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BIOCOMPATIBILITY STUDIES

Original Research

Effects of S53P4 bioactive glass on osteoblastic cell and biomaterial surface interaction

R. Pérez-Tanoira[1,2] · T. J. Kinnari[1] · T. Hyyrynen[1] · A. Soininen[3,4] · L. Pietola[1] · V.-M. Tiainen[3,4] · Y. T. Konttinen[3,4,5,6] · A. A. Aarnisalo[1]

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Abstract To study the effect of bioactive glass bone substitute granules (S53P4) and hypoxic atmospheric conditions on human osteoblastic cell adhesion on different biomaterials. Cellular adhesion and cytoskeletal organization were studied on titanium, polytetrafluoroethylene, polydimethylsiloxane and S53P4 plates in the presence or absence of S53P4 granules. Cells used were human osteoblast-like SaOS-2 cells. The experiments were done either in normal atmospheric conditions or in hypoxia which simulates conditions prevailing in chronically infected bone or bone cavities. Vinculin-containing focal adhesions, organization of actin cytoskeleton and nuclear staining of cells on biomaterial surfaces were studied at 4.5 h, 2 and 4 days. In normoxic conditions S53P4 granules alkalinized the cell culture medium but cellular adhesion and cytoskeletal organization were usually not affected by their presence. Hypoxic conditions associated with lower pH and impaired cellular adhesion, vinculin-containing focal adhesion formation and rearrangement of the actin filaments to actin cytoskeleton. On most materials studied in hypoxic conditions, however, S53P4 granules prevented this impairment of cellular adhesion and cytoskeletal reorganization. The S53P4 granules promote the adhesion of SaOS-2 cells to various biomaterial surfaces especially in hypoxic conditions, in which S53P4 granules increase pH. The presence of S53P4 granules may protect biomaterial surface from bacterial colonization and promote osteointegration of implants used together with S53P4 granules for fixation and weight bearing.

✉ R. Pérez-Tanoira
ramontanoira@hotmail.com

[1] Otorhinolaryngology-Head and Neck Surgery, University of Helsinki and Helsinki University Hospital, Haartmaninkatu 4 E, 00029 HUS Helsinki, Finland

[2] Department of Clinical Microbiology, IIS-Fundación Jiménez Díaz, Madrid, Spain

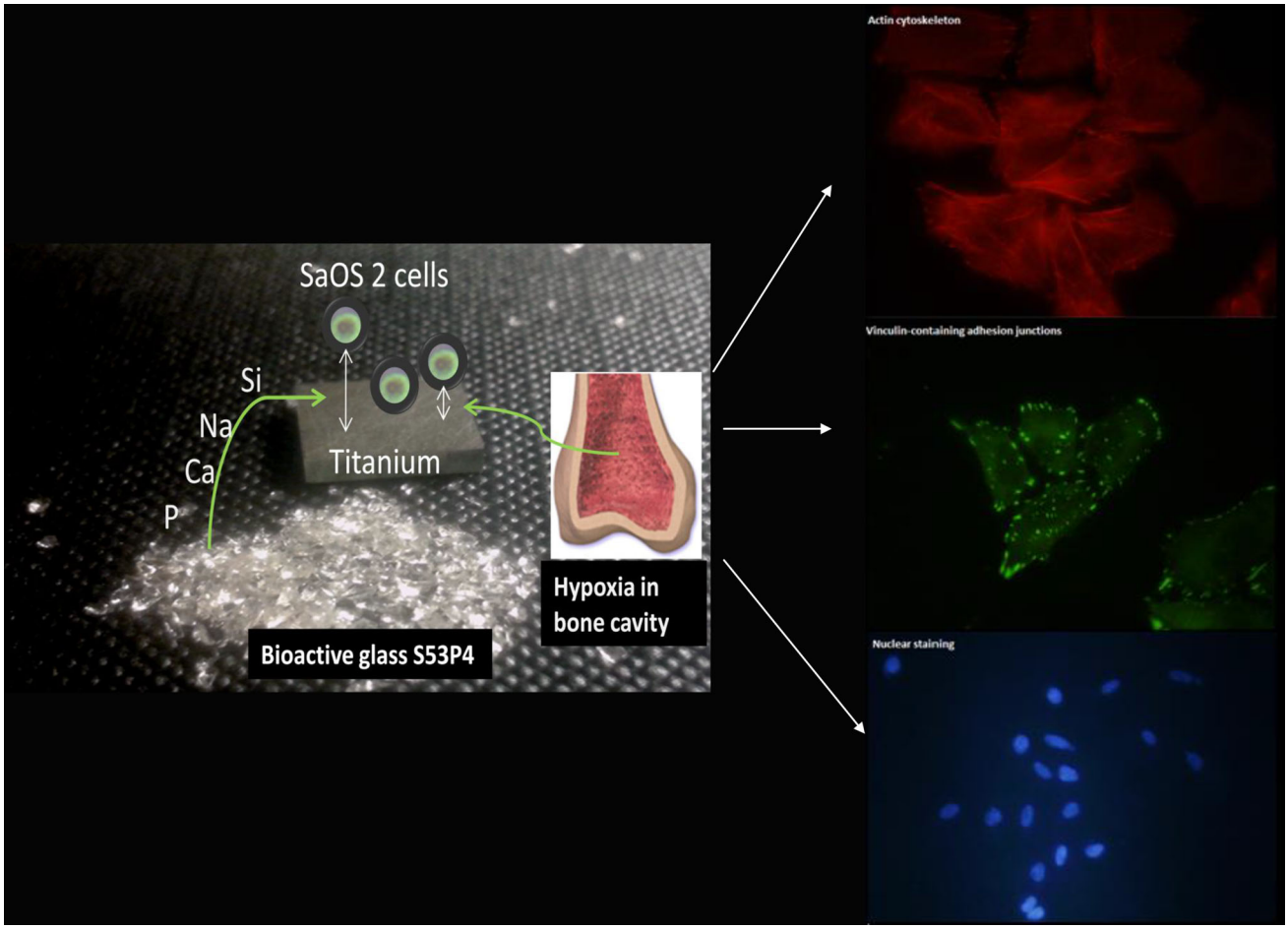
[3] ORTON Research Institute, Helsinki, Finland

[4] ORTON Orthopedic Hospital, Helsinki, Finland

[5] Department of Medicine, University of Helsinki and Helsinki University Hospital, Helsinki, Finland

[6] COXA Hospital for Joint Replacement, Tampere, Finland

Graphical Abstract



1 Introduction

In obliteration of an infected bone sequester, the contaminated bone is revised and the resulting cavity is filled using autologous material or bone substitutes such as bioactive glass (BAG) [1–3]. Usually rigid fixation implant materials are needed to restore the weight bearing properties of the bone or conductive hearing in middle-ear surgery [4]. Bioactive glasses of different compositions have been studied for decades for clinical use and several applications are today in use in dentistry, orthopedic surgery and otorhinolaryngology [5, 6].

