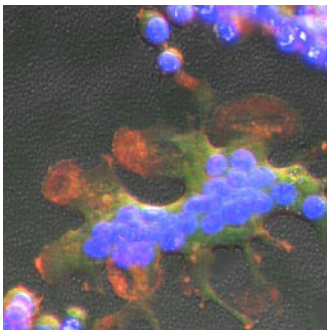
Primary Human Osteoclast Differentiation and Function on Dentine Discs and Corning® Osteo Assay Surface



SnAPPShots

A brief technical report from the Corning Development Group

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Introduction

In vitro assays that can measure both the formation and function of osteoclasts are critical for furthering our understanding of the biology of normal bone remodeling and diseases. Some of the more notable disease processes caused by increased bone resorption include osteoporosis, metastatic cancer, and Paget's disease. The gold standard system for assessing osteoclast activity is both time consuming and difficult to quantify. The typical assay involves primary osteoclasts and utilizes bone or dentine slices to evaluate osteoclast differentiation and function. An inorganic coating has been developed on standard polystyrene multiple well plates by Corning that can be used for in vitro assays and begins to address the challenges typically associated with osteoclast activity experiments. A comparison was performed with the Corning Osteo Assay Surface multiple plates and dentine discs using cultures of primary human osteoclast precursors cultured with macrophage colony stimulating factor (MCSF), and receptor activator of nuclear factor-ΧB ligand (RANKL) which stimulates osteoclast differentiation and activity. The results demonstrate that the osteoclast differentiation can be assessed rapidly and accurately on the Corning Osteo Assay surface using tartrate-resistant acid phosphatase (TRAP) staining. Visualization of TRAP positive multinucleated cells on dentine discs with normal light microscopy is difficult. Osteoclast activity can be assessed by measuring the area of the mineral coating removal as a model of pit formation on Corning Osteo Assay Surface, but this activity is extremely difficult to visualize on dentine discs. In summary, both osteoclast differentiation and activity can be readily measured with the Corning Osteo Assay Surface coated multiple well polystyrene plates, and the results are consistent and reproducible for assays using osteoclast precursors whereas use of dentine discs for similar experiments can be problematic.

Materials and Methods:

Human Osteoclast Precursor Cells

Human osteoclast precursor cells (Lonza Cat. No. 2T-110) were rapidly thawed and washed with Osteoclast Precursor Basal Medium (Lonza Cat. No. PT-8201) which had been supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 units/mL penicillin and streptomycin (Pen/Strep). Cells were counted and viability determined by using trypan blue exclusion. Primary human osteoclast precursors cannot be passaged however the cells can be differentiated into osteoclasts in the presence of RANK ligand (66 ng/mL) and M-CSF (33 ng/mL). Precursor cells grown in the presence of M-CSF but lacking soluble RANK ligand will expand in number but will not differentiate into functional osteoclasts. All cells, including a non-differentiation control, were resuspended to a final concentration of 50,000 cells/mL. 100 µL of cell suspension was plated per well in a 96 well Corning Osteo Assay Surface plate (Corning Cat. No. 3988XX1) or polystyrene 96 well plate to which had been added a single dentine disc (Immunodiagnostic Systems Cat. No. AE-8050). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. After 5 to 7 days of culture, the osteoclasts could be identified as large multinucleate cells when visualized by phase microscopy. Media was changed on day 3 of culture.

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IDS Dentine Discs

The dentine discs were supplied sterile and handled in a biosafety cabinet to maintain sterility. The contents of the sterile container were emptied on a Petri dish to facilitate handling. Forceps were used to carefully transfer the discs to a 96 well multiple well plate. The discs were pre-wetted for at least one hour with phosphate buffered saline and the solution was removed prior to adding cells.

Tartrate-Resistant Acid Phosphatase Quantitation and Cell Staining

In order to analyze for the presence of the osteoclast specific marker TRAP, a staining kit from B-Bridge International (Cat. No. AK04) was used. Briefly, 30 µL of culture supernatant was added to 170 µL of the chromogenic substrate which had been diluted in Tartrate-containing buffer (3 mg/5 mL as described in the kit). Plates were incubated for 3 hours at 37°C and absorbance read at 540 nm in a microplate reader (PerkinElmer® Victor3). For TRAP staining, the cells were washed with 100 µL of PBS and fixed for 5 minutes with the fixative reagent at room temperature. The cells were washed three times with distilled water and then stained for 20 to 60 minutes at 37°C with the chromogenic substrate and finally washed with distilled water to stop the reaction when optimum color was achieved. Longer incubations with the stain caused precipitation and were avoided.

