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POTENTIAL ACTIVATION OF THE IMMUNE SYSTEM ON METALLIC MATERIALS FOR BONE IMPLANTS

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Titanium and stainless steel are metallic materials that have been in use for a long time in orthopedics, traumatology and stomatology. These metals are strong, corrosion-resistant and biocompatible. However, metallic materials have some disadvantages in comparison with the natural bone, particularly their relatively high specific weight and toughness. For example, the Young's modulus of AISI 316L stainless steel, Co-Cr alloys and Ti-6Al-4V alloy, i.e. materials frequently used for implantation into bone, ranges between 110-220 GPa, while the Young's modulus of bone tissue is 10-40 GPa [1]. In addition, these metals can release cytotoxic, allergenic and immunogenic ions, which can affect their biocompatibility [2, 3]. Implantation is a special type of transplantation process, in which the implant is inserted into the body, usually in order to replace an irreversibly damaged tissue. However, the immune system recognizes the implant as a foreign substance and attacks it with its effector mechanisms. Just as it can reject other types of transplants, the immune system can reject an artificial implant. To prevent rejection of an implant, it is important to study the potential activation of the immune system.

This study has investigated the biocompatibility of samples made of pure titanium (according to quality standard ISO 5832-2) and corrosion-resistant steel (quality standards ISO 5832-1 and AISI 316L), obtained from Beznoska Ltd. (Kladno, Czech Republic), and the potential activation of the immune system by these materials. In addition to Fe, the steel samples contained C (max. 0.025 wt.%), Si (0.6 wt.%), Mn (1.7 wt.%), P (max. 0.025 wt.%), S (max. 0.003 wt.%), Cr (17.5 wt.%), Ni (13.5 wt.%), Mo (2.8 wt.%), and Cu (max. 0.1 wt.%). The materials were used in the form of square samples (9x9 mm or 30x30 mm, thickness 1 mm). Both the Ti samples and the steel samples were ground with SiO2. The surface of the steel samples was then treated by polishing with Al_2O_3 paste (grain size up to 1 um), while the surface of the Ti samples, i.e. a material not suitable for polishing, was finished by brushing using another type of Al_2O_3 paste with slightly larger grains. Thus, the surface of the steel samples was finally smoother and glossy, while the Ti surface was rougher and matte.

For the in vitro biocompatibility tests, human osteoblast-like MG 63 cells (European Collection of Cell Cultures, Salisbury, UK) were used. The smaller samples (9x9 mm) were inserted into polystyrene 24-well cell culture plates (TPP, Trasadingen, Switzerland; well diameter 1.5 cm). Each well contained 25 000 cells (approx. 14 150 cells/cm^2) and 1.5 ml of Dulbecco's Modified Eagle Minimum Essential Medium (DMEM; Sigma, USA, Cat. No. 10270-106) supplemented with 10% foetal bovine serum (FBS; Gibco, Cat. No. 10270-106) and gentamicin (40 µg/ml, LEK, Slovenia). These samples were used for evaluating the size of the cell spreading area (day 1), and for evaluating cell shape and cell viability (days 1, 4 and 7 after seeding). The size of the cell spreading area was measured using Atlas Software (Tescan Ltd., Brno, Czech Republic). The viability of the cells was determined by the LIVE/DEAD viability/cytotoxicity kit for mammalian cells (Invitrogen, Molecular Probes, USA).

The larger samples (30x30 mm) were inserted into GAMA polystyrene dishes (diameter 5 cm; GAMA Group Joint-Stock Company, Ceske Budejovice, Czech Republic) and seeded with 300 000 cells/dish (approx. 15 300 cells/cm^2) suspended in 9 ml of the above mentioned culture medium. These samples were used for evaluating the cell number on days 1, 4 and 7 after seeding, using a Beckman Vi-CELL XR Cell Analyser automatic cell counter.

For the in vitro analysis of markers of osteogenic differentiation and cell immune activation, human osteoblast-like MG 63 cells (European Collection of Cell Cultures, Salisbury, UK) were used. The samples (9x9 mm) were inserted into polystyrene 24-well cell culture plates (TPP, Trasadingen, Switzerland; well diameter 1.5 cm). Each well contained 25 000 cells (approx. 14 150 cells/cm^2) and 1.5 ml of Dulbecco's Modified Eagle Minimum Essential Medium (DMEM; Sigma, USA, Cat. No. 10270-106) supplemented with 10% foetal bovine serum (FBS; Gibco, Cat. No. 10270-106) and gentamicin (40 µg/ml, LEK, Slovenia). The cells were cultured for 1, 4, or 7 days at 37°C in a humidified atmosphere of 5% of CO_2 in the air. On day 4 after seeding, the medium was changed; one half of the samples contained standard medium DMEM with 10% foetal bovine serum and gentamicin (40 µg/ml) mentioned above, and the second half contained osteogenic medium, i.e. the standard medium further supplemented with β-glycerophosphate, L-glutamin, ascorbic acid, dihydroxyvitamin D3, dexamethason, 10% foetal bovine serum and gentamicin (40 µg/ml).

