PP112-S

Influence of Melatonin on Murine Osteoclast Formation in Culture – Automated Detection and Quantification via a Novel Image Analysis System

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Introduction

The multinucleated, bone resorbing osteoclasts (OC) are key elements in bone remodelling and mineral homeostasis. Their formation is induced by the osteoblast-produced factors receptor activator of nuclear factor κb ligand (RANKL) and macrophage colony stimulating factor (MCSF) [1]. Excessive OC-formation and -activity is involved in the pathogenesis of various diseases like osteoporosis. Therefore, investigation of the influence of substances on osteoclastogenesis is an important step in the development of new treatments for such diseases.

Melatonin, the principal hormone of the pineal gland, was recently discovered to have osteoanabolic potential [2] and to decrease bone remodelling [3]. Multiple indirect influences on OC-formation via effects of melatonin on osteoblasts have been proposed, including inhibition of RANKL secretion [3, 4], or upregulation of osteoprotegerin [5]. These effects of melatonin on osteoblasts could be mediated via melatonin receptor 1 (MT1) that has been localized in these cells [3]. So far, it remains unknown whether melatonin can also directly influence OC-formation and whether OCs express MT1.

Aim

Analyse in murine OC cultures:

1) the direct effect of melatonin treatment on OC formation via a novel automated image analysis system (presented on poster PP082-S)
2) and the expression of melatonin receptor 1 (MT1) in OC and their precursor cells.

Osteoclast quantification

We have developed a new automated quantification system for murine OC in culture, which uses images of immunofluorescent stained cell cultures (Figure 1A). These images are automatically analysed to identify all cells in the image (Figure 1B) and the OC are discriminated from their precursor cells via a complex 3-step algorithm (Figure 1C). From these masks, a variety of paramaters (e.g. OC or precursor cell number and area, OC-/precursor-associated MT1 protein expression), can then be calculated.

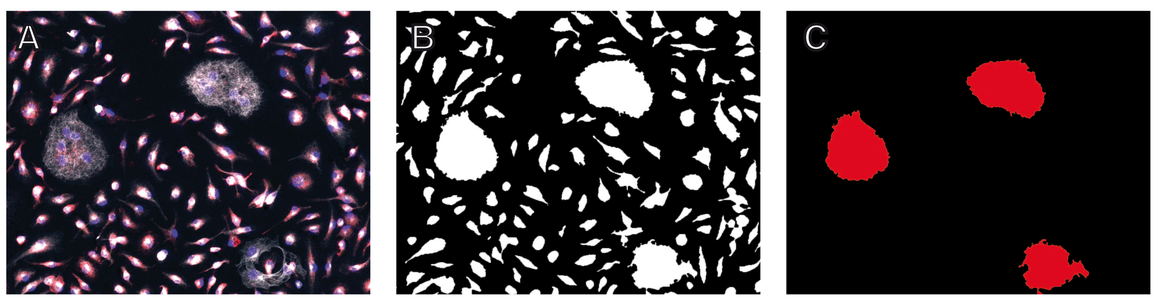

Figure 1: Automated image analysis of OC in culture. **A**: Immunofluorescence image (overlay) of OC and precursor cells. Red = F4/80 macrophage marker. White = alpha-tubulin and calcitonin-receptor. Blue = nuclei. **B**: Detection of all cells by the automated system. **C**: Identification of the OC among those cells.

Materials and Methods

OC formation is stimulated after isolation of cells from murine bone marrow by the addition of 25 ng/ml RANKL and 15 ng/ml MCSF to the medium. Culture is maintained for seven days with the medium changed every other day.

A quadruple immunofluorescence staining is performed to segment all cells, identify the OC and explore MT1 expression levels. See Table 1 for details.

Table 1: Immunofluorescence staining protocol. The anti calcitonin receptor antibody and the anti alpha-tubulin antibody are used to make all cells visible and therefore available for detection. Lack of precursor-cell specific F4/80 macrophage marker is one of the parameters for OC identification. An anti MT1 antibody is used to quantifiy MT1 expression in OC and precursors. DAPI is used to stain nuclei, as the amount of nuclei is another parameter for OC identification.

Primary antibody	Secondary antibody	Application
Anti calcitonin receptor	Alexa Fluor 750	Cell segmentation
Anti α-tubulin	Alexa Fluor 750	
Anti F4/80 macrophage marker	Alexa Fluor 568	OC Identification
Anti MT1	Alexa Fluor 647	MT1 expression
DAPI (nucleic acid dye)		OC identification

Images (Fields of view; FOV) of the stained cells are acquired using an automated Axio Imager epifluorescence microscope (Zeiss) equipped with TissueFAXS hard- and software (TissueGnostics GmbH) using a 40x (oil) objective. The acquired FOVs are aligned and stitched to form one big image (for each fluorescence channel) which is then fed into our novel software where all single cells are automatically separated and identified as OC or precursors. The image can then be quantified by a number of parameters; for this study, the amount of OC per 1 million (Mio) pixels (about 0.065 mm²) and the percentage of total OC area per analysed area were chosen. The expression of MT1 protein was quantified as mean fluorescence intensity per OC- and precursor area. See Figure 2 for an overview of the workflow.