Resorption Pit Assay

To analyze the surface for pit formation the medium was aspirated from the wells on day 5 and 100 µL of 10% bleach solution was added. Cells were incubated with the bleach solution for 5 minutes at room temperature. The wells were washed twice with distilled water and allowed to dry at room temperature for 3 to 5 hours. Individual pits or multiple pit clusters were observed using a microscope at 25 to 100x magnification. In order to observe the resorption pits on dentine, the discs were first stained with 1% toluidine blue for 5 minutes (1).

Imaging on Dentine

The TRAP stained cells and resorption pits on dentine discs were visualized and images captured using an upright microscope (Nikon® Eclipse 50iPOL) equipped with a ColorView soft imaging system.

Results and Discussion

The bone slice assay, in which cultured osteoclasts form pits on a mineralized substrate, has been widely used to investigate the effects of many factors on bone resorption and is considered to be the "gold standard" method (2,3). It has been widely used to study resorption by human osteoclasts either isolated directly from bone or cultured from marrow. One recent advance in the field has been the development of a technique to induce osteoclast formation from peripheral blood cells by the addition of RANK ligand (4). Using this technique large numbers of functional osteoclasts can be generated which will form resorption pits in mineralized substrates.

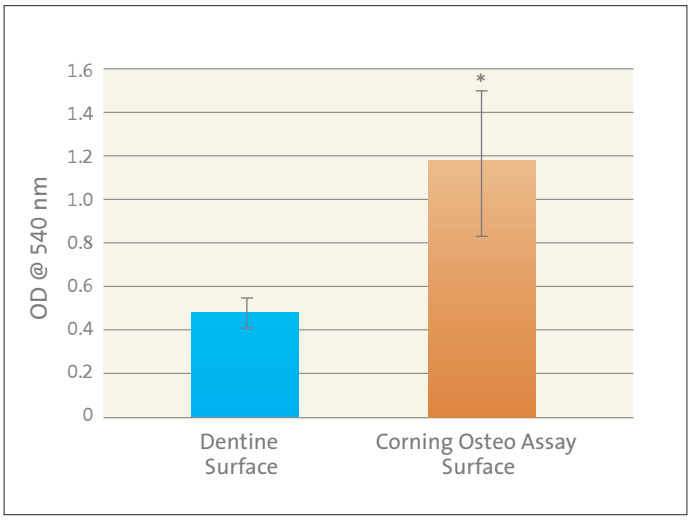

Figure 1. Soluble TRAP activity found in the medium of human osteoclast precursor cells which had been cultured for 5 days on the Corning Osteo Assay Surface or dentine surface (*p < 0.05 relative to dentine).

Poietics® human osteoclast precursors, which had been expanded using media containing M-CSF and plated onto Corning® Osteo Assay Surface 96 well plates or IDS dentine discs were differentiated for 5 to 7 days in the presence of RANK ligand, then tested for the production of soluble TRAP using the B-Bridge kit (described above). Figure 1 shows a comparison of TRAP activity in the culture medium derived from human osteoclast precursors cultured on Corning Osteo Assay Surface or on dentine discs. Cells grown on the Osteo Assay Surface express more than twice the TRAP activity in the medium compared with dentine discs. Dentine discs present several challenges for tissue culture, as the discs are easily damaged during handling and culturing of cells. Additionally, the discs are prone to floating and do not completely fill the bottom of an individual well (using 96 multiple well plates). These factors could contribute to cell loss on the dentine surface as the cells attach to the plastic well instead, and ultimately a lower TRAP value.