In general, bioactive materials are defined as materials, which elicit a specific biological response at the interface of material and tissue, resulting in the formation of a bond between them by forming a bone mineral-like calcium phosphate layer on their surfaces [5–7]. In vivo and in vitro studies have reported that BAGs are able to stimulate more bone regeneration than other bioactive ceramics due to their dissolution products [7, 8].

Osteointegration of different biomaterials depends on the behavior of cells at the bone-implant interface and, particularly, on their initial attachment, adhesion and spreading [9]. Cell adhesion is mediated by several different types of transmembrane receptor proteins connecting the cytoskeleton of the cell to the extracellular matrix. Adhesion to a substrate, either surface adsorbed proteins or extracellular matrix, is mediated by attachment organs, so called focal adhesions, which make up focal contacts between the cell and matrix. These cell adhesion complexes consist of various integrin and non-integrin receptors, which link or "integrate" the cellular actin cytoskeleton first to a provisional matrix consisting of adsorbed proteins and later to cell-synthesized more mature extracellular matrix. This allows attachment and adhesion of the cell to the external surface. The intracellular components of the focal adhesion complex include talin, α-actinin, vinculin and paxillin as well as focal adhesion kinase, a signal transduction molecule. This dynamically links the transmembrane receptors attached to extracellular

ligands to the actin rearranging cytoskeleton of the cell and regulates various cellular functions via outside-in and inside-out signaling [10, 11].Furthermore, the build-up of actin microfilament bundles produces tension for cellular spreading and migration and via mechanotransduction regulates various other cellular functions [10].

The BAG S53P4 has been used as a substitute for the reconstruction of different kinds of non-weight bearing bone defects [3, 12–15]. It has bone conductive properties and serves as a reconstruction material in revision surgery of osteomyelitis, craniofacial defects and sinus or mastoid cavity obliteration.

The partial pressure of oxygen at the sea level is 160 mmHg (oxygen volume percentage 20.95 %), in arterial blood 75–100 mmHg, but in the middle ear only 46 mmHg which in a gas mixture would make 6 % oxygen by volume [16–19]. Intravascularly in bone marrow the partial pressure of oxygen is 20.4 mmHg and extravascularly in bone marrow 13.3 mmHg (2.7 and 1.7 % percentage by volume respectively) [20]. Several bone pathologies and diseases are associated with an oxygen deficit, which favors anaerobic bacterial growth and may decrease host cell adhesion impairing their capacity in the race for the surface with bacteria [21]. This introduces a critical problem in surgery of infected bone. However, almost all in vitro research of implant biomaterials has been done in cellular incubators in atmospheric conditions (20 % oxygen and 5 % CO_2).

S53P4 has antimicrobial properties. This effect is often related to an increase in the local pH and osmotic pressure through the release of ions from S53P4 [5, 6, 22–24]. However, it is not known whether this bioactive bone filling material could also affect host cell behavior and antimicrobial host defense. The hypothesis was that S53P4 bioactive glass granules affect cellular behavior especially in hypoxic conditions such as prevail in the chronically infected middle ear. Osteoblast-like cells were chosen for testing because they are the main cells S53P4 comes into contact with after revision and obliteration of a bone cavity.

2 Materials and methods

2.1 Materials

2.1.1 S53P4 bioactive glass

S53P4 bioactive glass granules (BonAlive Biomaterials Ltd., Turku, Finland) and S53P4 bulk bioactive glass plates were used. The composition of the bioactive glass in weight percentages is SiO_2 53 %, Na_2O 23 %, CaO 20 % and P_2O_5 4 %. S53P4 plates were cut using a low speed diamond saw by the Process Chemistry Centre of Åbo Akademi University in Turku, Finland. Before the experiments all surfaces studied were finished by polishing with SiC abrasive papers (120 grit) to obtain R_a values of 300–400 nm. The size of these S53P4 plates was of 5 mm × 5 mm × 1.5 mm.

2.1.2 Titanium samples

Titanium samples were produced by coating of 2 mm thick (8 cm × 10 cm surface area) regular glass substrate. Before the titanium deposition, sample surfaces were cleaned with argon sputter (SAM-7 kV, Minsk, Belarus) in a vacuum. The initial vacuum chamber pressure was 8×10^{-4} Pa and during argon sputtering the vacuum chamber pressure was 10^{-2} Pa. The sputtering time was 2 times 5 min and the voltage and current used were 4 kV and 20 mA, respectively. The purity of argon was 99.999 % (Instrument Argon 5.0, Oy AGA Ab, Espoo, Finland). Titanium surface deposition process was continued directly after the argon sputtering without breaking the vacuum. Magnetron sputtering system (Stiletto Series ST20, AJA International Inc., North Scituate, MA, USA) was used to produce titanium-coated samples. In this magnetron sputtering system a negative target potential of 530 V with 1 A current was used to accelerate the positively charged Ar ions to the high purity (+99.7 %) elemental titanium target from where titanium was deposited on the substrate to be coated by titanium (Goodfellow Cambridge Ltd, Huntingdon, England). For titanium deposition process the vacuum chamber pressure was 4×10^{-2} Pa, current 1 A and the deposition time was 4 times 1 min (with 2 min intervals). After the coating process the titanium coated glass was cut to 9 mm × 9 mm sample size by using EXAKT-cutting and grinding system (EXAKT-Apparatebau, Hamburg, Germany). The roughness value R_a of the titanium plates was 300–400 nm.

2.1.3 PDMS and PTFE samples

The bulk PTFE and PDMS polymers were obtained from a commercial supplier of industrial polymers (ETRA, Helsinki, Finland). PTFE samples were cut from 1 mm thick polymer sheets and PDMS samples from 2 mm thick sheets. Average surface roughness R_a was approx. 300–400 nm for both materials.

