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Influence of manganese ions on cellular behavior of human osteoblasts *in vitro*

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Abstract

Divalent cations like Mn^{2+} are known to strongly influence the integrin affinity to ligands and – in consequence – cell adhesion to extracellular matrix proteins. Therefore, divalent cation supplementation of biomaterials could be a promising approach to improve the ingrowth and the integration of implants. We were interested, whether manganese ions affect cellular functions like spreading, proliferation as well as gene expression in human osteoblasts. MG-63 osteoblastic cells were cultured in DMEM with 10% FCS. $MnCl_2$ was added at a concentration range of 0.01–0.5 mM for 24 h and 48 h. Spreading (cell area in μm^2) of PKH26-stained cells (cell membrane dye) was analyzed using confocal microscopy. Cell proliferation was measured by flow cytometry. Quantification of the phosphorylation status of signaling proteins was estimated using the Bio-Plex 200 system. Gene expression of osteogenic markers at the mRNA and protein level was analyzed by quantitative real time RT-PCR and Western blot, respectively. The results demonstrated that at higher concentrations of Mn^{2+} cells revealed a spindle shaped morphology. Further analyses indicated a reduced spreading, proliferation as well as phosphorylation of signaling proteins due to the influence of Mn^{2+} in a concentration-dependent manner. Although expression of bone sialo protein (BSP) at the mRNA level increased both after 24 h and 48 h in the presence of manganese, no increased expression of BSP was detected at the protein level. The expression of alkaline phosphatase (ALP) and collagen 1 (Col 1) mRNA decreased at >0.1 mM $MnCl_2$. We speculate that the effect of manganese cations on cell functions is strongly concentration-dependent and the release of manganese when incorporated in a biomaterial surface has to be thoroughly adjusted.

Keywords: Manganese ions; Human osteoblasts; Proliferation; Gene expression

1. Introduction

The biologically important metal manganese is an essential key cofactor for metalloenzymes (oxidases and dehydrogenases), DNA polymerases and kinases (Culotta et al., 2005). Furthermore, this divalent cation is known to strongly influence the integrin avidity and the integrin affinity to ligands and – in consequence – cell adhesion to extracellular matrix proteins (Byzova et al., 2000; Mould et al., 1995; Zreiqat et al., 2002). In addition, the stimulating effect on the affinity maturation of αvβ1-integrins is accompanied by focal adhesion organization and actin stress fiber formation (Dormond et al., 2004) which is accompanied by enhanced cell migration (Byzova et al., 2000). Therefore, divalent cation supplementation of biomaterials could be a promising approach to improve the ingrowth and the integration of implants. We were interested, whether manganese ions affect cellular functions like spreading, proliferation as well as gene expression in human osteoblasts to get insights about the effectiveness as well as the concentration necessary for the immobilization of divalent cations in the process of biomaterial surface functionalization.

2. Materials and methods

2.1. Cell culture

Human MG-63 osteoblastic cells (osteosarcoma cell line, ATCC, LGC Promochem) were cultured in six-well chambers (Greiner) in DMEM supplemented with 10% fetal calf serum (FCS Gold, PAA) with 1% gentamicin (Ratiopharm) at 37 °C and in a 5% CO_2 atmosphere. In general, cells were seeded with a density of 3×10^5 cells/well. $MnCl_2$ were directly added to the cell suspension at a concentration range of 0.01–0.5 mM. The cultivation time of osteoblasts was 24 h and 48 h (Lüthen and Nebe, 2005). Cell morphology was investigated under the confocal microscope using brightfield-image modus (LSM 410, Carl Zeiss).

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2.2. Spreading

Human MG-63 osteoblasts were cultured for 24 h, trypsinated, washed in PBS and the cell membrane stained with the red fluorescent linker PKH26 (PKH26 General Cell Linker Kit, Sigma) for 5 min in suspension as already described (Lüthen et al., 2005). The cells were then seeded into the wells and cultured for 3 h, 16 h, 24 h, and 40 h. After fixation with 4% paraformaldehyde (PFA, Merck) the cells were embedded with a cover slip. Spreading (cell area in μm^2) of 40 cells/specimen was measured using the software 'area measurement' of the confocal microscope LSM 410 (Carl Zeiss).

2.3. Actin cytoskeleton

MG-63 cells were cultured in the presence of $MnCl_2$ (0.1 mM) for 24 h. Cells were fixed with 4% PFA (10 min, room temperature RT). After washing with PBS, cells were permeabilized with 0.1% TritonX-100 (10 min, RT) (Merck), incubated with phalloidine-TRITC (diluted 1:100, Sigma) for 30 min in the dark at RT, washed again, embedded and examined at the LSM 410 (exc. 488 nm, Carl Zeiss) using a 63× oil immersion objective 1.25 oil/0.17.