Active and fully differentiated osteoclasts are characterized as large multinucleate cells which display positive TRAP staining. Figure 2A shows human osteoclast precursors which had been cultured for 5 days on Corning Osteo Assay Surface fixed and stained for TRAP as described above. The cells were uniformly stained for TRAP and had greater than 3 nuclei per cell, the standard metric for differentiated osteoclasts. Cells prepared in exactly the same manner can not be visualized directly on dentine disks, as the dentine

discs are not transparent. To visualize fixed and TRAP stained cells on dentine discs required the use of an upright microscope and brightfield illumination. Figure 2B shows TRAP positively stained cells on dentine discs. The size and number of TRAP positive staining cells on the dentine surface is much lower than that observed on the Corning® Osteo Assay Surface. The dentine surface is quite uneven and displays numerous scratches and knife marks. While it is possible to visualize TRAP stained cells on the discs, it is very difficult – if not impossible – to distinguish individual nuclei, making the quantification of cells with 3 or more. Resorption of a mineralized substrate such as bone slices and dentine discs are considered the "gold standard" for measuring osteoclast function. Figure 3A shows resorption pits produced by human osteoclast precursors cultured on Corning Osteo Assay Surface for 5 days and then removed by bleaching the well. The pits are numerous and readily visible. As has been noted above, it is not possible to visualize resorption pits in dentine discs using a standard cell culture inverted microscope. In fact, using an upright microscope and brightfield illumination was not sufficient to visualize resorption pits on dentine discs. Figure 3B shows resorption pits on dentine discs which had been stained with toluidine blue. The staining resulted in dark

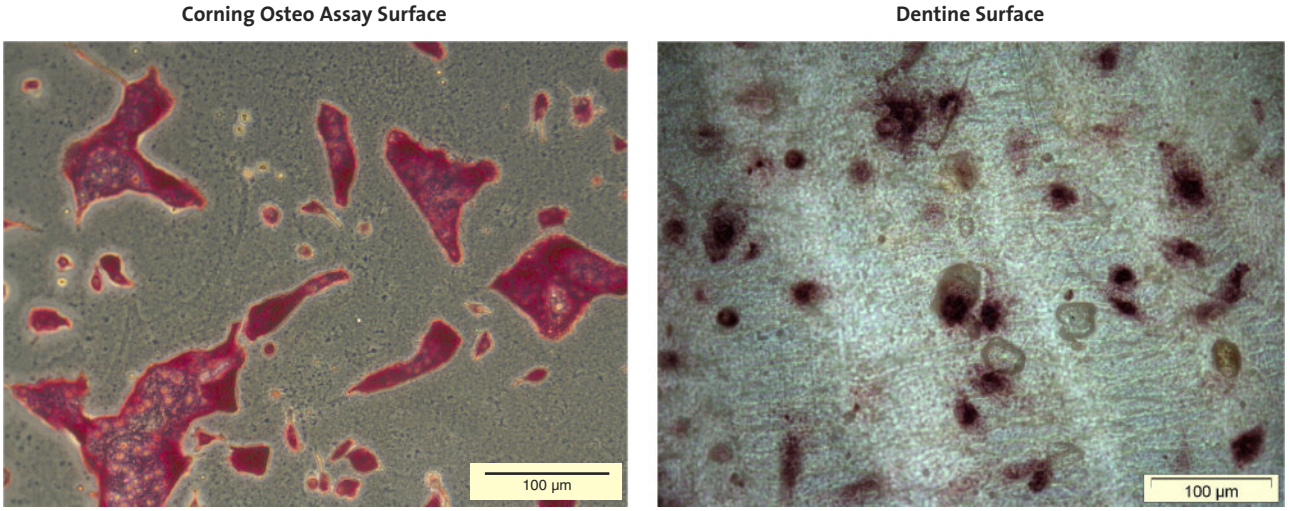

Figure 2. Human osteoclast precursors were fixed after 5 days of growth on the Corning Osteo Assay Surface or dentine surface and stained for the osteoclast marker TRAP.

