A novel method for automated quantification of osteoclasts in culture – Advantages, workflow and application

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Introduction

Bone is subjected to intensive turnover which is necessary to maintain structural stability and mineral homeostasis. Remodeling of bone is a highly coordinated process consisting of bone resorption by osteoclasts (OC) and subsequent formation of new bone by osteoblasts [1].

Various hormones and cytokines influence osteoclast growth, and since osteoclast formation plays an important role in the genesis of diseases like osteoporosis, these influences are of great interest.

Osteoclast formation can be investigated in an *in vitro* model using murine bone marrow cultures supplemented with hormones and/or cytokines. In culture, mature multi-nucleated osteoclasts are identified by their number of nuclei (>3 nuclei) and the expression of tartrate resistant acid phosphatase (TRAP) [2,3]. Previous studies suggested that melatonin is able to increase bone mass in animal models and this may result from its inhibitory effects on osteoclastic activities [4].

Aim

Use of a novel immunofluorescence microscopy-based automated image analysis system to quantify the effect of melatonin treatment on osteoclast formation *in vitro*.

A new osteoclast quantification system

Until now, cultured osteoclasts are being mainly identified by the histochemical TRAP-staining and are then quantified by manual counting of TRAP+ cells. However, this is a tedious and unreliable task which will often produce highly variable results, depending on factors like vigilance. Also, the counts of two different persons may vary up to the extent of 20 % (test with two human experts, each counting the same regions). Finally, it is next to impossible to manually quantify the amount of precursor cells for calculation of a relative osteoclast/precursor ratio.

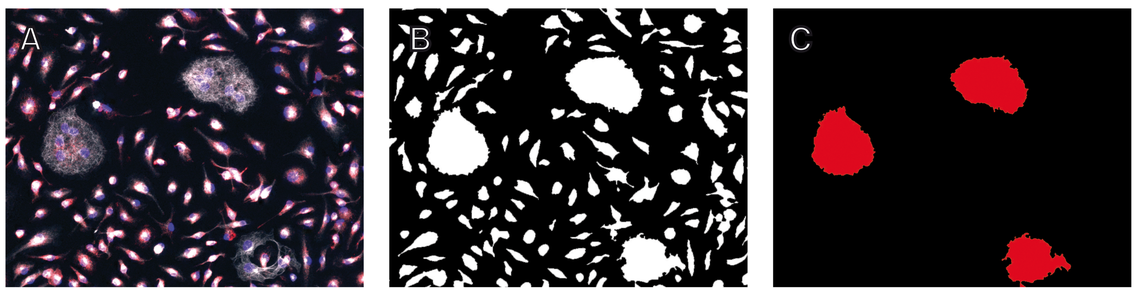

Figure 1: Automated image analysis of osteoclasts in culture. A: Immunofluorescence image (overlay) of OC and precursor cells. Red = F4/80 macrophage marker. White = alpha-tubulin and calcitonin-receptor. Blue = nuclei. B: Detection of all cells by the automated system. C: Identification of the OC among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

To overcome these problems, we are developing an automated quantification system for OC in culture. Starting from acquired immunofluorescence images of cell cultures (Figure 1A) and combining classical digital image processing with pattern recognition approaches, the system segments all cells in the image (Figure 1B). Afterwards, discrimination of the OC from all other cells (Figure 1C) is based on two criteria: 1) area of nuclei staining, 2) intensity of staining of a precursor-cell specific marker. See Workflow for details.

Key advantages of the new automated system

- Consistent, operator independent results
- 20 times faster than manual counting – unlimited sample size
- Many different quantification parameters, e.g. cell area, cell number, number of nuclei,...
- Quantification of associated proteins

Workflow

Biological preparation

Mouse (mus musculus) bone marrow is extracted with minimum essential medium and osteoclast formation is stimulated by the addition of 25 ng/ml RANKL and 15 ng/ml M-CSF. The cells are being bred in 24-well plates, each well containing a glass coverslip. Culture is maintained for eight days with the medium changed every other day. The mature cells are fixated and then stained via immunofluorescence with the antibodies applied directly in the culture plate.

To enable automated detection and separation of the cells, they have to be visible in their entirety in the fluorescence images. Therefore, all cells are stained using two antibodies: one directed against the calcitonin receptor (Acris), staining the border of cells, and another one directed against alpha-tubulin (SigmaAldrich), staining the cytoskeleton. Both antibodies are labeled using Alexa Fluor 647 (Invitrogen) secondary antibodies. The OC are discriminated from the precursor cells by staining the latter with an antibody directed against F4/80 macrophage marker (eBioscience.com) which is labeled with an AlexaFluor 568 (Invitrogen) secondary antibody. DAPI is used to stain the nuclei.

Images (fields of view, FOVs) of the stained cells are acquired using an automated Axio Imager epifluorescence microscope (Zeiss) equipped with TissueFAXS hard- and software (TissueGnostics GmbH) using a 20x objective. To ensure correct focus in all images, pictures of each FOV for each channel are automatically taken on seven different heights of the microscope stage (2 µm steps) and merged into one critically sharp image.