RTq-PCR for MT1 and MT2 mRNA expression was performed using commercially available TaqMan probes. MT1 expression was quantified as ΔCT in relation to beta-actin levels.

See poster **PP082-S**, presented by Heindl et al., for details on the image analysis procedure.

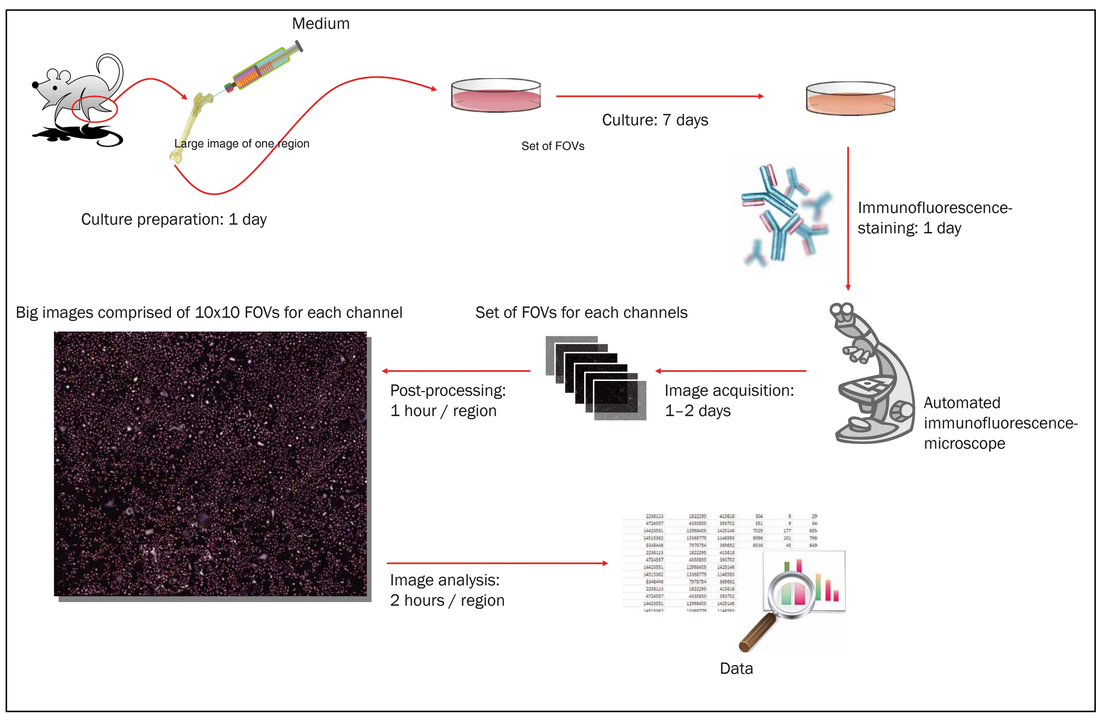

Figure 2: Abridged workflow of our method: about 10 days are necessary from bone marrow isolation to the ready data.

Results

Osteoclastogenic potential was tested in murine bone marrow cultures treated with 10 and 100 nM of melatonin, or vehicle only. Visual evaluation already indicated that OC growth was reduced with melatonin (Figure 3).

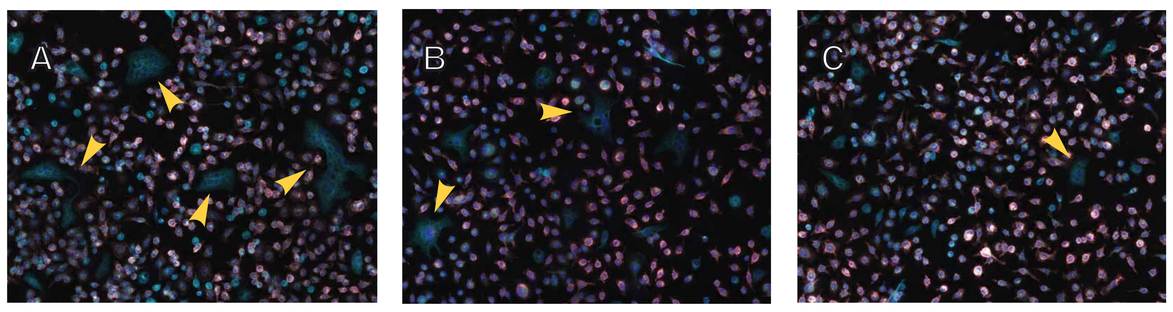

Figure 3: Melatonin influence on OC-formation. Representative regions are shown. OC are indicated with arrows and appear green due to their lack of F4/80 macrophage marker. Red = F4/80 macrophage marker. Green= alpha-tubulin and calcitonin-receptor. Blue = nuclei. **A**: Control (RANKL and MCSF only). **B**: 100 nM melatonin. **C**: 10 nM melatonin.

