Three-dimensional characterization of osteoclast bone-resorbing activity in the resorption lacunae

Niroshani Surangika Soysa¹, Neil Alles¹ ², Kazuhiro Aoki¹ and Keiichi Ohya¹

1) Section of Pharmacology, Department of Hard Tissue Engineering, Graduate School, Tokyo Medical and Dental University, Japan.
2) Global COE program, Tokyo Medical and Dental University, Japan.

Confocal laser microscopy is a well-recognized research tool in the fields of biological and material science which enables high-resolution images of samples with minimum requirements for specimen preparation. Here we introduce an innovative technique for the 3-D description and measurement of resorption pits using Super Depth Surface Profile Measurement Microscope based on the principle of confocal microscope. We show one example of culturing for 48 h with an established NF-κB inhibitor named NBD-peptide after plating mature osteoclasts on dentine slices with osteoblasts. The activity of osteoclasts is measured by determining the volume of resorbed portion of dentine by osteoclasts in vitro. The 3-D surface profile could be obtained by detecting the position at which the reflected laser intensity from the target becomes the maximum on z-axis. The volume and depth of resorption lacunae by stimulated osteoclasts is significantly increased compared to the un-stimulated group without changing of resorption area. The increase in volume and depth are dose-dependently inhibited by the NBD-peptide. Comparing to the classical method by measuring 2-D area of pits, analysis based on this technique could provide reliable quantitative assessment reflecting the osteoclast activity.

Key words: Confocal microscope, osteoclast, 3-D measurement

Introduction

Over recent years a variety of approaches have been developed to quantify the activity of isolated osteoclasts (OC). The simple demonstration that a resorptive event occurred does not necessarily give an idea about its activity. So far most of in vitro resorption studies to evaluate OC-resorbing activity are limited to counting pits and measuring the surface area. The measuring pit area reflects the spreading and attachment rather than resorptive function of OC. Area resorbed may be misleading because an increase in pitted area may occur with a decrease in pit depth and volume. Though the volume of bone resorption represents the work done by OC, no valid estimate of resorption can be made without reliable 3-dimensional (3-D) measurement and characterization¹². Many researchers are using scanning electron microscopy (SEM) to identify and quantitate the excavations and although SEM gives excellent images it requires expensive equipment and considerable sample preparation in which the extent to which stain is taken up by pits is variable and sometimes it is not easy to distinguish pits from background³.

Hence a new procedure for the 3-D description and measurement of resorption pits using a reflective laser confocal microscopy, Super Depth Surface Profile Measurement Microscope (SDSPMM) has been established. SDSPMM divides the field of view into approximately 786,000 points (H: 1024 x V: 768 points) for observation and measurement. The purpose of the current study is to clarify the feasibility of measurement of pits resorbed by OC cultured on
dentine using the SDSPMM and to reliably identify parameters to accurately predict the OC bone resorbing activity. For that we used an established NF-κB inhibitor of inflammatory bone resorption named NBD peptide.

Materials and Methods

Animals

Mice were obtained from the Japan Charles River Breeding Laboratories (Kanagawa, Japan) and were maintained in our animal care facilities. The experimental procedures were reviewed and approved by the Animal Care and Use Committee in the Tokyo Medical and Dental University (Tokyo, Japan).

Osteoblast Isolation

Calvariae of 1 day old mice were aseptically isolated, washed with calcium/magnesium-free phosphate buffered saline (PBS), and treated five times with 10 ml of digestion medium composed of 0.1% collagenase and 0.2% dispase in PBS at 37°C. The cells retrieved by the five digestions of 10 min each were pooled, and sub cultured in a flask with α-minimum essential medium (α-MEM) with 10% fetal bovine serum (FBS).

Bone Marrow Isolation

The femurs and tibias of ICR mice were aseptically removed and adherent soft tissues were dissected away. The bones were collected into 10 cm dishes, minced in culture media containing α-MEM and 10% FBS. Then media containing bone marrow cells were collected into 50 ml tube without the bone particles by tilting the dishes and centrifuged. The cell pellet was resuspended in culture media containing α-MEM, 10% FBS, penicillin and streptomycin after removing the supernatant.

