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TIME COURSE OF RECRUITMENT, PIT FORMATION AND APOPTOSIS OF OSTEOCLAST POPULATIONS ON DENTIN *IN VITRO*

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Abstract

The resorptive capacity of osteoclasts *in vitro* has been used as an indicator of bone resorption. However, the kinetics of osteoclast development and senescence is not well understood. It has been noted that mononuclear precursors migrate to calcified substrate and after multinucleation become competent bone resorbing cells. Osteoclasts, once finished a wave of resorption, become senescent. In order to determine the survival characteristics of osteoclast populations involved in calcified tissue destruction, we have investigated the time course of the recruitment to dentin and apoptosis of osteoclasts. We have established the linkage between these measurements and the time course of the appearance of pits using both chick and rat osteoclasts *in vitro*. Osteoclasts from both species caused increases in pit number associated with decreases in cell number on dentin. In the rat model, we have used a fluorescent linker to mark cells before they migrate to dentin and have shown that cells can be followed from the culture dish onto the dentin disc. In the chick model, we have used time lapse cinematography and fluorescent nuclear staining to observe death of osteoclasts on dentin and have established an osteoclast half live *in vitro*.

Key Words: Osteoclasts, dentin, resorption, pits, apoptosis, osteoclast recruitment.

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Introduction

The osteoclast resorption pit assay has been used with both the preformed, isolated mature osteoclasts as well as with a developing mononuclear cell population to determine the destructive capacity of these bone cells (Fenton *et al.*, 1993; Lewinson *et al.*, 1993; Zheng *et al.*, 1993). However, estimates of osteoclast life span *in vitro* have not been extensively studied. In the isolated osteoclast pit assay, the assessment of bone resorption is usually performed one to several days after plating. Isolated cells, either mononuclear or mixed mononuclear and multinuclear are plated in wells containing a dentin slice. During the time interval between plating and the fixation of osteoclast-like cells on dentin, osteoclast precursors become competent bone resorbing cells (Baron *et al.*, 1986). Following adhesion to dentin, these cells become multinucleate and develop resorptive capacity (Krukowski and Kahn, 1982; Chambers *et al.*, 1984; Jones *et al.*, 1984). On the other hand, preexisting multinuclear cells may finish the resorptive cycle and cease to be active. Using the fluorescent linker, PKH26 (Greenwood and Croy, 1993; Lansdorp, 1993; Van de Langerijt *et al.*, 1994), we have followed the migration of cohorts of neonatal rat mononuclear cells to dentin. The process of recruitment of osteoclast-like cells continues for at least 48 hours (Baron *et al.*, 1986; Kukita *et al.*, 1993). In our studies, we have also followed the parallel increase in pit numbers on dentin. We have analyzed the numbers of osteoclasts with increasing *in vitro* culture time and found that cell numbers do not follow the same kinetics as pit numbers. This discrepancy in cell numbers versus pit numbers can be accounted for by the loss of mature cells over time. To test this hypothesis, we have carried out both static time point experiments and studies using time lapse cinematography to demonstrate the gradual disappearance of osteoclast populations on dentin *in vitro*. In addition, we have quantified nuclear staining changes consistent with apoptosis in osteoclasts on dentin.

Materials and Methods

Isolation of avian osteoclasts

A modification of the Oursler *et al.* (1991) method was utilized to isolate the cell populations (IACUC approval #1446). The tibiae of 3 week old chicks which had been on a 0% calcium diet for 2.5 weeks were harvested in cold (4°C) Moscona's low calcium medium containing 200 units/l heparin (Solopak Laboratories, Inc., Elk Grove, IL). The bone was digested with collagenase (125 units/ml, Sigma, St. Louis, MO) and trypsin (1%, Gibco, Grand Island, NY) in sequential digestions of 15 minutes each at 37°C. Vigorous manual agitation for 4 minutes dislodged the cells from the partially digested matrix. Cells were chilled during filtration (Nytex, 350/36, Tetko, Inc., Braircliff Manor, NY), when resuspended in Moscona's low calcium medium, and when layered on a 6% Percoll (Sigma) for 1 hour at 4°C. Three layers were collected, pelleted at 1500 x g for 15 minutes at 4°C, resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, counted and plated at a density of 5 x 10^5 cells/well (24 well plate) in one milliliter of medium. Initial pH of the medium was 7.4, but pH decreased to 6.8 to 7.0 over the incubation period. The fastest sedimenting Percoll fractions were used to provide the cell populations for these experiments. Cell suspensions were placed in a culture well (16 mm diameter) containing a glass coverslip (12 mm diameter) and a polished human dentin disc (area 0.2 cm^2). Polishing was obtained by using the lowest speed and no additional weight on the Isomet® (Buehler, Lake Bluff, IL) saw arm. Osteoclast cultures were maintained *in vitro* for up to 96 hours.