2.2 Study conditions

S53P4 granules were added to McCoy‘s 5A medium (containing 10 % of fetal calf serum (FCS) and 1 % penicillin/streptomycin) in a ratio of 1/5 (200 mg/mL). The pH of the solution was measured at 4.5 h, 2 and 4 days in

atmospheric (20.9 % O_2 and 0.35 % CO_2) and hypoxic (6 % O_2 and 7 % CO_2) [16–19] conditions. The hypoxic conditions were maintained with Invivo 2 Hypoxia Workstation (Ruskinn Technologies, Ltd., Sanford, Maine, USA).

2.3 Cell culture

Human primary osteogenic sarcoma SaOS-2 (ECACC 890500205) cells were cultured in 10-cm-diameter Petri dishes (Corning Inc., Corning, NY, USA) using McCoy's 5A culture medium containing GlutaMAX™ (Gibco BRL/Life Technologies Inc., Gaithersburg, MD, USA) and supplemented with 10 % v/v fetal calf serum (FCS), 100 IU/mL of penicillin and 100 µg/mL of streptomycin. Cells were grown to 80 % confluence at +37 °C in a humidified incubator with 5 % CO_2 in air. At 80 % confluence, the culture medium was carefully decanted and the cell monolayer was washed twice with 5 mL of 10 mM phosphate buffered, 140 mM saline (PBS, pH 7.4). Cells were removed from plates by applying 1–2 mL fresh trypsin diluted 1:10 (v/v) in PBS-ethylenediaminetetraacetic acid (EDTA, Sigma, Steinheim, Germany) to cover the monolayer and incubated for 5 min at +37 °C until cells detached. Medium was added to the plates, and the suspension was centrifuged at 400*g* for 5 min, after which cells were resuspended in culture medium and seeded onto the biomaterial surfaces at a density of 2×10^4 cells per well and cultured for 4.5 h, 2 days and 4 days under four different conditions: (1) in normoxia, (2) in normoxia in the presence of S53P4 granules (1:5 w/w) with a culture medium pretreated 2 h with S53P4 granules, (3) in hypoxia and 4) in hypoxia at the presence of S53P4 granules as in condition 2 above. Polystyrene (PS) 24 well plates (Nunc, Roskilde, Denmark) were used in all experiments.

2.4 Staining of vinculin, actin and nuclei

Culture medium was removed and the biomaterial plates were rinsed 2 times with sterile PBS to remove any non-adherent cell. Cells were fixed in 4 % paraformaldehyde (ChemCruz™, Heidelberg, Germany) in PBS and permeabilized using 0.1 % Triton® X-100 (Polyethylene glycol tert-octylphenyl ether) (Sigma, Steinheim, Germany) in PBS. For blocking of non-specific staining, samples were incubated in normal goat serum (Invitrogen Corporation, Frederick, USA) diluted 1:30 in 0.1 % bovine serum albumin (BSA) (Bovogen biological, Victoria, Australia) in PBS for 1 h. After washing three times with PBS, samples were incubated in monoclonal mouse anti-human vinculin IgG [25, 26] (1:400 in 0.1 % BSA in PBS) for 1 h. Cells were rinsed three times in PBS before Alexafluor 488-conjugated secondary antibody (1:400 in BSA-PBS; Molecular Probes, Eugene, OR, USA) was applied for 30 min. To simultaneously detect actin cytoskeleton, Alexafluor 568-conjugated phalloidin (Invitrogen, Carlsbad, CA, USA) was added (final dilution 1:30).Samples were rinsed three times with PBS and cell nuclei were stained with nuclear DAPI stain (blue color) (4′,6-diamidino-2-phenylindole) (Invitrogen, Eugene, Oregon, USA) (1:40 dilution in PBS) for 5 min. Samples were rinsed 3 times in PBS, once with milli-Q water and mounted on objective slides before mounting in Vectashield mounting medium (Vector Laboratories, INC. Burlingame, USA).

2.5 Immunofluorescence analysis of cell numbers and focal adhesions

DAPI was used as marker for cell number, vinculin for focal contact and actin for cellular cytoskeleton. On each plate, ten images were viewed and photographed with a fluorescence microscope at ×400 magnification (AX70,Olympus, Tokyo, Japan), in each condition and in each time point (4.5 h, 2 and 4 days). After 4 days the data could not be used for reliable analysis due to overlapping fluorescence of confluent cells. All experiments were performed in triplicate. First cell number (DAPI), then focal adhesions (vinculin), then actin cytoskeleton (actin) and last spreading.

2.6 Measurement of the number and spreading area of the attached cells

To determine the number of attached cells, the DAPI stained nuclei were counted in each image. Fluorescence microscope (AX70, Olympus, Tokyo, Japan) was used to inspect the samples at ×200–400 magnification. Photomicrographs were taken using a CCD camera (Nikon Coolpix 8400 (Nikon, Melville, NY, USA) coupled to the fluorescence microscope. All experiments were performed in triplicates. In total forty cells were outlined from 30 representative images per material and condition and the cell area was measured using ImageJ software (National Institute of Health, Bethesda, MD). To quantify cell spreading, the size of cells was analyzed using actin staining.

2.7 Statistical analysis

Statistical analysis was performed using the SPSS® software version 15.0 (SPSS Inc. Chicago, IL, USA). Mann–Whitney test was used for the statistical analysis.

3 Results

3.1 pH study

The effect of S53P4 granules in normoxia and hypoxia on pH values of cell culture medium at different time points is shown in Fig. 1. In normoxia S53P4 granules increased the pH compared with the other conditions ($P < 0.05$, Mann–Whitney test). In contrast, hypoxic conditions without any S53P4 granules decreased, the pH values statistically significantly compared with the other conditions ($P < 0.05$, Mann–Whitney test) but the significance of this difference was lost over time compared to normoxia without S53P4 and hypoxia with S53P4 granules ($P = 0.1840$, Mann–Whitney test) (Fig. 1).

3.2 Measurement of attached cells

The numbers of cells attached on each biomaterial surface in different conditions and time points are shown in Fig. 2. If the S53P4 granules were not present hypoxia decreased the number of attached cells. S53P4 granules increased the number of attached cells in normoxic conditions on PTFE at 2 and 4 days ($P = 0.037$ and 0.001, respectively, Mann–Whitney test) and on PDMS at 4 days ($P = 0.049$, Mann–Whitney test). In hypoxia S53P4 granules increased cellular attachment on titanium and, except for at 4.5 h, PTFE and PDMS. In hypoxia on S53P4 surfaces, S53P4 granules increased the attachment of cells at 4.5 h and 4 days ($P = 0.014$ and 0.007, respectively, Mann–Whitney test). Table 1 shows all the *P*-values.