Osteoclast isolation and purification

Bone marrow cells (1.5 × 10⁷ cells) were co-cultured with osteoblasts (1.5 × 10⁶ cells) on collagen gel-coated 10 cm dishes in the presence of 10⁻⁸ mol/l 1 α, 25(OH)₂ D₃ (VitD3) and 10⁻⁶ mol/l PGE₂ for 5 days (Fig.1). The half of the culture medium was changed after 3 days. Cultures were then treated with 0.3% collagenase in α-MEM and incubated at 37°C for 20 min to recover cells from the dishes. Cells were collected into 3ml of FBS and centrifuged for 5 min and resuspended in α-MEM+10% FBS.

Pit formation assay by OC

For resorption pit assay, aliquots of OC preparations in α-MEM+10% FBS (300μL) were put on dentine slices, which had been placed in 48-well plates. After pre-incubation for 1 h, half of the medium was replaced with or without VitD3 and cultured for 48 h. To clarify the inhibitory effect of NBD peptide on OC activity, OC were cultured in the presence of VitD3 with NBD peptide (10μM and 20μM). OPG was used as a positive control. At the end of the culture period, the dentine slices were removed from the culture wells, immersed in water containing 0.03 M NaOCl for 5 min, and sonicated for 10 sec to remove cells. Separate set of dentine slices were stained for tartrate resistance acid phosphatase (TRAP) to count the number of mature OC at the end of culture period.

Preparation of dentine slices

Dentine slices (6x6x0.4 mm) were cut from previously prepared rectangular rods on a low-speed saw and smoothened with sand paper. The slices were sonicated for 10 minutes in PBS and washed twice in two changes of PBS. The slices were sterilized with 70% ethanol and placed and left overnight under ultraviolet light.

Pit measurement by SDSPMM

OC resorption lacunae were measured using SDSPMM (Fig.2)(VK-8510K; Keyence Corp, Osaka, Japan) interfaced via a CCD camera to an image-analyzing computer as described previously. A separate computer was used to analyze the data using a software program called Win Roof (Mitani Corp, Fukui, Japan). In brief, resorption pits were identified using 50 objective. Total depth range was determined by through focusing and this could be performed once for the whole dentine slice. First, the laser performed the scanning in the horizontal and the vertical direction to generate a surface-scanning beam. When surface scanning was completed, the lens shifts in the Z axis direction by one step according to the predetermined pitch and moves onto the next surface scanning. After data gathering was completed it was possible to attain a color image in which focus was obtained at every height and a black and white image.
3-D characterization of resorption pits

Fig. 2: Super Depth Surface Profile Measurement Microscope (SDSPMM).

Fig. 3: (A) Appearance of pits under microscope. (B) Topographical map of the same dentine slice showing the excavation made by OC. Bars represent 50 μm. (C) In this image the 3-D data set in B is re-projected from an elevation angle of 30°. Color contours represent the depths of the resorption pits.
generated with reflected laser light quantity (Fig.3A). To identify the pit on a surface that is known to be reasonably flat but may be tilted, we may artificially level the detected surface by determining its height in three areas around the rim of the pit and applying an appropriate correction. Having the complete 3-D data set, the topographical map could be projected from any angle. Number of color combinations could be used to visualize the varying depths of the pit (Fig.3B and C).

Statistical analysis
All data are presented as means ± SD. The statistical significance of difference among groups was assessed using ANOVA. When the significant $F$ value was detected, Fisher’s PLSD post hoc test was performed for comparing assay groups. The difference was considered significant when $p < 0.05$.

Results
Fig.1 shows the schematic diagram of the co-culture system used in this study to obtain mature OC. At the end of the culture period the number of TRAP+ multinucleated cells after the 48 h culture was similar in all the groups (Fig.4A). The dentine slices were cleaned of cells and subjected to analysis by SDSPMM. First, the number of pits was counted. Pit number was similar in un-stimulated and stimulated controls (Fig.4B). NBD $20\mu M$ and OPG could decrease the pit number. The SDSPMM uses the area and volume data to measure the depths of the pits. In this study we observed significantly increased pit volume and average depth in stimulated OC with VitD3 for 48 h compared to the control group without stimulation (Fig.4D and E) where as the pit area is similar in both control and stimulated groups (Fig.4C). NBD peptide dose-dependently the pit area, volume and depths brought by decreased VitD3 stimulation (Fig.4C, D and E). Similar tendency was observed with OPG.