Isolation of rat osteoclast precursors

Cells were isolated from the femoral shafts of 8-12 day old Sprague Dawley CD rat pups (Charles River Laboratories, Stoneridge, NY) (IACUC approval #1446). As described above, the method was modified from Oursler *et al.* (1991). Femoral shafts were dissected out and minced with scissors or scalpel and placed in a 50 ml centrifuge tube on ice. Ten ml of collagenase (125 units/ml) was added and the suspension incubated at 37°C for 7 minutes. The supernatant was decanted and 10 ml of 1% trypsin added to the tube. After incubating for 7 minutes at 37°C, the tube was vigorously agitated manually for 4 minutes to dislodge cells. Trypsin was quenched with 1 ml fetal bovine serum (FBS), the suspension filtered through Nytex and the suspension centrifuged at 1200 x g for 10 minutes. The cell pellet was osmotically shocked with 1 ml of doubly distilled water for 30 seconds to lyse red blood cells. Centrifugation was again performed at 1200 x g for 10 minutes to pellet the cells. The cells were resuspended in DMEM + 10% FBS, preplated for 20 minutes at 37°C, then aliquoted to a density of 5 x 10^5 cells per well (24 well plate, well diameter, 16 mm) with a glass coverslip (12 mm diameter) in 1 ml of medium. Previous experiments have shown that this preplating decreases the number of acid phosphatase negative cells present in the culture (Pollice *et al.*, 1995). After incubating for 2 hours at 37°C, the media was aspirated leaving behind cells adherent to the glass coverslips. The freshly isolated osteoclast precursors, were divided into experimental groups (n = at least 4 wells per group). At this point, dentin slices were added to the wells. The initial adhesion step was performed solely on coverslip, so that any cells found on dentin had migrated from the coverslip to the dentin slice. After each incubation period, the cells on dentin as well as on the coverslips were fixed with citrate-acetone (200 mM sodium citrate, pH 7.4, 3:2 with acetone), stained for acid phosphatase and counted. Tartrate-resistant acid phosphatase (TRAP) staining was positive for 50-80% of the osteoclast precursors on dentin at 24 and 48 hours. To investigate possible migration of osteoclast precursors from dentin to coverslips, dentin slices were moved to new wells with a cell-free coverslip. Cells were then maintained *in vitro* for 24 hours before analysis. Each experiment was repeated at least 3 times with different isolations of osteoclast precursors.

Polished human dentin slices

Human premolars were provided by the University of Pennsylvania School of Dental Medicine after human review board expedited approval. Teeth were cut and partially polished with an Isomet® low speed saw and a diamond blade without additional weight. Slices were washed in 70% ethanol and stored in fresh 70% ethanol. The slices were 100 μm in thickness. The discs were examined for defects and comparable cross sectional areas were cut with a surgical scissors. The average area of discs was 0.2 cm^2. Slices were air dried under the isolation hood for 30 minutes before use. Slices are washed with medium before placing in the well.

Acid phosphatase staining

Acid phosphatase staining was utilized to identify rat osteoclast precursors in some experiments. All of the migrating rat osteoclast precursor cells on dentin were positive with acid phosphatase staining, whereas 50-80% were positive after tartrate inhibition at 24 to 72 hours. Since TRAP is a late appearing enzyme in osteoclast development, acid phosphatase was more helpful in visualizing the initial wave of migration to dentin from coverslip. Most of these cells were mononuclear until after 96 hours *in vitro*. When followed for up to 120 hours, the rat osteoclast populations on dentin slices became predominately multinucleate and 100% TRAP positive.

