Osteoporosis is one of the most common causes of disability in the elderly, leading to both hip and vertebral fractures (1). The prevention of osteoporosis and its associated fractures is essential to maintaining health, quality of life, and independence in the elderly (2). Bone is constantly destroyed or resorbed by osteoclasts and then replaced by osteoblasts during bone remodeling. Most bone disorders, including osteoporosis, are caused by increased bone resorption by osteoclasts (3). Therefore, osteoclasts serve as key target cells for anti-osteoporosis therapies.

Osteoclast formation requires the presence of RANKL (receptor activator of nuclear factor κB ligand) and M-CSF/CSF-1 (macrophage colony-stimulating factor/colony-stimulating factor-1). Both of these membrane-bound proteins are necessary for osteoclastogenesis and are heavily involved in the differentiation of monocyte/macrophage derived cells. Indeed M-CSF/CSF-1 stimulates the fusion process in osteoclasts and/or their precursors (4) and also increases bone resorption by increasing osteoclast size (5). It is important to understand osteoclast morphology to develop new drug treatments for bone disease, but it is difficult to study osteoclast morphology continuously (i.e., in real-time). Instead, osteoclasts are typically fixed and stained with a dye such as tartrate-resistant acid phosphatase (TRAP), and the stained cells are examined by light microscopy (6). These assays as described are end-point assays that provide a ‘snapshot’ of the morphology process (7).

Recently, the xCELLigence real-time cell analysis DP (Dual Plate) system (RTCA; Scrum Inc., Tokyo, Japan) was developed to observe cell morphology and cell adhesion using electrical impedance in vitro (7, 8). This RTCA instrument is a proprietary microelectronic biosensor system for cell-based assays. The core of the system is composed microelectronic cell sensor arrays that are integrated into the bottom of E-Plate 16. Measurements of the electronic impedance of these sensor electrodes allow changes in the cell to be detected and monitored. Cell viability, cell number, cell morphology, and the degree of adhesion affect electrode impedance. Impedance measured between electrodes in an individual well depends on the electrode geometry, the ionic concentration in the well, and whether cells are attached to the electrodes. In the absence of cells, electrode impedance primarily depends on the electrode geometry, the ionic concentration in the well, and whether cells are attached to the electrodes. In the presence of cells, the cells attached to the electrode sensor surfaces act as insulators and thereby alter the
Fig. 1. Osteoclast precursor cells of 1.5 x 10⁵ cells/well were cultured for 3–8 days in the presence (A–F) or absence (negative control; G–I) of M-CSF/CSF-1 and RANKL. Media were changed after 1 and 3 days. Osteoclasts were identified via tartrate-resistant acid phosphatase (TRAP) staining (original magnification x100). C) The red arrow indicates TRAP-positive multinucleated giant osteoclasts. E) The yellow arrow indicates osteoclasts apoptosis. Osteoclast precursor cells of 1.0 x 10⁵ cells/well were cultured for 3–8 days in the presence (J–O) or absence (negative control; P–R) of M-CSF/CSF-1 and RANKL. Media were changed after 1 and 3 days. Osteoclasts were identified via TRAP staining (original magnification x100). M) The red arrow indicates TRAP-positive multinucleated giant osteoclasts. N) The yellow arrow indicates osteoclasts apoptosis.
local ionic environment at the electrode/solution interface, leading to increased impedance (7). No studies examined real-time changes in osteoclast morphology have been published. In this study, we examined a new method to capture the differentiation of osteoclasts in real-time. Moreover, we previously reported that (−)-epigallocatechin-3-gallate (EGCG) inhibits the differentiation of osteoclasts (6, 9). Therefore, we also investigated whether this new RTCA method could monitor the effect of EGCG on osteoclasts.

We used 5- to 8 week-old ddY male mice (Sankyo Labo Service Corporation, Inc., Tokyo, Japan). All experiments were conducted according to the “Guiding Principles for the Care and Use of Laboratory Animals” of The Japanese Pharmacological Society and with the approval of the Showa University Animal Care and Use Committee (certificate number: 13041). M-CSF/CSF-1 and RANKL were acquired from Pepro Tech (Rocky Hill, NJ, USA.). EGCG was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

We obtained bone marrow cells from the femur and tibia bones of male mice and cultured them (1.0 × 10⁵ cells/well or 1.5 × 10⁵ cells/well) in an E-plate for RTCA and in a 96-well plate with medium that contained 15% serum, 25 ng/ml M-CSF/CSF-1, and 100 ng/ml RANKL. Media were changed after 1d and 3 d. We also cultured cells in medium without M-CSF/CSF-1 and RANKL as negative controls (10). Moreover, we cultured bone marrow cells (1.0 × 10⁶ cells/well) in an E-plate for RTCA and a 96-well plate with or without 10 and 100 μM EGCG.

