Invited lecture/Scientific contribution/Original research

Scanning Electron Microscope Images of HUVEC Cells Treated with Materials Used for Processing of Orthopaedic and Dental Implants

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Abstract:
Use of orthopedic implants (OI) and dental implants (DI) is increasing due to obesity and ageing of the population. To increase the bio-functionality of metallic biomaterials, used for OI and DI, it is important to modify their surface composition, roughness, and structure without altering their mechanical properties. Different materials, such as minerals and inorganic compounds are used for coating OI and DI, however, they may cause response of the cells that are in contact with them in the body. To optimize the use of the materials in implant design, it is of interest to study the effect of the materials on cells. Here we present observations of micron-sized particles of milled Al_2O_3, TiO_2 and hydroxyapatite (HA) on human umbilical vein endothelial cells (HUVEC) by scanning electron microscope. We observed morphological changes of the cells – budding of the cell membrane. Comparing to the control, more cells were detached from the glass they were grown on, indicating possibility of increased cell death or inability of the cells to attach to the surface. Described changes can be due to oxidative stress and inflammatory response of the treated cells.

Keywords: Orthopedic implants; Inorganic coatings**;** Dental implants; in vitro cell lines; Inflammatory response; Oxidative stress

1. Introduction

1.1. Coating of orthopeadic and dental implants

With bio ceramic coatings and coatings made of minerals and inorganic compounds the life-time stability and biomineralization of metallic OI and DI with bone can be enhanced. Despite the progress made in fabrication of particles of different sizes, morphologies, and chemical properties, we do not fully understand how particle properties modulate immune responses in human body. Previous reports indicate that ceramics is not as bio-inert as suggested (Lee et al., 2017; Malem et al., 2013; Campbell et al., 2017). Materials used for coatings are called bioactive and are important for clinical use as bone-repairing materials (Dolinar et al., 2018). Implant surfaces achieve faster osseointegration and a stronger bone to implant interface, therefore it is important that used materials are being improved continuously (Ting et al., 2017). Bioactive ceramic coatings are utilised due to their chemical stability and ionic dissolution yet understanding of the interactions between implanted materials and host cells is of interest in recent decades. Small defects and injury in maxillofacial region can heal on their own in healthy people, but DI are necessary to treat big defects in hard and soft tissues (Zeng et al., 2018). Hydroxyapatite (Ahn et al., 2018) and titanium and titanium alloys (for example TiO_2) are currently most used implant materials in clinical dentistry (Saito et al., 2021) and in orthopaedy of deteriorated hip and knee joints, due to their favourable mechanical properties and biocompatibility (Siebers et al., 2005). Hydroxyapatite spontaneously forms a bone-like apatite layer on its surface and bond with the bone once OI or DI is placed in human body (Kokubo et al., 2004). Survival rate of joint replacement is high yet risk for inflammation without fatal outcome and therefore need for revision surgeries is common. Prevalent reason for revision surgery is aseptic loosening which often occurs as collateral to osteolysis caused by immune-mediated inflammation responses to debris from materials, used in OI and DI (Tsaousi et al., 2010).

1.2. Implant debris associated inflammation, oxidative stress and cytotoxicity

Cells of the innate immune system (e.g. macrophages, dendritic cells, neutrophils) are believed to be the first to response to implantation of a biomaterial, with the phenotype of these cells modulated by the structure and composition of the implant (Anderson et al., 2008). Macrophages respond around implanted material (Sussman et al., 2014) and dendritic cells recognize response of other cells to implantation, when they are damaged, stressed or necrotic and produce danger-associated molecular patterns (Gallo and Gallucci, 2013). Also, the type of biomaterial implanted can impact the maturation of dendritic cells which activate the adaptive immune system (Carroll et al., 2016). Metal debris from COI has previously been linked to the development of inflammatory pseudotumours (Jamieson et al., 2021). Pseudotumours are made of soft tissue mass composed of different inflammatory cells such as macrophages and T cells which are localized near the COI (Hart et al., 2012). Because of the common use of COI, investigation of potential inflammatory responses to ceramics is becoming more and more important. Previous results indicate that needle-shaped and smaller HA particles significantly enhance cytokine secretion, while larger smooth spherical particles did not. These findings indicate that HA particles have the ability to regulate immune responses that are induced after biomaterial implantation (Lebre et al., 2017). Titanium oxide (TiO_2) is one of the most used materials in clinical dentistry. Osseointergration is achieved when implanted TiO_2 attaches to alveolar bone (Brånemark, 1983). It is chemically stabile and is suggested to be bioinert – not causing inflammation and cytotoxicity in periodontal tissue (Saito et al. 2021). However, highly concentrated fluorides used for caries prevention (Schiff et al., 2002) can corrode TiO_2 and titanium ions can elute into the body (Rodrigues et al., 2013) which can induce peri-implantitis and allergic reaction(Delgado-Ruiz and Romanos, 2018). This means that titanium loses biocompatibility in acidic environments (Saito et al., 2021). TiO_2 nanoparticles (NPs) stimulate a wide array of oxidative stress related pathways. The use of TiO2 nanotube-coated titanium implants is on the other hand suggested to reduce oxidative stress and promote osteogenesis in bone remodeling (Abdulhameed et al., 2022).