3.3 Vinculin containing adhesion junctions

Vinculin was visible in the peripheral regions of cells attached on titanium and PTFE surfaces (Fig. 3). Due to heavy autofluorescence of the PDMS and S53P4 surfaces, they could not be analyzed for vinculin containing adhesion junctions. The overall vinculin labeling was more pronounced in normoxic conditions compared to hypoxic conditions for both titanium and PTFE at all measured time points.

At 4.5 h almost no vinculin stained focal contacts were observed in cells cultured on PTFE, only weak labeling was seen next to the nuclei. However, on cells cultured on titanium surface vinculin stained focal contacts were seen already at 4.5 h at the periphery of the cytoplasm. Vinculin was localized mainly at the main edges of the cells but not in their cellular extensions. Figure 4b shows the number of vinculin stained focal contacts per cell in different conditions. At 4.5 h the presence of S53P4 granules was not associated with any changes in the number of vinculin containing focal contacts, whereas hypoxia produced a clear decrease in the number of vinculin stained focal contacts at 4.5 h on titanium ($P = 0.005$).

At 2 days vinculin containing focal contacts were more numerous that at 4.5 h in cells attached on titanium surface. Vinculin staining could now be seen throughout the whole cell contours, but at a higher density at the periphery of cells at the end contact points of the actin filament fibers. At 2 days hypoxia produced a decrease in the number of vinculin stained focal contacts on both materials ($P = 0.026$ for titanium and $P < 0.001$ for PTFE). The presence of S53P4 granules did not associate with any changes in the number of vinculin stained focal contacts in normoxia, but in hypoxia their numbers decreased on titanium and an increased on PTFE surfaces ($P < 0,001$ for both materials).

At 4 days (Fig. 4a) the situation was rather similar as at 2 days. Hypoxic conditions produced a clear decrease in the number of vinculin stained focal contacts on both materials ($P < 0.001$ for both). In normoxic conditions the presence of S53P4 granules did not affect focal contacts on titanium but produced a decrease in the number of vinculin stained focal contacts on PTFE surface ($P = 0.002$). At day 4 the presence of S53P4 granules in hypoxia produced a clear increase in the number of vinculin stained focal contacts mainly at the cell periphery for both titanium ($P = 0.004$) and PTFE ($P < 0.001$) (Mann–Whitney test).

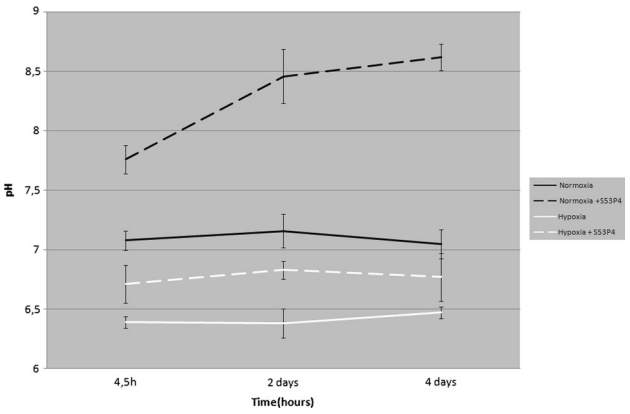


Fig. 1 The pH of the culture medium (McCoy's 5A) at 4.5 h, 2 and 4 days in normoxia and hypoxia, with or without S53P4 granules. The *error bars* represent the standard deviation

3.4 Arrangement of the actin cytoskeleton

The ability of SaOS-2 cells to organize their actin cytoskeleton upon culture was investigated (Fig. 3). At 4.5 h in normoxia two patterns of organization of actin cytoskeleton were observed: most typically in cells cultured on PTFE, PDMS and S53P4 still had an immature