We stained cells with TRAP at 37 °C for 20 min on days 3, 4, 5, 6, 7, and 8. We counted osteoclasts that were TRAP-positive and had three or more nuclei (6, 10).

The results were expressed as the mean ± standard error of the mean (S.E.M). The data were analyzed using the Mann–Whitney U-test. Correlations were calculated by Pearson’s product moment correlation coefficient. P values less than 0.05 were considered significant.

Osteoclast precursor cells cultured at 1.5 × 10⁶ cells/well in medium with M-CSF/CSF-1 and RANKL increased in size over the course of 3 d, however, no osteoclasts were observed at 3 d (Fig. 1A). Osteoclasts first appeared at 4 d (Fig. 1B). The largest osteoclast (greater than 1 mm) was observed at 5 d (Fig. 1C; red arrow). Afterwards, osteoclasts became smaller due to apoptosis (Fig. 1D, yellow arrow; 1E, F). However, osteoclast precursor cells cultured at 1.0 × 10⁵ cells/well in medium with M-CSF/CSF-1 and RANKL started to increase in size at 3 d (Fig. 1J), but few osteoclasts were observed.

![Figure 2](image_url)

**Fig. 2.** A) TRAP-positive multinucleated cells with more than three nuclei as determined by light microscopy were counted as osteoclasts. Media were changed after 1d and 3 d. The blue line represents osteoclast cells cultured at 1.5 × 10⁶ cells/well with M-CSF/CSF-1 and RANKL. The red line represents osteoclast precursor cells cultured at 1.0 × 10⁵ cells/well with M-CSF/CSF-1 and RANKL. Values are expressed as the mean ± S.E.M from 4 independent experiments. *P < 0.05, significantly different from 5 d for 1.5 × 10⁶ cells/well. **P < 0.05, compared with 6 d in 1.0 × 10⁵ cells/well. B) The CI of osteoclastogenesis for 1.0 or 1.5 × 10⁵ cells/well with/without M-CSF/CSF-1 and RANKL. Media were changed after 1d and 3 d. Values are expressed the mean ± S.E.M of 6 independent experiments. *P < 0.05, **P < 0.01, compared with controls of 1.5 × 10⁶ cells/well. ***P < 0.01, compared with controls of 1.0 × 10⁵ cells/well. C) Correlation between the CI and the number of osteoclasts. A highly significant positive correlation (r = 0.9423, p < 4.072 × 10⁻⁷) between the CI and the number of osteoclasts was observed.
by 4 d (Fig. 1K). The largest osteoclast was observed at 6 d (red arrow; Fig. 1M), and became smaller at 7 d (yellow arrow; Fig. 1N–O). Osteoclasts did not appear in the medium without M-CSF/CSF-1 and RANKL until 8 days (Fig. 1G–I, and P–R).

Fig. 2A shows the number of multinucleated (more than three) osteoclasts in each well. In the medium cultured with 1.5 x 10⁵ cells/well, the number of osteoclasts increased to 71.5 ± 4.9 at 4 days and reached 331.5 ± 10.2 at 5 days. Thereafter, the number of osteoclasts decreased over time. Similarly, in the medium cultured with 1.0 x 10⁵ cells/well, the number of osteoclasts increased to 24.8 ± 0.7 at 4 d, reached 242.8 ± 5.5 at 6 d, and then decreased over time.

A unitless parameter termed cell index (CI) was used to measure the relative change in electrical impedance as a marker of cell status. The CI is a relative and dimensionless value because it represents the impedance change divided by a background value (7, 8).