It is the aim of this work to contribute to better understanding of the physicochemical and biological effects taking place at the implant-tissue interface. Previous studies indicate that the surface properties of the implant including particle debris that forms in processing of the implants may have important impact on the adjacent cells when implanted in the body (Jenko et al., 2017, Feizpour et al., 2019). It is of particular interest to analyse the effect of small particles that are formed in the processing of the implants and particles found on the surface of retrieved implants that underwent fail-

ure (Avsec et al., 2019). We focused on the effect of micron-sized particles of Al_2O_3, TiO_2 and hydroxyapatite (HA) on inflammatory, oxidative stress-related features in two cultures of human umbilical vein endothelial cells - HUVEC cells.

2. Methods

2.1. Treatment of the Cells

HUVEC cells were placed in 6-well plate with glass disc on the bottom of each well. 20 x 10^4 cells/well were in 6-well plate for 24 hours for cells to attach to the glass surface. Cells were than exposed to TiO_2 and hydroxy apatite (HA) particles and three different corundum ceramic particles: u.Al_2O_3 – used white fused alumina, Al_2O_3 – unused white fused alumina, Al_2O_3-$SiZrO_4$ nanocomposites: aluminium oxide and zirconium silicate for 24 hours.

Original-sized particles, provided by Institute of Metals and Technology (IMT) were milled in smaller particles using mill (Milimix 20, Domel, Slovenia). Cells were exposed to micron-sized particles at concentrations of 10, 50 and 100 μg/mL for 24 hours.

2.3. Scanning electron microscopy

Small cellular particles (SCPs) formation by cells is considered a physiological process (Hurley et al,. 2010) that can be accelerated by oxidative stress (Borras, et al. 2020) and by inflammation process (Chaar et al., 2011). (Yarana and St Clair, 2017)) suggested that during oxidative stress, oxidized proteins are formed, and cells release SCPs as a compensatory mechanism to maintain homeostasis. To observe the processes leading to SCP release, after treatment cell samples were fixed using standard protocol. Cells were fixed in Karnovski fixative (1 mL of 25% glutaraldehyde, 0,5 mL of 8% paraformaldehyde, 8,5 mL of Na-P buffer) for 12 hours at 4 °C, washed 3 times for 10 minutes with Na-P buffer (36% of component A*, 14% of component B**, 50 % of dH_2O); *3,561g $Na_2HPO_4 \times 2H_2O$ + 100 ml dH_2O, **3,131g $NaH_2PO_4 \times 2H_2O$ + 100 ml dH_2O, incubated with added 1% OsO_4 for 1 hour, washed 3 times for 10 minutes with dH_2O, incubated with added TCH in dH_2O for 15 minutes, washed 3 times for 10 minutes with dH_2O, incubated with added 1% OsO_4 for 1 hour, washed 3 times for 10 minutes with dH_2O, dehydrated in graded ethanol (EtOH): 30%, 50%, 70%, 80% and 90% for 10 minutes at each concentration and in absolute EtOH 2-times for 10 minutes, incubated with added absolute EtOH and HMDS (ratio 3:7) for 10 minutes, incubated in added absolute EtOH and HMDS (ratio 1:1) for 10 minutes, incubated in added 100% HMDS for 10 minutes and depleted of HMDS by evaporation in exicator with silica gel for 12 hours. After fixation, samples were gold-sputtered and observed by the scanning electron microscope (SEM, JEOL JSM-6500F).