An adaptation of the Barka (1960) method was used for acid phosphatase (Silverton and Kaye, 1987). Solution A was prepared with 5 ml of sodium acetate buffer, 12 ml of water and 1 ml of substrate solution (10 mg of napthol AS-TR phosphate + N, N, dimethylformamide). Solution B was prepared with 0.8 ml of pararosaniline solution and 1.2 ml of sodium nitrite solution. Combined solution A and solution B were used to stain citrate-acetone fixed cells on dentin and on coverslips for 45 minutes to an hour at room temperature. Dentin slices and coverslips were then washed with distilled water and air-dried.

Tartrate resistant acid phosphatase (TRAP)

TRAP staining was carried out on chick cells for identification of osteoclasts. Cells on dentin and coverslip were fixed in citrate-acetone for 1 minute at room temperature, washed twice with distilled water and air-dried. TRAP staining (Sigma kit) was over 15 to 30 minutes at 37°C.

Cell count analysis

Acid phosphatase positive or TRAP positive cells on dentin and coverslips were counted under a model TMS inverted Nikon microscope (Optical Apparatus Co., Inc., Ardmore, PA) at a 250x magnification. The fields of interest were also viewed using a color monitor which was connected to a color television camera mounted on the microscope. For rat experiments, each group contained at least 4 replicates and each experiment was repeated at least 3 times with different cell isolations. Rat osteoclasts and precursors were mainly mononuclear at the time intervals used in these experiments. For chick experiments, each group contained at least 3 replicates and each experiment was repeated at least 3 times with different cell isolations. Chick osteoclasts on dentin slices were TRAP positive and both mononuclear and multinuclear. Chick TRAP positive cells on coverslips were mononuclear and multinuclear. Mononuclear and multinuclear cells were counted separately in chick experiments. Results from chick multinuclear cells only are given. For an individual experiment, the significance was determined using signed rank (Snedecor and Cochran, 1980). For analyses which included all experiments, the distribution of results was analyzed and Gaussian statistics utilized as deemed appropriate.

Pit count analysis

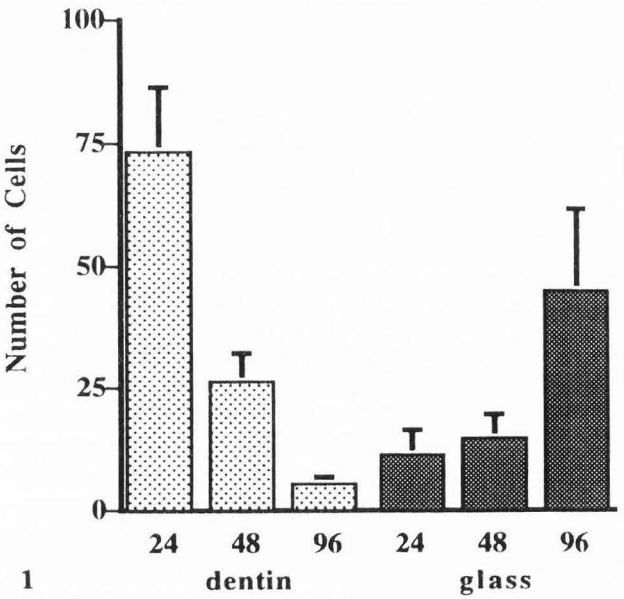
After the cell counting, dentin slices were soaked in 0.1% SDS (sodium dodecyl-sulfate; Sigma) overnight and then gently rubbed with a laboratory tissue to dislodge cells. Dentin slices were immersed in 1% toluidine blue in 1% borate buffer for four minutes (Murrils and Dempster, 1990) followed by a 5 second wash in 0.1 N HCl, then rinsed in distilled water. Pits were counted and analyzed as described above. There was a noticeable variation in pit size in the chick model (Shapiro *et al.*, 1994).