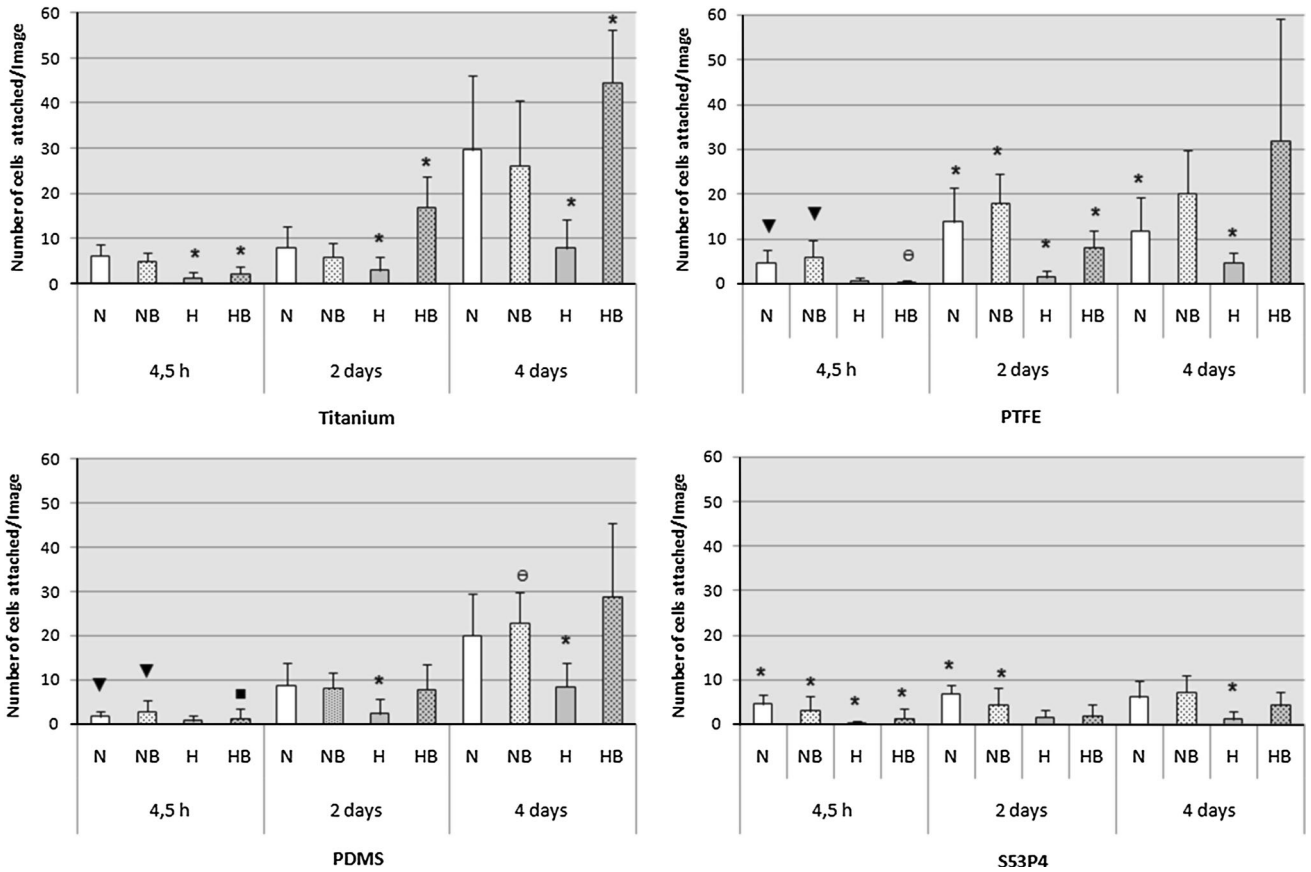


Fig. 2 The mean number of cells attached to four different biomaterials in normoxia and hypoxia, with and without S53P4 granules, at 4.5 h, 2 and 4 days. *N* normoxic conditions without S53P4 granules, *NB* normoxic conditions with S53P4 granules, *H* hypoxic conditions without S53P4 granules, *HB* hypoxic conditions with S53P4 granules. The *error bars* represent the standard deviation. *Asterisk* statistically significant difference. *Theta* statistically significant difference compared to normoxic conditions without S53P4 granules. *Filled down pointing triangle* statistically significant difference compared to hypoxic conditions without S53P4 granules. *Filled square* statistically significant difference compared to normoxic conditions with S53P4 granules

Table 1 *P* values for comparing the cell attachment using Mann–Whitney test

	Conditions	Titanium	PTFE	PDMS	S53P4
4, 5 h	N vs. NB	0.107	0.201	0.124	0.014
	H vs. HB	0.001	0.076	0.406	0.005
	N vs. H	<0.001	<0.001	0.008	<0.001
2 days	N vs. NB	0.066	0.037	0.843	0.07
	H vs. HB	<0.001	<0.001	<0.001	0.262
	N vs. H	<0.001	<0.001	<0.001	<0.001
4 days	N vs. NB	0.459	≤0.001	0.049	0.271
	H vs. HB	<0.001	<0.001	<0.001	<0.001
	N vs. H	<0.001	<0.001	<0.001	<0.001

N Normoxic conditions without S53P4 granules, *NB* Normoxic conditions with S53P4 granules, *H* Hypoxic conditions without S53P4 granules, *HB* Hypoxic conditions with S53P4 granules

round shape without any apparent organization of the actin cytoskeleton or spreading, whereas the cells cultured on titanium had assumed a polygonal shape and actin cytoskeleton was seen in the periphery of the cells, but without much evidence of active spreading yet. Organized actin fiber bundles were not seen at this time point yet.

At 2 days, SaOS-2 cells showed a polygonal morphology. The parallel orientation of actin filaments was most evident on titanium surface, less evident on PTFE surface and least evident on PDMS surface. This ranking order was less evident in hypoxic conditions without S53P4 granules. In some cases actin filaments formed long and thin filopodia or microspikes extending from the surface of the cells further away on titanium surface. Frequent cortical filament bundles were seen at the cell periphery.

At 4 days the situation on titanium surfaces did not change compared to day 2. However, on PTFE and PDMS surfaces more parallel orientation and polygonal morphology of actin filament and well defined stress fiber bundles could now be seen. In hypoxic conditions in the presence of S53P4 this situation was particularly evident on PTFE and PDMS surfaces.