For first results, three regions, of 10x10–13x13 FOVs per concentration were analyzed using our novel image analysis software. Confirming the visual impression, OC-number per analyzed area (calculated as 1 Mio pixels, corresponding roughly to 0.065 mm²) as well as % of total OC area per total analyzed area were reduced compared to control (Figure 4). This shows that melatonin has indeed, possibly additional to the indirect effects mentioned in the introduction, a direct inhibiting influence on osteoclastogenesis, as RANKL, as well as MCSF were supplied excessively in the growth medium.

However, cell number as well as area was not as much reduced with 100 nM as with 10 nM of melatonin. This indicates that in addition to an inhibiting – possibly receptor mediated – effect on OC-growth, there could be a second effect that stimulates OC growth in higher melatonin concentrations. We speculate that this might be attributed to melatonin's antioxidative capacity.

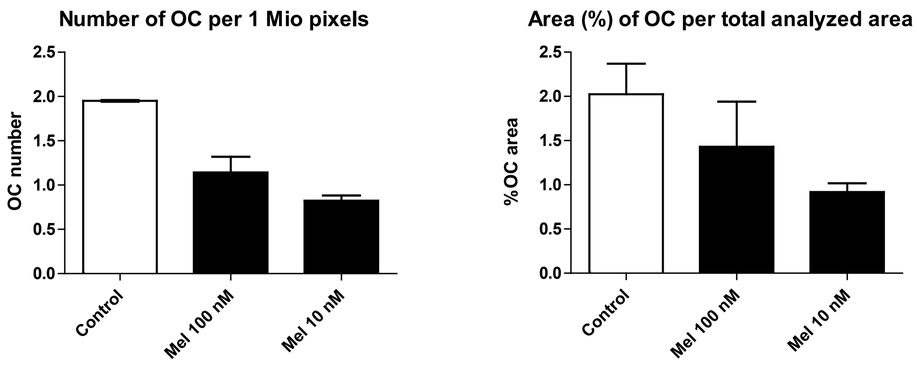

Figure 4: OC-number and -area in melatonin-treated and control cultures. **Left**: Number of OC per 1 Mio pixels (roughly 0.065 mm²) of analyzed image area. **Right**: Percentage of total OC-area per total analyzed image area. Mean + SEM.

Investigation of MT1 protein expression in melatonin treated OC and precursors by automatic quantification of their average immunofluorescence intensity showed that MT1 is expressed in OC as well as precursor cells, but that OC express significantly lower amounts of MT1 than precursor cells (Figure 5, left). RT-qPCR analysis confirmed expression of MT1 mRNA in all groups of cells (Figure 5, right). No MT2 mRNA expression could be detected.

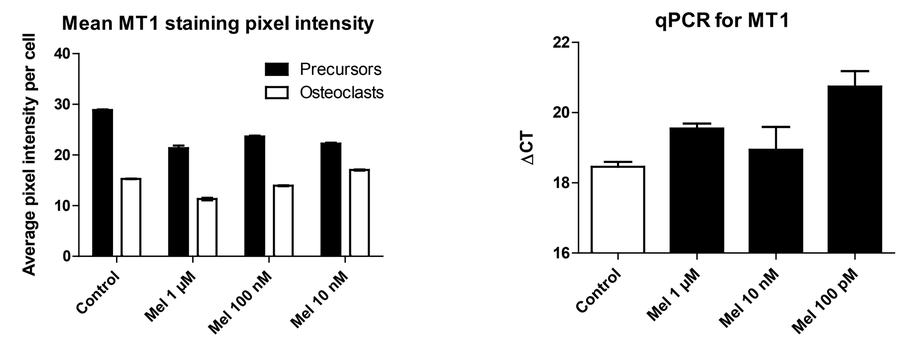

Figure 5: MT1 expression on OC-cultures. **Left**: Mean pixel intensity of MT1 immunofluorescence staining. **Right**: RT-qPCR analysis of MT1 mRNA expression, shown as ΔCT in realtion to beta-actin. Mean + SEM.

Literature

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Summary

Melatonin has a direct inhibiting effect on OC formation in murine bone marrow cultures. This effect could be mediated via MT1, which was found to be expressed in OC and their precursor cells at mRNA and protein level.

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