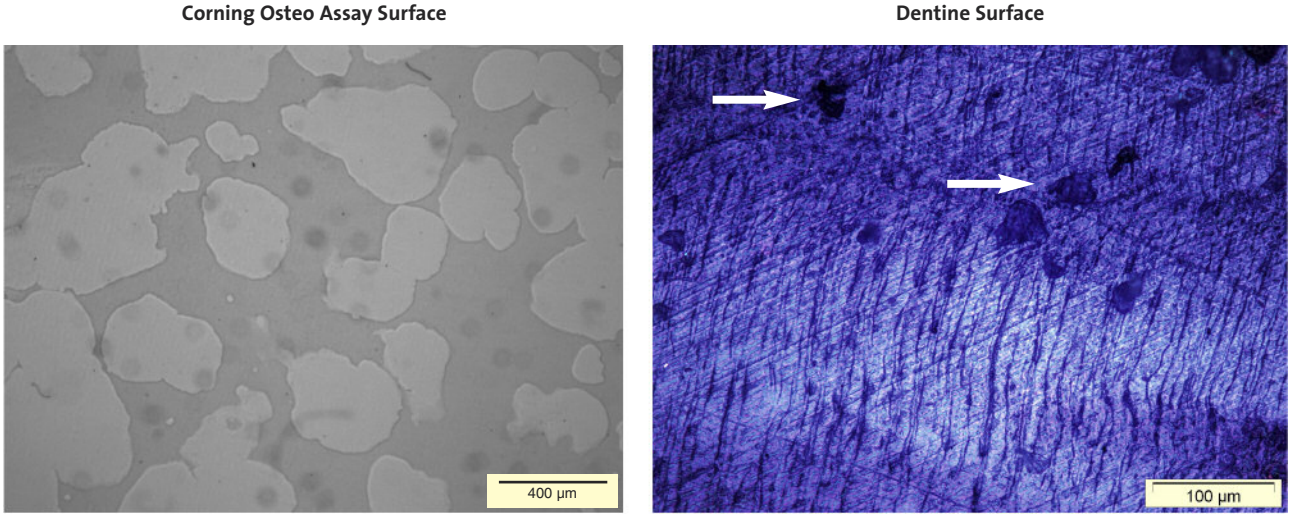

Figure 3. Resorption pits observed on the Corning Osteo Assay Surface and dentine surface after 5 days of culture of human osteoclast precursor cells. The dentine surface was stained with toluidine blue to visualize the pits.

blue pits which were visible in spite of a high level of background staining which also brought out the uneven surface and numerous scratches and knife marks. The high background and numerous imperfections of the dentine discs make the quantification of resorption pit area all but impossible. It can be argued that Corning® Osteo Assay Surface represents a robust, convenient, and reproducible substitute for bone slices or dentine discs.

Conclusions

It was demonstrated that the Corning Osteo Assay Surface can be used to easily measure soluble TRAP activity, to visualize and quantify multinucleated TRAP positive stained differentiating osteoclasts, and to visualize resorption pits created by differentiated osteoclasts. In contrast, dentine discs were difficult to handle, easily damaged and do not cover the entire bottom of the well. We speculate that these handling issues result in fewer cells on the dentine discs and therefore a much lower soluble TRAP value. In fact, the soluble TRAP value on Corning Osteo Assay Surface was more than twice that of what was observed for the dentine discs.

Corning Osteo Assay Surface is a convenient tool for the measure of the number of differentiated osteoclasts, because TRAP staining cells can be easily visualized using an inverted microscope. The uniformity and clarity of the surface in the Corning multiple well plates makes the counting of nuclei quite straightforward. On the other hand, fixed and stained cells can not be visualized on dentine discs using the standard cell culture inverted microscope. When TRAP stained cells on the dentine discs are examined using an upright microscope and brightfield illumination, the cells are much smaller and fewer in number compared to what was observed on Corning Osteo Assay Surface. Additionally, the unevenness of the background and numerous imperfections make counting the number of nuclei for the individual cells all but impossible.

The visualization and quantification of resorption pits formed by differentiated osteoclasts is also easily accomplished on Corning Osteo Assay Surface after the cells have been removed. The pits are numerous and quite large when viewed under an inverted microscope without the need for additional staining. As discussed above, visualization of pits on dentine discs required the use of an upright microscope and brightfield illumination. It was also necessary to stain the dentine discs with toluidine blue in order to visualize the pits. There were not as many pits on the dentine discs and they appeared smaller in size. Because of the high background of the stained discs, it would be very difficult to quantify the area of resorption pits on the discs.

References

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