For osteoclast precursor cells cultured at 1.5 x 10⁵ cells/well in the medium with M-CSF/CSF-1 and RANKL, the CI increased significantly for 71 h and reached maximum values (1.01 ± 0.09) at 5 d (Fig. 2B). For osteoclast precursor cells cultured at 1.0 x 10⁵ cells/well with M-CSF/CSF-1 and RANKL, the CI increased significantly for 76 h and reached maximum values (0.71 ± 0.07) at 6 d. The CI did not increase in osteoclast precursor cells cultured at 1.0 or 1.0 x 10⁵ cells/well without M-CSF/CSF-1 and RANKL.

**Fig. 3.** Osteoclast precursor cells were cultured at 1.0 x 10⁵ cells/well for 72, 144, and 164 h without (A–C) or with (D–F) 10 µM and (G–I) 100 µM EGCG. Media were changed after 1 and 3 days. Osteoclasts were identified via TRAP staining (original magnification x100). E) The white arrow indicates TRAP-positive multinucleated small osteoclasts. G) TRAP-positive multinucleated cells more than three nuclei as determined by light microscopy were counted as osteoclasts. Media were changed after 1d and 3 d. The blue line represents osteoclast cells cultured at 1.0 x 10⁵ cells/well with M-CSF/CSF-1 and RANKL (control). J) The light blue line represents osteoclast precursor cells cultured at 1.0 x 10⁵ cells/well without M-CSF/CSF-1 and RANKL. The green and broken red line represents osteoclast cells cultured at 1.0 x 10⁵ cells/well with 10 and 100 µM EGCG, respectively. Values are expressed as the mean ± S.E.M of 4 independent experiments. **P < 0.01, compared with control. K) This graph shows the CI of osteoclastogenesis of 1.0 x 10⁵ cells/well with/without M-CSF/CSF-1 and RANKL or with 10 and 100 µM EGCG. Media were changed after 1d and 3 d. Values are expressed as the mean ± S.E.M of 8 independent experiments. *P < 0.05, **P < 0.01, compared with control.
1.5 x 10^5 cells/well in medium without M-CSF/CSF-1 and RANKL. The number of osteoclasts observed via TRAP staining and the CI by RTCA were similar (Fig. 2A, B). A highly significant positive correlation (r = 0.94, p < 4.1 x 10^-7) between CI and the number of osteoclasts is presented in Fig. 2C.

Fig. 3 presents cultured osteoclasts with or without 10 and 100 μM EGCG. EGCG (100 μM) strongly suppressed the cell—cell fusion and proliferation of osteoclast precursor cells. A small number of multinucleated osteoclasts (white arrow; Fig. 3E) and many small osteoclasts (uncountable level at the macroscopic level) were observed at 144 h when cultured with 10 μM EGCG. Fig. 3J and K presents the number of osteoclasts via TRAP staining and according to the CI by RTCA with or without 10 and 100 μM EGCG. The discrepancy between the figures is due to EGCG inhibition. RTCA measures the CI values for the minute uncountable osteoclasts at the macroscopic level. The CI level does not increase when osteoclasts do not differentiate. Therefore, CI levels may be more accurate for the measurement of osteoclasts compared to macroscopic measurements. However, we observed a significant positive correlation (r = 0.7957, p < 7.850 x 10^-5) between the CI and the number of osteoclasts in the 10 μM EGCG and control groups.

We established a new method for real-time monitoring of osteoclastogenesis using RTCA. It is difficult to monitor osteoclastogenesis in real time using traditional endpoint assays. We modified RTCA to allow for real-time monitoring of osteoclastogenesis. This new method offers the following advantages:

1) Real time monitoring
2) High reproducibility
3) Relatively simple and easy
4) High correlation with classic endpoint assays

Overall, these advantages appear to outweigh the limitations, and we believe that RTCA will be advantageous for determining fixed-quantities of osteoclasts because RTCA has been proven useful for assessing a fixed-quantities of cells during migration and invasion (11-14). However, it is difficult to use RTCA to assess fixed-quantities of osteoclasts during migration and invasion because osteoclasts exhibit a low CI. RTCA is useful for measuring the effect of a compound (8, 11, 12). In fact, using RTCA to analyze changes in osteoclasts was also useful for measuring the effects of EGCG in the present study. Various reports have detailed numerous methods of studying osteoclasts in vitro (6, 14, 15), but RTCA will likely become a new essential tool.

It is potentially possible to determine the effects of various compounds on osteoclasts using this new system. Consequently it is possible that this new system can contribute to the development of osteoporotic medications.

Conflicts of interest

The authors indicate no potential conflicts of interest.

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References


