Biomaterials 44 (2015) 36–44

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

A dual function of copper in designing regenerative implants

Ines Burghardt [a], Frank Lüthen [a], Cornelia Prinz [b], Bernd Kreikemeyer [c], Carmen Zietz [d], Hans-Georg Neumann [b], Joachim Rychly [a, *]

[a] Laboratory of Cell Biology, Rostock University Medical Center, Schillingallee 69, 18057 Rostock, Germany
[b] DOT GmbH, 18059 Rostock, Germany
[c] Institute of Medical Microbiology, Virology and Hygiene, Rostock University Medical Center, 18057 Rostock, Germany
[d] Department of Orthopaedics, Biomechanics and Implant Technology Research Laboratory, Rostock University Medical Center, 18057 Rostock, Germany

ARTICLE INFO

Article history:
Received 28 August 2014
Accepted 20 December 2014
Available online 10 January 2015

Keywords:
Copper
Bacteria
Antimicrobial
Mesenchymal stem cell
Titanium

ABSTRACT

The supply of titanium implants which are widely used in orthopaedics with both regenerative and antimicrobial properties will achieve a great progress in bone regeneration. We asked, whether by appropriate concentrations of copper ions it will be possible both to inhibit growth of bacteria and stimulate biological responses in mesenchymal stem cells (MSC). Using titanium material which released galvanically deposited copper at concentrations from 0.3 to 1.75 mM, growth of planktonic *Staphylococcus aureus* was blocked and more importantly adherent bacteria were cleared from the material surface within 24 h. To test biological responses of human bone marrow derived MSC due to copper ions, we found that copper stimulated the proliferation of MSC in a narrow concentration range around 0.1 mM. Similar copper concentrations enhanced osteogenic differentiation of MSC when cells were cultured in osteogenic differentiation medium. We observed increased activity of alkaline phosphatase (ALP), higher expression of collagen I, osteoprotegerin, osteopontin and finally mineralization of the cells. We conclude that titanium implants that release copper ions can be effective against bacterial infections at higher concentrations of copper near the implant surface and can promote bone regeneration when its concentration becomes lower due to diffusion.

1. Introduction

Titanium represents a main material used for orthopaedic implants which make up the majority of medical devices implanted [1,2]. While such permanent implants, like artificial hip prostheses, are typically inert and replace an irreversibly damaged tissue, the current challenge in designing biomaterials is to modify physical and chemical characteristics to stimulate regenerative processes in the tissue [3–5]. To test a regenerative potential of an implant material, biological responses of mesenchymal stem cells, which are the progenitor cells of the anabolic osteoblasts in bone, are evaluated in vitro [6]. Recent research provides evidence that both physical and chemical modifications of material surfaces support the osteogenic differentiation of mesenchymal stem cells in vitro and promote osteogenesis *in vivo* [7–12]. Of the chemical modifications, grafting of molecules of the extracellular matrix to support cell adhesion or growth factors to be released from the surface are characteristic approaches to generate a bioactive material surface [13]. In addition, as we and others have shown, also more simple coatings using calcium phosphate, promote the osteogenic differentiation of mesenchymal stem cells [14–16]. While progress has been achieved in the development of implants which may be effective in tissue regeneration, in about 3% of the patients with joint endoprostheses a periprosthetic joint infection represents a devastating complication, which indicates a revision arthroplasty and includes substantial economic consequences [17]. In biomaterial-associated infections, bacteria interact with the surface of the material and after an initial reversible adhesion a firm layer of bacteria is formed together with the secreted glycoproteins and polysaccharides, known as biofilm [18–20]. Such biofilms enable bacteria to evade antibiotics and immune responses [21].

To combine the two challenges, i.e. designing a material surface with regenerative properties and which simultaneously prevents periprosthetic infections by a cost-effective approach we tested the use of copper ions. Copper ions like other heavy metal ions can be cytotoxic and a primary mechanism in killing bacteria includes the

* Corresponding author. Tel.: +49 381 494 5730; fax: +49 381 494 5739.
E-mail address: joachim.rychly@med.uni-rostock.de (J. Rychly).

http://dx.doi.org/10.1016/j.biomaterials.2014.12.022
0142-9612/

inactivation of central catabolic and biosynthetic pathways [22]. On the other hand, copper plays a role in physiological processes of the cell and defects in copper homeostasis are directly responsible for human diseases [23,24]. A number of copper binding intracellular proteins are known in which copper is required for the functional activities of these proteins [25]. Due to the broad biochemical requirements for copper in cellular processes, the significance of copper for proliferation and development is obvious. We provide evidence that implantation of a copper salt into a titanium implant material is suitable, both to kill bacteria and stimulate biological responses in mesenchymal stem cells like proliferation and osteogenic differentiation.