Image analysis

The acquired FOVs are aligned and stitched to form one big image (for each fluorescence channel) of the whole region that is to be analyzed. This image is then fed into our novel software which separates all single cells and identifies them automatically as OC or non-OC. The image can then be quantified by any number of parameters: number of OC / non-OC, area of OC / non-OC, number of OC-nuclei / non-OC nuclei, relative OC / non-OC ratio, etc. For this study, the percentage of OC number and area with respect to total cells was chosen as method of quantification. See Figure 2 for an overview of the workflow.

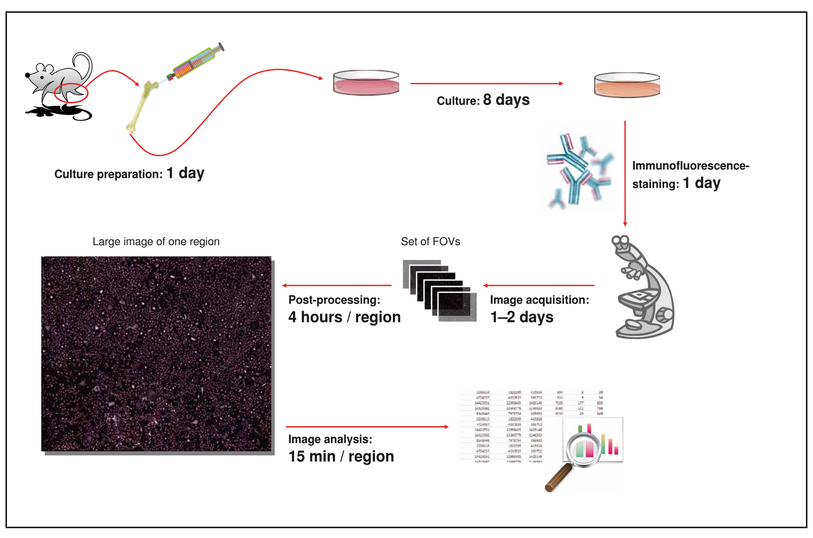

Figure 2: Abridged workflow of our method: about 2 weeks are necessary from bone marrow isolation to the ready data.

Results

OC formation under control conditions and in the presence of pharmacological (1 µM) and physiological (10–100 nM) concentrations of melatonin was tested. Visual evaluation indicated that OC growth was reduced with pharmacological, but even more so with physiological doses of melatonin. Representative FOVs of selected groups are shown in Figure 3.

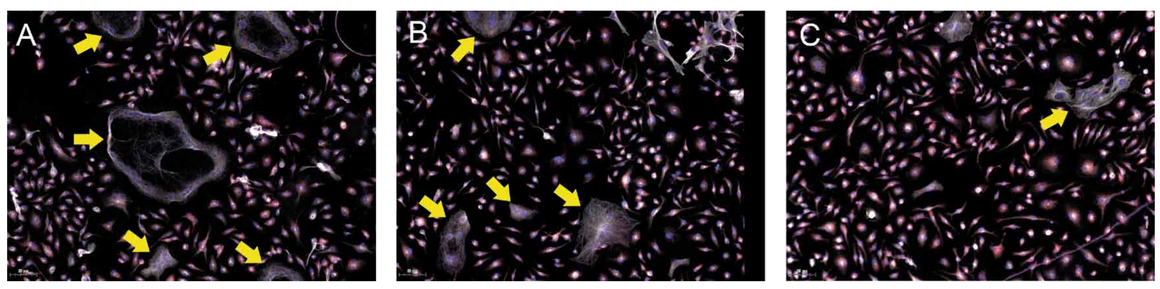

Figure 3: Influence of melatonin on OC formation. OC are indicated by yellow arrows. A: Control. B: 1 µM melatonin. C: 10 nM melatonin.

To quantify the observed differences in OC formation, we subjected the samples to our new automated quantification system. An example of a whole region, consisting of 10 x 10 FOVs and containing roughly 7000 cells can be seen in Figure 4A. The resulting segmentation of the cells and the identified osteoclasts are shown in Figure 4B.