Discussion
To clearly identify the indices which show OC activity we used a reflective laser confocal microscope called
SDSPMM. Confocal microscopy provides accurate 3-D surface map which can be used for all possible 3-D measurements. Although resorption pits are 3-D in nature, 2-D analyses are commonly performed, due to the difficulty in accurately measuring the volume of the pits. Two dimensional analyses have a drawback when comparing the resorbability of a substrate, because even pits with the same area could have different volumes, due to differences in their depths.

The uncomplicated specimen preparation is a substantial attribute of this confocal microscopy. OC resorption lacunae could be visualized without prior staining of pits as used before. It is possible to conduct sub-surface examination of pits, but the interpretation is difficult due to the irregularities on the surface. This can be achieved by polishing the sample flat and use this surface as a reproducible reference point.

To clarify the inhibitory effect of NBD peptide on OC activity, OC were cultured in the presence of 10μM and 20μM of NBD-peptide. OC stimulation with VitD3 for 48 h could not increase the pit numbers whereas pit number of NBD 10μM was similar to the control and stimulated control. NBD 20μM could decrease the number of pits (Fig.4B). Counting numbers of discrete pit is the simplest assay of bone resorption which could be easily done even with a light microscope. This may not be accurate as pit area, volume and depth can be change with similar pit numbers. Pit number (along with pit area) may reflect the OC spreading and attachment rather than resorptive function. The pit count may also indicate the pattern of movement and resorption rather than the quantity of resorption. In addition, several pits could be coalesced together resulting in erroneous counting.

In this study we observed similar pit areas in both control and stimulated control groups (Fig.4C). To exclude the effect of OC differentiation we counted the number of TRAP+ OC present on the dentine slices at the end of the culture period. There was no significant difference in the number of TRAP+ OC in the two groups, proving that the survival and differentiation of OC were comparable (Fig.4A). NBD peptide could dose-dependently decrease the area of pits. Further our study emphasizes the inadequacy of using pit area as a measure of change in an isolated osteoclast assay. This area the least sensitive to change of all the parameters, and its use is open to wrong interpretation for not only can the change remain undetected, but an increase in area may be associated with a decrease in the volume of tissue resorbed. The cells may have spread more and/or have more extensive ruffled border zone, yet have done less work. In addition, pit area may reflect the OC size rather than OC activity. A simpler approach of measuring the width of the pits has been discussed. Though it may give some idea of the OC activity its reliability is still questionable.

SDSPMM could illustrate both pit area and pit volume at the same time and NBD peptide could inhibit pit volume in a dose-dependent manner (Fig.4D). The work done by the OC is best estimated by measuring the volume of dentine destroyed. Estimation of volume from formulae using area and depths at the deepest point in a pit is unlikely to be accurate as done in previous reports. Therefore volume and mean depths are better determined by confocal laser microscopy as SDSPMM.

Several lines of evidence indicate the importance of pit depth. Asagiri et al. shows that pit depths could be different in the presence of the same pit area. Consistent with this observation, our study also shows dose-dependent inhibition of pit depths by NBD-peptide (Fig.4E). Volume and area parameters however, reflect the number of OC on the dentine slice. Hence in the presence of similar number of OC, pit depth could be reliably used to measure the OC activity which is independent of OC differentiation. Therefore the measurement of pit area, volume and depth should be considered together to evaluate the osteoclastic activity in a given situation. This is necessary when checking the therapeutic value of inhibitors of osteoclastic activity.

No valid estimate of resorption can be made without reliable 3-D (Fig.3B and C) which shows the true picture of OC resorption lacunae. The variety of configurations of lacunae suggests some individuality in the way OC begin and end resorptive episodes. Therefore 3-D reconstruction of pits is important especially in calcified connective tissues where the demineralization of the substance gives rise to a collagen fringe which could mimic the resorbing surface.

The same kind of microscope has been used to evaluate the analysis of resorption pits on synthetic biomaterials showing the importance of comparing the resorbing ability of biomaterials by mature OC. Our established method reveals the real bone resorptive activity of OC is not influenced by OC numbers and/or pit area as used in previous studies.

In summary, SDSPMM is a reliable tool to measure the bone resorption lacunae and to produce a 3-D view of pits. The SDSPMM can be used to measure the volume of resorption lacunae in addition to area and depths which gives the true picture of OC activity.
Acknowledgements

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References