2. Materials and methods

2.1. Generation of material surfaces

Ti6Al4V plates were used to prepare copper containing surfaces. The technique for copper implantation is described elsewhere [26] (European patent EP 2 204 199 B1). Briefly, a porous oxide layer was created by plasma electrolytic oxidation. In a second step, copper ions were galvanically deposited onto the surface using a bath saturated with copper (II) acetate ($CuCO_2CH_3)_2$. The Cu/Ti oxide composite layer was then blasted using glass spheres resulting in a surface with an average copper load of 1 μg/mm^2 (termed "PeO-gCu"). Images of the distribution of copper on the surface are previously described [26]. Ti6Vl4V plates treated by oxidization and glass sphere blasting, but without coating with copper were used as controls (termed "PeO"). Both samples were sterilized by gamma irradiation using a dosage of 25 KGy.

2.2. Determination of copper concentration

Quantitative measurements of copper ions in aqueous solutions were performed using atomic absorption spectrophotometry in a ZEEnit 650 (Analytik Jena AG, Jena, Germany). To measure the Cu^{2+} release from the material surface, titanium plates were incubated in 1.3 ml of osteogenic differentiation medium at 37 °C in a humidified atmosphere containing 5% CO_2. After various time points, samples of 495 μl of the medium were taken, acidified with 65% nitric acid and analysed using a standard protocol.

2.3. Cultivation of Staphylococcus aureus

The *Staphylococcus aureus* strain ATCC 25923 (Pro-Lab Diagnostics) was used in this study. A freeze dried bacteria pellet was incubated in 1 ml phosphate buffered saline (PBS) in a shaking water bath at 37 °C for 30 min. Then 9 ml of LB-medium, Miller (Merck, Darmstadt, Germany) were added to the bacteria and incubated at 37 °C for 16 h. For the experiments, a bacterial suspension using osteogenic differentiation medium without antibiotics was diluted 1:100. After inoculation of the bacteria at time 0, colonies were counted at the indicated time points. In the experiment with the implant material, bacteria both in the supernatant and adherent to the material surface were analysed. Adherent bacteria were detached from the surface by sonication for 3 min. For counting colonies at several time points after inoculation, 100 μl of the bacterial suspension, as well as serial dilutions were plated on mannitol salt phenol-red agar for 48 h at 37 °C.

2.4. Determination of bacterial viability

To test the viability of adherent bacteria on the material surface, a Live/Dead® BacLight™ Bacterial Viability Kit (Molecular Probes, Eugene, OR) was used. Titanium plates were washed twice with 0.85% NaCl and then incubated in a 1:1 diluted staining solution for 15 min at room temperature in the dark. Fluorescence images were taken in a confocal laser scanning microscope (LSM 780, Zeiss, Oberkochen, Germany) to visualize green (viable) and red (dead) bacteria.

2.5. Culture of mesenchymal stem cells

Human bone marrow derived mesenchymal stem cells (MSC) isolated from the posterior iliac crest were purchased from Lonza (Walkersville, MD) and used for the experiments after the third to 6th passage. MSC were cultured in MSCGM™ Mesenchymal Stem Cell Growth Medium (MSCGM BulletKit, Lonza, Walkersville, MD) containing 10% foetal bovine serum, L-glutamine, gentamicin sulfate and amphotericin-B (termed "growth medium"). Cells were grown in a 5% CO_2 humidified atmosphere at 37 °C. For osteogenic differentiation of MSC, cells were cultured in hMSC Mesenchymal Stem Cell Osteogenic Differentiation Medium (hMSC Osteogenic BulletKit, Lonza, Walkersville, MD) containing foetal bovine serum, L-glutamine, dexamethasone, ascorbate, β-glycerophosphate, penicillin and streptomycin (termed "osteogenic differentiation medium"). In the experiments, cells were seeded at a density of 6×10^4 cells per cm^2. Medium was changed up two to three times a week.

In experiments to determine the influence of different copper concentrations on bacteria and MSC, copper sulfate ($CuSO_4$) at concentrations indicated in the results was added to the culture medium.

