful for the organism because could cause destruction of it's own tissues. Confirmation, that increased secretion of some pro-inflammatory cytokines was to initiate inflammatory reaction, is increased production of pro-inflammatory cytokine IL-10. This cytokine is automatically secreted by cells to control the lowering of inflammatory reaction through inhibition of migration of lymphocytes destructing a foreign body [11]. Differences mentioned above, observed only after 7 days of cell culture and non observed after 3 days, point out the lack of acute intensive reaction. The lack of acute phase of inflammation seems to be beneficial, although it should be remembered that titanium alloy are placed in the tissue for period much longer than 1 week.

Conclusion

Obtained results indicate that used surface modifications of titanium alloy appeared to be beneficial because they improved the adherence of osteoblast-like cells and didn't stimulate the production of cytokines inducing immune response. Application of coatings allows to maintain mechanical properties, high strength-to-weight ratio, and high corrosion resistance of titanium alloy and simultaneously improve its biocompatibility.

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