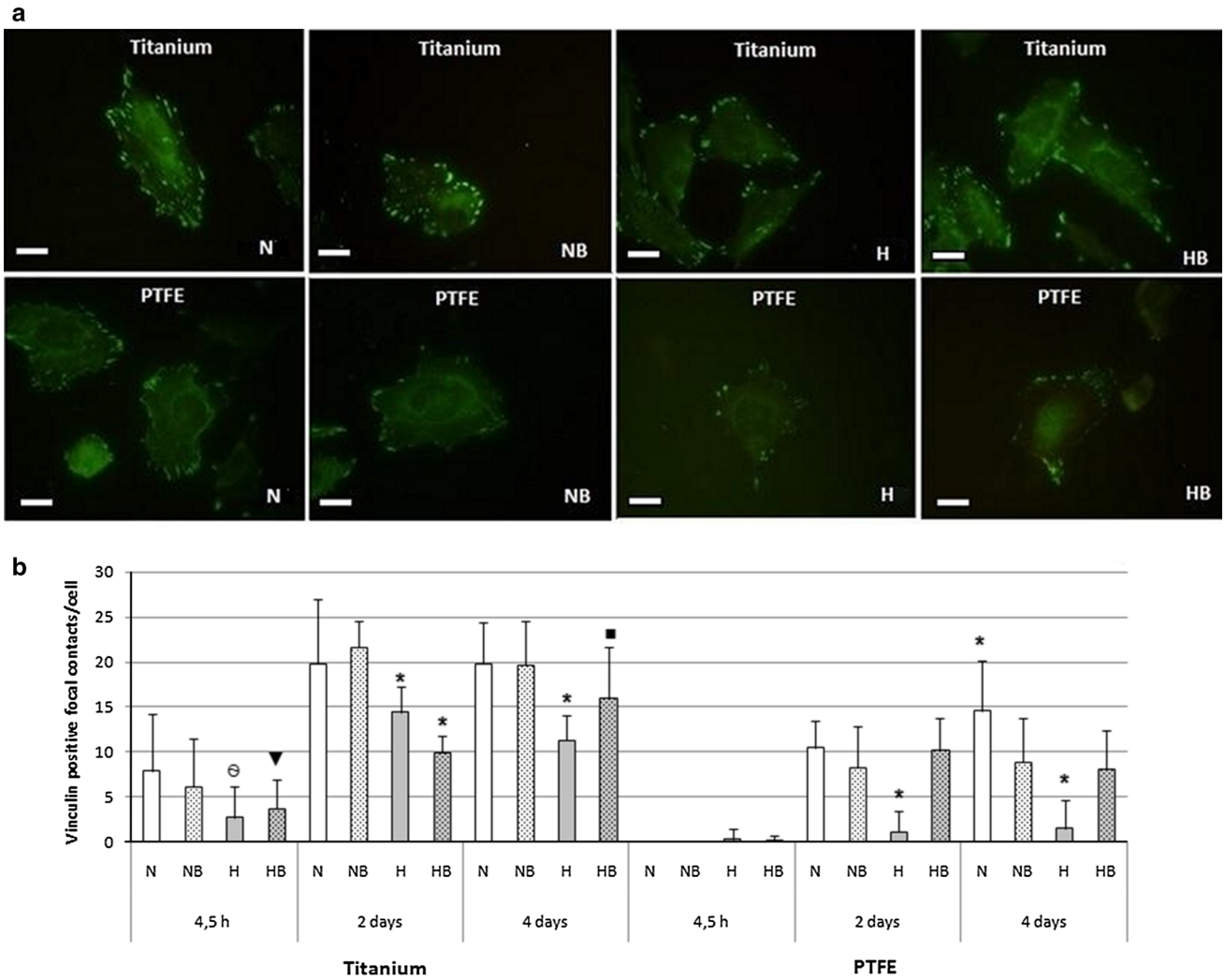


Fig. 3 **a** Arrangement of vinculin stained focal contacts in SaOS-2cells adherent to titanium or PTFE in different conditions at day 4 (×400 magnification). The samples were stained with Alexafluor 488-conjugated secondary antibody (Molecular Probes, Eugene, OR, USA). The *white bar* represents 20 μm length. **b** The mean percentage of vinculin stained focal contacts per each SaOS-2 cell adhered to titanium or PTFE surface. *N* normoxic conditions without S53P4 granules, *NB* normoxic conditions with S53P4 granules, *H* hypoxic conditions without S53P4 granules, *HB* hypoxic conditions with S53P4 granules. The *error bars* represent the standard deviation. *Asterisk* statistically significant difference. *Theta* statistically significant difference compared to both normoxic conditions. *Filled down pointing triangle* statistically significant difference compared to normoxic conditions without S53P4 granules. *Filled square* statistically significant difference compared to normoxic conditions with S53P4 granules

3.5 Cellular spreading

Figure 5a shows images of red phalloidin stained actin cytoskeleton and nuclei stained with blue DAPI counter stain for each condition on titanium surface at 4 days. For most conditions and biomaterials, the hypoxic conditions or the presence of S53P4 granules were not associated with statistically significant differences in cellular spreading with some exceptions (Fig. 5b).

At 4.5 h, the presence of S53P4 granules produced an increase in the cell spreading in hypoxia on titanium ($P = 0.013$) and a decrease in the cell spreading in normoxia on PTFE and S53P4 plates ($P = 0.032$ and 0.021, respectively). Hypoxic conditions did not produce any change in cell spreading.

At 2 days hypoxic conditions produced an increase in the cell spreading on PTFE ($P = 0.042$) and S53P4 plates ($P \leq 0.001$). The presence of S53P4 granules in normoxic conditions produced an increase in cell spreading on S53P4 plates ($P = 0.002$) and in hypoxic conditions a decrease in the cell spreading on titanium ($P = 0.030$) and an increase in the cell spreading on PDMS ($P = 0.019$).

At 4 days the hypoxic conditions produced a decrease of the cell spreading on PTFE ($P = 0.042$) and an increase on S53P4 plates ($P = 0.008$). At 4 days the presence of S53P4 granules produced a decrease in the cell spreading

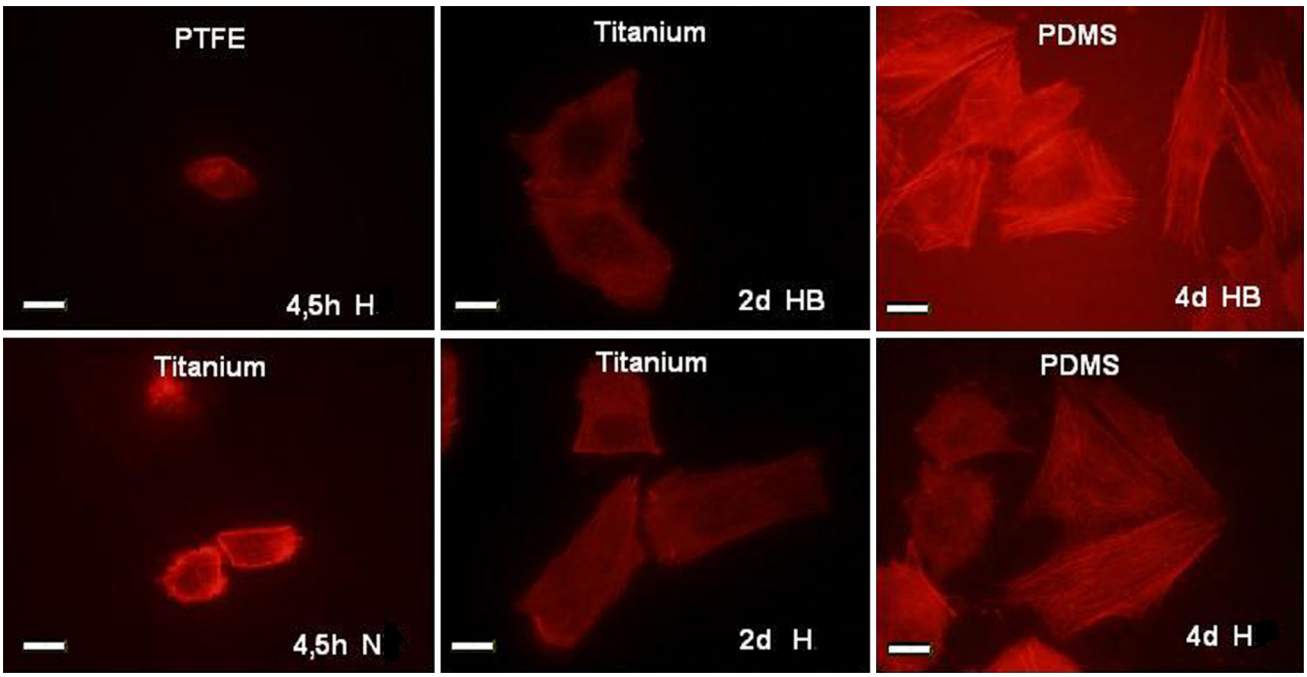


Fig. 4 Arrangement of actin cytoskeleton of SaOS-2 cells cultured on polytetrafluoroethylene (PTFE), titanium and polydimethylsiloxande (PDMS) (×400 magnification). *N* Normoxic conditions without S53P4 granules, *H* Hypoxic conditions without S53P4 granules, *HB* Hypoxic conditions with S53P4 granules. The samples were stained with Alexafluor 568-conjugated phalloidin (Invitrogen, Carlsbad, CA, USA). The *white bar* represents 20 μm length

on PTFE in both normoxic and hypoxic conditions ($P \leq 0.001$ and $P = 0.002$, respectively) and on S53P4 plates in normoxic conditions ($P = 0.008$). The *P*-values are shown in Table 2.