2.4. Proliferation

The cell monolayer was trypsinated after 24 h of cultivation with 0.05% trypsin/0.02% EDTA for 5 min. Cells in suspension were washed in PBS and fixed with 70% ethanol over night at −20 °C. After washing twice cells were treated with RNase (1 mg/ml, Sigma) at 37 °C for 20 min and incubated with propidium iodide (PI) (50 μg/ml, Sigma) for at least 3 h on ice. Up to 20,000 events per sample were acquired by the flow cytometer FACSCalibur (BD Biosciences). For the analysis of cell proliferation the cell cycle phases G0/G1, S and G2/M were calculated in percent using ModFIT LT 3.0 for Power Mac G4 (BD Biosciences). For statistical evaluation S- and G2/M-phase cells were defined as proliferative cells.

2.5. Phosphorylation of signaling proteins

Quantification of the phosphorylation status of signaling proteins was estimated using the Bio-Plex 200 system (Bio-Rad Laboratories GmbH). Cell lysates were simultaneously quantitatively analyzed by the Bio-Plex suspension array system, a flow-based 96-well fluorescent microplate assay reader. Five hundred μl/ml of each cell lysate were incubated with antibody-conjugated beads (Phospho-ERK1/2 Assay #171V22238, Phospho-Akt Assay #171V221075, Bio-Rad) in a microplate well to react with the specific phosphorylated proteins as described in the manual.

2.6. Real time RT-PCR

Gene expression of osteogenic markers at the mRNA level was analyzed by quantitative real time RT-PCR (ABI Prism® 7000 Sequence Detection System, Applied Biosystems). Cells were washed twice with PBS and total RNA was isolated using the NucleoSpin RNA II Kit (Macherey-Nagel) with DNase treatment.

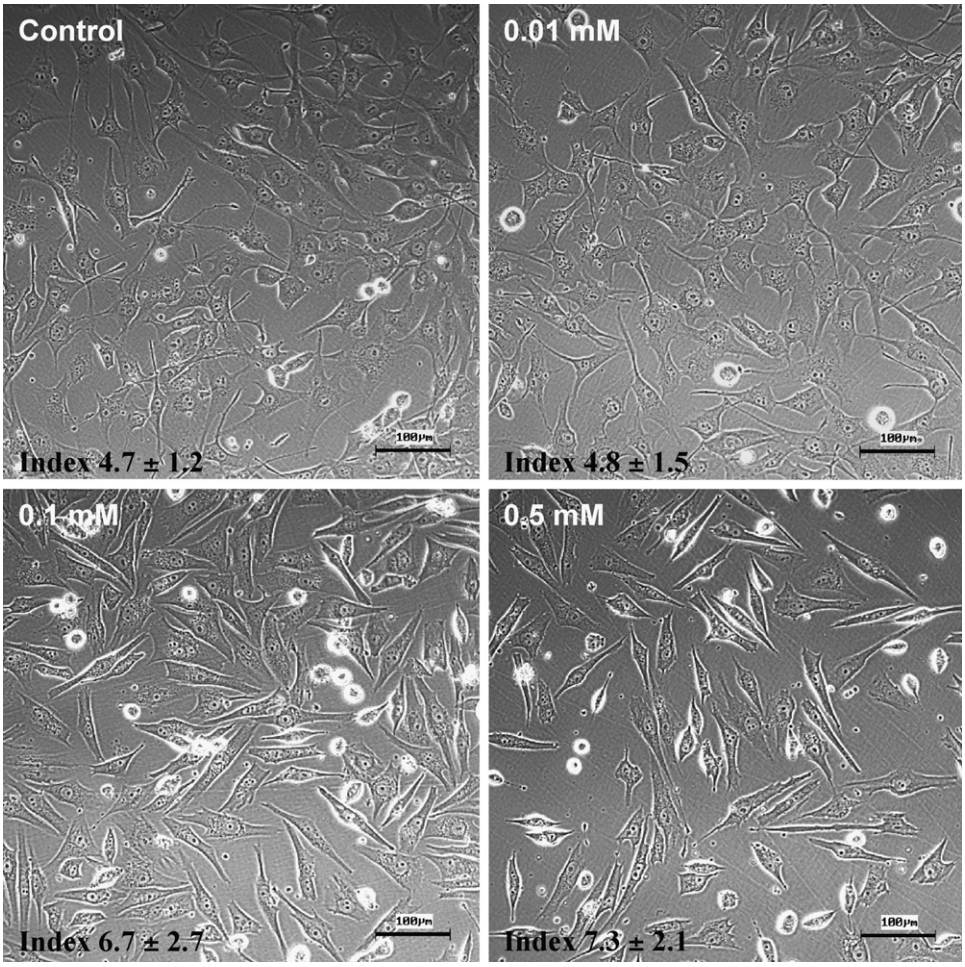

Fig. 1. Morphology of MG-63 osteoblasts. In the monolayer culture the cells switch to a more spindle like morphology when incubated with 0.1 mM and 0.5 mM Mn^{2+}. The index indicates the quotient of the cell length and the cell width: index of 1 = rounded cell, index of >1 = grade of longitudinal spreading (mean ± S.D., $n = 40$) (Bright-field image, magnification 10×, LSM 410, Carl Zeiss).