3. Results

SEM images revealed morphological features of the treated cells – budding of the cell membrane and detachment of the cells from the glass disk surface. No such features were observed in control – untreated cells (**Figure 1**). Untreated cells were better attached to the glass disk surface while treated cells were detached at several places. Also, budding of the membrane can be seen in treated cells (pointed to with white arrows in **Figure 1**).

4. Conclusions

Membrane budding of the cells that can be seen after treatment micron-sized particles, used for OI and DI is a key step in vesicular transport, multivesicular body and exosome biogenesis (Hurley et al., 2010). We noticed that untreated cells are more attached to the glass disk surface, on the other hand, treated cells are at many parts detached, indicating cell death or inability of cells adhesion to the surface. Cell adhesion is essential for cell integrity, cell growth, and communication with other cells, therefore detachment from the surface represents adverse effects of treatment to the HUVEC cells. Inflammation process and oxidative stress are two important factors that can contribute to reduction of cell adhesion. Cell inflammatory and oxidative stress response corelate with vesiculation process (Jan et al., 2021), indicated by budding of the membrane.

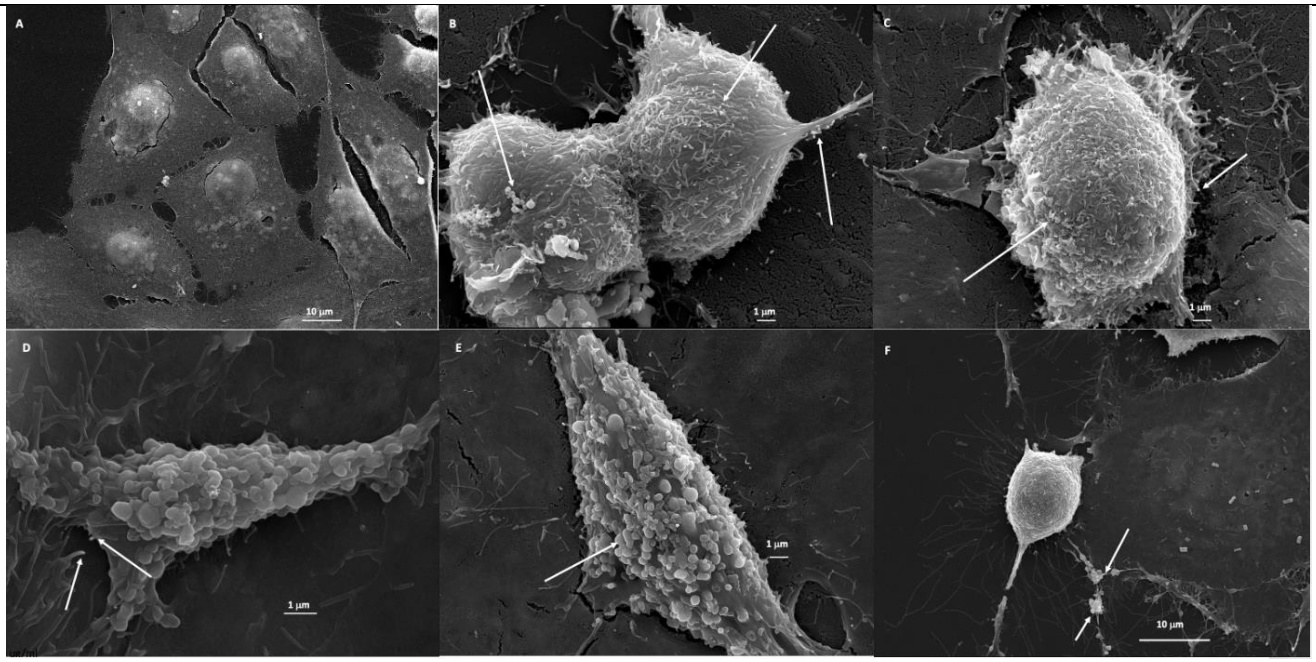

Figure 1. Scanning electron microscope image of untreated (**A**) and treated HUVEC *in vitro* cells with different particles with concentration of 100 µg/m. **B**: TiO_2, **C**: Hydroxy apatite (HA), **D**: Al_2O_3-$SiZrO_4$, **E**: used Al_2O_3 and **F**: unused Al_2O_3.

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