4 Discussion

Physical and chemical surface properties of the implant surface as well as the properties of the surrounding fluid medium are of prime importance in establishing the interaction of cells and tissues with artificial materials [27, 28]. Cells adhere to substrates by means of organized protein clusters, called focal adhesions, which anchor the cytoskeleton to the extracellular matrix.

In the present work the effects of S53P4 granules and low oxygen levels were evaluated on human osteoblastic cell–biomaterial surface interaction. We chose the hypoxic conditions that mimic the situation of oxygen level in bone and bone cavities as the implant materials are clinically mainly used in such tissues. Although normoxia is often used to refer to the normal levels of oxygen in atmosphere or in a cell incubator, such high oxygen levels are actually hyperbaric to most bone cells, including osteoblasts. This may affect the cellular metabolism and behavior and cause various artefacts, but has still been the norm in cell culture experiments. When such cells are cultured under so called hypoxic conditions e.g. in 6 % O_2 the conditions actually mimic more closely those prevailing in healthy and pathological bone. For clarity normoxia is in this report used to refer to 20.9 % cell incubator oxygen and hypoxia to 6 % hypoxia chamber oxygen although they might be renamed as hyperbaric oxygen and normoxia respectively as the normal atmospheric oxygen concentration is quite unusual in human body. Changes in cell adhesion in response to different substrates and conditions are linked intimately with modifications of cellular attachment organs, rearrangement of the actin cytoskeleton and cellular spreading. These cellular features were therefore used in the present work to probe for the eventual effects of oxygen content and S53P4 granules on osteoblast-like cells.

A switch in protein conformation can be triggered by a change in temperature, pH or ion concentration [29]. The presence of S53P4 granules in normoxia has been described to produce significant alkalinization of cell culture media and also to exert considerable osmotic effects. These effects are related with the antimicrobial properties associated to S53P4 [14, 23, 30, 31].

Also in this study S53P4 granules alkalinized the McCoy medium. After 4 days in normoxia the pH was 8.62 due the S53P4 granules and in medium without the granules 7.05. In hypoxia the pH was much lower, 6.77 in medium with S53P4 granules and 6.47 in medium without