2.6. Scanning electron microscopy

Cell morphology was visualized by scanning electron microscopy. Cells were washed in PBS and then fixed with 2.5% glutaraldehyde in PBS for 1 h. After fixation, cells were dehydrated and dried in a critical point dryer (K 850, Emitech, Taunusstein, Germany). Samples were then sputtered with gold using a sputter coater (SCD 004, Bal-Tec, Balzers, Liechtenstein). Samples were examined in a scanning electron microscope (DSM 960A, Carl Zeiss, Germany) and images were edited by using the software DISS 4.26.19.

2.7. Cell counting

To determine cell proliferation, cells were counted after different time periods using CASY cell counting technology (CASY Model TT cell counter, Schärfe System Reutlingen, Germany). Measurements were performed in triplicate.

2.8. Flow cytometry

Flow cytometry was used to determine the percentage of cells in cell cycle phases, as well as apoptotic cells. Adherent cells were detached using trypsin/EDTA (0.05%/0.02%) and washed twice in PBS. For staining, cells were resuspended in propidium iodide solution and stored at 4 °C for 24 h. For the measurement of the propidium iodide fluorescence which correlates with the DNA content, a flow cytometer FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA) was used. For data acquisition and analysis, the softwares CellQuestPro® Vers. 4.0.1, ModFitLT® V3.0 and FlowJo version 7.6.5. were used.

2.9. Alkaline phosphatase activity assay

Cells were washed in PBS and fixed in 4% paraformaldehyde (PFA) for 5 min. After washing again, cells were incubated with 0.1% naphthol AS-MX phosphate and 0.1% fast red violet LB salt in a 2-amino-2-methyl-1,3-propanediol buffer (56 mM) for 10 min. Quantitative analysis of alkaline phosphatase (ALP) activity was performed by measuring the fluorescence intensity in a Tecan infinite M 200 fluorescence reader (excitation 543 nm/emission 620 nm) and normalized to the staining of cells using DAPI.

2.10. Western blot analyses

Western blot analyses were performed for proliferating cell nuclear antigen (PCNA) and collagen I. Briefly, adherent cells were lysed using the Bio-Plex™ cell lysis kit (Bio-Rad Laboratories, CA, Hercules, USA). For immunoblotting, 80 μg of total protein were separated by SDS-PAGE and then transferred onto PVDF membranes (Roche, Mannheim, Germany). The membranes were blocked and incubated overnight at 4° C with a rabbit monoclonal anti-collagen I antibody (Rockland, Gilbertsville, PA) or mouse monoclonal anti-PCNA antibody (Santa Cruz Biotechnology Inc., Dallas, Tx), and as control a mouse monoclonal anti-vinculin antibody (Sigma, St. Louis, MO). As secondary antibody a HRP-conjugated monoclonal anti-rabbit IgG or anti-mouse IgG (DakoDenmark A/S, Glostrup, Denmark) was used. Protein expression was detected by chemiluminescence using CDP Star reagent (Roche). The secondary antibody was detected by using the SuperSignal West Femto Maximum Sensitivity Substrate according to the manufacture's instructions (Thermo Scientific, Rockford, IL). The resulting chemiluminescent signals were recorded using a Molecular Imager® ChemiDoc™ XR system (Biorad, Hercules, CA). Data analysis was performed with Quantity One® 1-D software version 4.6.7 and Image Lab™ software. Immunoblots prepared from individual donors were repeated at least three times to ensure reproducibility.

2.11. Luminex assay

The Luminex technique (Bio-Plex-200 system, Bio-Rad, Herkules, CA) was used to quantitatively detect the expression of osteopontin and osteoprotegerin. In brief, cells were prepared using the Bio-Plex™ cell lysis kit and the protein content was measured using a Qubit® protein assay kit (Invitrogen, Karlsruhe, Germany). A 96 well plate was loaded with aliquots of protein. To quantify osteopontin and osteoprotegerin a Millipore's MILLIPLEX® MAP Human Bone Metabolism Panel 1B kit was used, first to incubate the cell lysate with anti-osteopontin and anti-osteoprotegerin coated beads overnight, followed by incubation with a secondary fluorescently labelled antibody. The samples were then measured in an array reader Bioplex-200 system (Bio-Rad).

2.12. Mineralization assay

After 28 days of osteogenic stimulation, cell monolayers were evaluated for calcium production by calcein staining. Cells were washed in PBS and incubated in a calcein staining solution for at least 4 h. To quantify calcein, the fluorescence was read in a Tecan i-control microplate reader (485 nm excitation, 530 nm emission). Staining of the nuclei by DAPI was used to normalize the fluorescence intensity. The