Using an Enzyme-Linked ImmunoSorbent Assay (ELISA), we measured the concentration of the Intercellular Adhesion Molecule-1 (ICAM-1, a marker of cell immune activation) and osteocalcin (a marker of osteogenic cell differentiation). These measurements were performed in homogenates of cells on days 4 and 7 after seeding, and the concentration of both

markers was measured per cell or per mg of protein. On day 7, the amount of osteocalcin was measured and compared in cells cultured in the standard and osteogenic media.

We also measured TNF-α and IL- 1β, i.e. other markers of cell immune activation. These cytokines are important mediators of the inflammatory response, and they are involved in a variety of cellular activities, including cell proliferation and differentiation. We measured the secretion of these markers into the cell culture medium in murine macrophage-like RAW 264.7 cells (American Type Culture Collection, Manassas, VA). The samples (9x9 mm) were inserted into polystyrene 24-well cell culture plates (TPP, Trasadingen, Switzerland; well diameter 1.5 cm). Each well contained 30,000 (approx. 16 980 cells/cm^2) cells and 1.5 ml of the culture medium. RAW 264.7 cells were cultured in the RPMI-1640 medium (Sigma; 10% fetal bovine serum, 40 μg/mL gentamicin).

After 7 days of cultivation, the cell culture medium was collected and used for measuring the concentration of TNF-α and IL-1β by a sandwich ELISA using commercially available kits. A mouse TNF-α kit and an IL- 1β Quantikine ELISA kit were used for the RAW 264.7 cells. Both kits were purchased from R and D Systems (Minneapolis, MN) and used according to the manufacturer's protocol.

The results indicated that the number of initially adhering MG 63 cells on day 1 after seeding was significantly lower on the titanium (5320±390 cells/cm2) and on the stainless steel (4110±370 cells/cm^2) than on the control polystyrene culture dishes (7740±350 cells/cm^2). However, on day 4 after seeding, the cell population density on both metallic materials became significantly higher than on the control polystyrene dishes (75200±2890 cells/cm^2 on Ti and 90 870±2350 cells/cm^2 on steel vs. 56440±1180 cells/cm^2 on polystyrene). This suggests faster cell proliferation on both metallic materials than on polystyrene. At the same time, the cell number on the stainless steel samples was significantly higher than on the Ti samples. On day 7, the differences in the number of adhered cells on the two metals and on the control polystyrene substrate was on an average similar (from 328780±680 cells/cm^2 to 362200±760 cells/cm^2). The cell viability on all tested materials was almost 100% in all culture intervals. The morphology of the cells adhered on the studied materials was similar to the morphology of the cells on the control polystyrene dishes, i.e. the cells were mostly flat and polygonal, and the size of their cell spreading areas was similar on all tested materials. The cells were distributed homogeneously on the entire material surface, and on day 4 they started to form confluent cell layers.

On day 4, we measured the amount of ICAM-1 by the ELISA test. This immunoglobulin molecule is typically expressed on cells of the immune system, but it is also expressed on other cell types, including MG 63, during their immune activation, e.g. by an artificial growth support. In this case, ICAM-1 molecules on cells are bound by β2-integrin receptors on inflammatory cells (for a review, see [4]). Surprisingly, titanium seemed to be more immunogenic than stainless steel, which was indicated by a higher concentration of ICAM-1 per cell and mg of protein in cells on day 4 after seeding. However, on day 7, there was no difference between the concentrations of ICAM-1 per cell and mg of protein in cells on titanium and on stainless steel.

The second molecule that we measured was osteocalcin, a calcium-binding extracellular matrix glycoprotein, an important marker of the bone formation process. The concentration of osteocalcin on day 4 in the standard culture medium was higher in MG 63 cells on the titanium and stainless steel than on the control polystyrene samples. This could be explained by the fact that the metals are harder than polystyrene. It is known that harder substrates promote osteogenic cell differentiation, while softer substrates direct the cell differentiation towards neural or muscle phenotype [5]. In addition, the osteogenic differentiation was further supported by the osteogenic medium, as indicated by a higher concentration of osteocalcin in cells grown in this medium compared to cells in the standard medium on day 7 after seeding.

On day 7 after seeding murine macrophage-like RAW 264.7 cells on the tested materials, the concentration of TNF-α in the culture medium ranged on an average from 57.10 to 79.39 pg per 2000000 cells. The concentration of TNF-α in the medium from Ti and Fe was significantly higher than in the medium from the control polystyrene dishes. The highest value (79.39 pg/2000000 cells) was found in the medium taken from RAW 264.7 cells on Ti.

The second molecule that we tested was IL-1β. No significant differences in the concentration of IL-1β were detected in the culture medium obtained from RAW 264.7 cells on all tested materials. In other words, neither type of metallic material, i.e. Ti and Fe, evoked significantly higher production of IL-1β by RAW 264.7 cells than standard polystyrene cell culture dishes.

It can be concluded that the tests of biocompatibility and immune activation confirmed that titanium and stainless are promising for construction of bone implants and for good integration with the surrounding bone tissue.

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