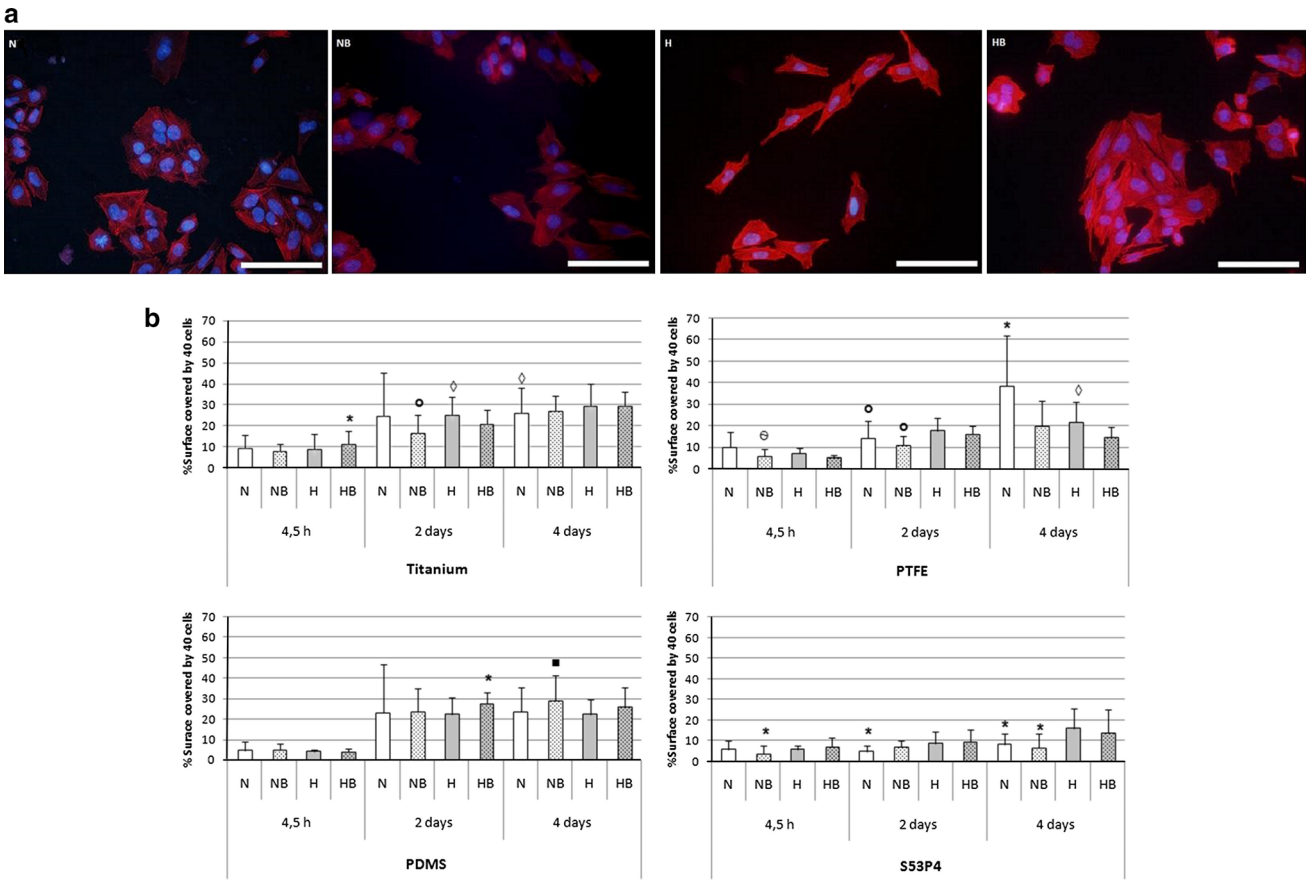


Fig. 5 **a** Cell spreading and arrangement of actin cytoskeleton in cells cultured on titanium (original magnification ×200). The samples were staining with Alexafluor 568-conjugated phalloidin for actin (*red*) and with DAPI for nuclei (*blue*) at 4 days on titanium in the different conditions. The *scale bar* is 100 μm. *N* normoxic conditions without S53P4 granules, *NB* normoxic conditions with S53P4 granules, *H* hypoxic conditions without S53P4 granules, *HB* hypoxic conditions with S53P4 granules. **b** The mean percentage of surface covered by 40 cells measured per image. The *error bars* represent the standard deviation. *Asterisk* Statistically significant difference. *Open circle* statistically significant difference compared to all the hypoxic conditions. *Open diamond* statistically significant difference compared to hypoxic conditions with S53P4 granules. *Theta* statistically significant difference compared to normoxic conditions without S53P4 granules. *Filled square* statistically significant difference compared to hypoxic conditions without S53P4 granules (Color figure online)

Table 2 *P*-values for comparison of the percentage of surface covered by 40 cells using Mann–Whitney test

	Conditions	Titanium	PTFE	PDMS	S53P4
4, 5 h	N vs. NB	0.584	0.032	0.052	0.021
	H vs. HB	0.013	0.074	0.277	0.571
	N vs. H	0.303	0.596	0.169	0.508
2 days	N vs. NB	0.451	0.283	0.139	0.002
	H vs. HB	0.030	0.491	0.019	0.400
	N vs. H	0.068	0.042	0.149	≤0.001
4 days	N vs. NB	0.169	<0.001	0.102	0.008
	H vs. HB	0.745	0.002	0.160	0.248
	N vs. H	0.139	<0.001	0.725	0.008

N Normoxic conditions without S53P4 granules, *NB* Normoxic conditions with S53P4 granules, *H* Hypoxic conditions without S53P4 granules, *HB* Hypoxic conditions with S53P4 granules

the granules. Although these differences look numerically minor, they are not because the pH scale is logarithmic. Hypoxic conditions produced a significant decrease in pH of the solution. It is possible that the high CO_2 levels of the hypoxia chamber (7 %) contribute to the pH change via the formation of weak carbonic acid thus decreasing the pH value.

Hypoxia caused a decrease in the number of attached cells and a decrease of the vinculin stained focal contacts, probably due to the acidification of the pH and an oxygen-level regulated shift in the cellular metabolism such as production of lactate from glutamine from glutaminolysis [32]. However, the cell spreading did not show a statistically significant decrease except on the anti-soiling PTFE surface at an early time point. It might be that the decrease in the number of vinculin-containing focal adhesion

contacts associated with hypoxia was not critical for those cells which were still able to adhere. Further, the metabolic switch of the cells upon a change from normoxic to hypoxic culture conditions may be enough to compensate the potential effects on initial cellular attachment and spreading although the number of focal adhesions was decreased.

Acidification of the medium under hypoxic conditions was to some extent neutralized by the presence of alkalinizing S53P4 bioactive glass granules. An increase in the number of attached cells was found compared to hypoxia without S53P4, but in contrast to increased cell numbers, there were no statistically significant differences in the cell spreading for most of the materials and time points suggesting that the effects of pH and ions on cellular adherence at this pH range between approximately 6.3 and 6.8 is rather small.

In normoxia the number of vinculin stained focal adhesion contacts increased on titanium from 4.5 h to 2 days but not further to day 4, probably because the attached cells were then already fully equipped with focal adhesions which may undergo subcellular recycling from the advancing edge to the trailing edge. In contrast, on PTFE focal adhesion contacts could not be seen at 4.5 h, but increased to day 2 and further to day 4. This suggests that titanium is more cell friendly than PTFE probably because the anti-soiling properties of PTFE slow down adsorption of proteins from cell culture medium on PTFE surface and impair adhesion of cell-made extracellular matrix.

There was a direct relation between the number of vinculin positive focal contacts and the rearrangement of actin stress microfibers. In hypoxic conditions less actin filaments were seen at all time points, suggesting that hypoxia impairs the formation of this dynamic cellular fiber network producing contractile force. The presence of S53P4 granules improved organization of the actin cytoskeleton in hypoxia suggesting that also the oxygen content and pH of the medium participates in the regulation of the actin cytoskeleton. In contrast to hypoxic conditions, in normoxic conditions bioactive glass impaired the formation of actin cytoskeleton and cell spreading at 4.5 h, but at later time points had no effect.

Based on the current findings S53P4 bioactive glass granules do not have adverse effects on the cell adhesion in close to normal atmospheric 20 % oxygen level regardless of the pH rise they cause. Furthermore, in hypoxic conditions in 6 % oxygen, S53P4 bioactive glass granules seem to partially neutralize the decreased pH which in part explains its positive effects on cellular adhesion.

Comparison of the arrangement of actin filaments and the vinculin stained focal contacts at 4.5 h suggests that titanium has the most favorable surface properties which could be observed already during the first hours. At later time points, the differences compared to other biomaterials were less pronounced suggesting that once the process has been initiated, cells growing on less favorable substrates catch up.

5 Conclusions

In normoxic conditions S53P4 bioactive glass granules cause alkalinization of the cell culture medium, but cellular adhesion and cytoskeletal organization were usually not affected by their presence. In hypoxic conditions pH did not similarly increase and focal adhesion contacts, rearrangement of the actin cytoskeleton and cellular spreading and adhesion were impaired. In the presence of S53P4 bioactive glass granules these changes were to a large extent reversed. This suggests that in addition to its alkalinization and osmotic antimicrobial effects, S53P4 bioactive glass granules contribute to host defense by protecting biomaterial surface from colonization by helping host cells in the race for the surface. The presence of S53P4 granules may promote osteointegration of implants, especially in hypoxia, such as in the middle ear gaseous condition. It is concluded that S53P4 bioactive glass granules have a potential as a good filling material of infected cavities.

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