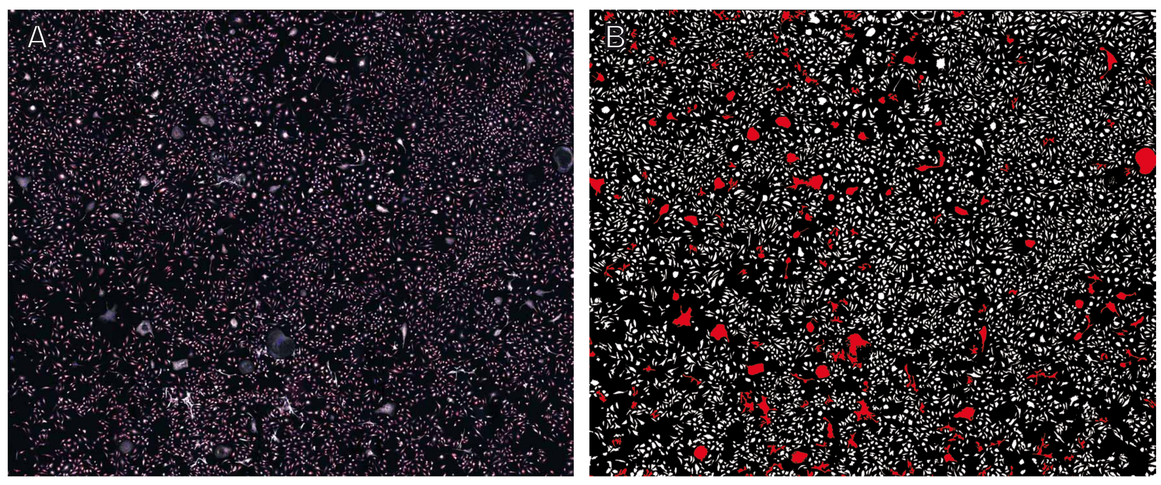

Figure 4: Image analysis of a whole sample-region consisting of 100 (10 x 10) FOVs. A: Immunofluorescence image. B: Result of cell segemntation and analysis: non-OC are marked in white, OC are marked in red.

Cell number as well as cell area of total cells, OC and non-OC were quantified and are presented in Table 1.

Table 1: Results of the automated analysis. # Cells = number of total cells, # OCs = number of OCs, # nOCs = number of non-OCs, % = percentage of OCs per number of total cells, A Cells = cumulated area of total cells, A OCs = cumulated area OCs, A nOCs = cumulated area of non-OCs, % OC of A = percentage of area of OCs per total area of cells

Sample	# Cells	# OCs	# nOCs	% OCs of #	A Cells	A OCs	A nOCs	% OCs of A
Control	5878	70	5808	1,19	6593279	832434	5760845	12,63
Mel 1 µM	7029	177	6852	2,52	14423551	1425146	12998405	9,88
Mel 100 nM	8066	101	7965	1,25	14515362	1146583	13368779	7,90
Mel 10 nM	6536	45	6491	0,69	8348446	369692	7978754	4,43

Evaluation of the cell numbers confirmed a significant reduction of OC formation at physiological (10–100 nM) melatonin levels compared to pharmacological levels (1 µM). In comparison with the untreated control sample, the number of OC was increased with 1µM melatonin, about equal with 100 nM melatonin and decreased with 10 nM melatonin (Figure 5A).

Assessement of the cell areas showed the same pattern of reduced OC formation with physiological melatonin levels compared to pharmacological levels. However, in contrast to the lower cell number, the cell area of the OC from the control was higher than the OC-area of all melatonin treated samples (Figure 5B).

This indicates that there are less, but bigger OC in the control group, and that evaluation of cell number only (e.g. by TRAP staining) might not always provide a correct estimation of the true OC state.

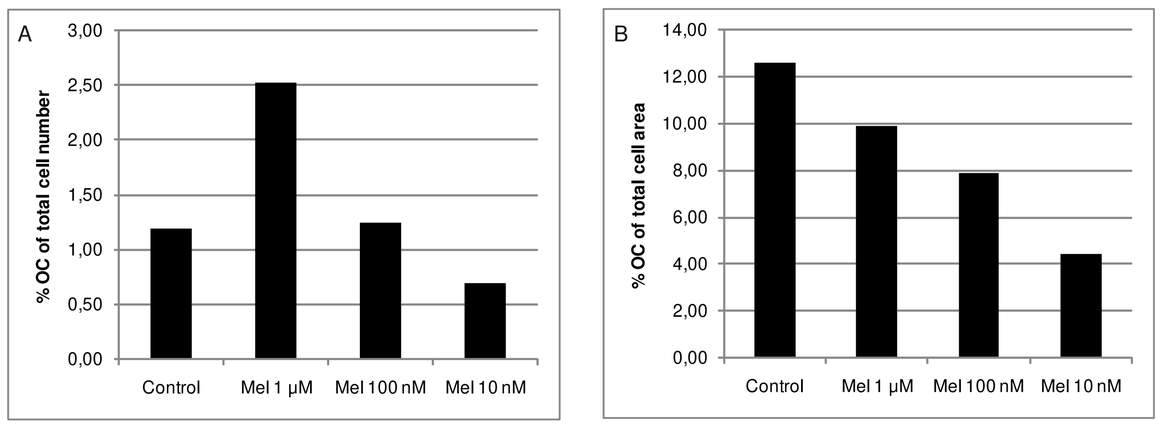

Figure 5: Inhibited OC formation by low levels of melatonin. A: Number of OC in percent of total cell number. B: Area of OC in percent of total cell area.

Summary

A new automated *in silico* method for quantification of cultured osteoclasts is in development, already yields promising results and opens new perspectives for evaluation of osteoclasts in culture. The system is currently applied on murine osteoclast cultures treated with the hormone melatonin. First preliminary data suggest that melatonin influences osteoclast formation.

References

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