Fluorescent linker labeling

In some experiments, isolated adherent cells on glass coverslips were labeled with red fluorescent cell linker (PKH26-HCl). Thus, all cells adherent to the glass coverslip were labeled 24 hours after initial plating. Coverslips containing adherent cells were removed from the well and washed with DMEM without added serum. Subsequently, the coverslips and adherent cells were incubated with 1 ml of fluorescent marker diluted in DMEM (PKH26 dye, 4×10^{-6} M, excitation 551 nm, emission 567 nm) for 2-5 minutes at room temperature, and then 1 ml of FBS was added for 1 minute. The cells were washed three times with complete DMEM and returned to the wells with the original dentin slice. For the next 48 to 72 hours, unlabeled rat osteoclast precursor cells on dentin slices which had already migrated to dentin, and labeled cells on glass coverslips which were expected to migrate to dentin were cultured in the same well. Experiments were terminated by fixing the cells on dentin and coverslips after 48 or 72 hours with 2% paraformaldehyde for 5 minutes at room temperature. Fluorescent and phase microscopy were used to view the fields on the dentin slices using a Zeiss microscope. The converse experiment of labeling cells on dentin and quantitating a decrease in the label as new cells migrated onto the dentin was not possible because treating the dentin with fluorescent marker resulted in non-specific labeling of approximately 20% of migrating cells. This was tested by exposing dentin slices without cells to the fluorescent label and subsequently incubating these labeled dentin slices with coverslips containing unlabeled cells (unpublished results). After completion of experiments, cells were fixed with 2% paraformaldehyde in phosphate buffered saline, pH 7.4 at room temperature for 10 minutes, washed with distilled water, air-dried and counted under fluorescence and phase using a Nikon fluorescence microscope.

Time-lapse cinematography

Isolated avian osteoclasts adherent to dentin were placed in a 35 mm petri dish located under the microscope attached to a video camera and video recorder. To prevent evaporation, a thin layer of mineral oil was gently layered over the surface of the fresh culture medium. The petri dish was maintained at a temperature of 37°C by a thermostatically controlled heat lamp. A grouping of attached cells on dentin was located under the microscope and the video recorder started at the minimal recording frame speed (Glasgow and Daniele, 1994). The cell groups were checked regularly for focus and for remaining cell number. After one group



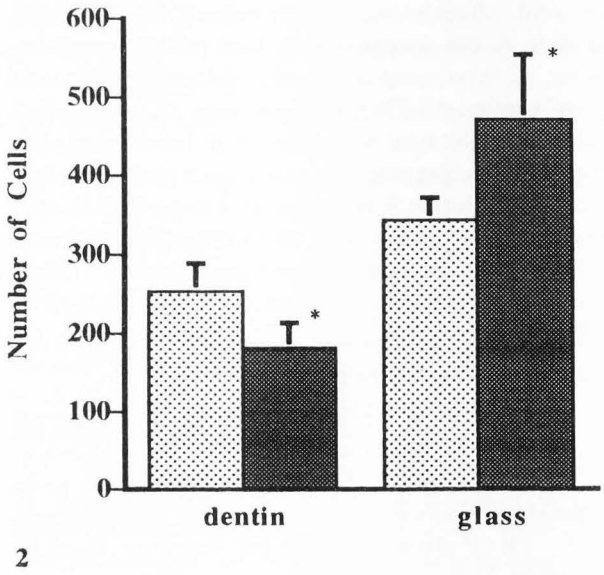


Figure 1. Avian osteoclast populations (TRAP positive cells) on dentin decrease in number during *in vitro* culture, while TRAP positive cell number on glass increases. Stippled bars show osteoclast number on dentin over 24-96 hours of *in vitro* culture. Gray bars show osteoclast-like, TRAP positive cell number on glass for the same culture wells. Error bars show the standard error. Significant ($p < 0.05$) differences in osteoclast number on dentin slices were seen after each 24 hour interval. There was a significant increase in TRAP positive cell number on glass between 48 hours and 96 hours ($p < 0.05$). Significance was tested using Wilcoxon (Snedecor and Cochran, 1980) signed rank testing.

Figure 2. Rat osteoclast precursors on dentin (TRAP positive cells) decreased significantly in number between 48 and 72 hours of *in vitro* culture ($p < 0.05$), while numbers of TRAP positive cells increased significantly ($p < 0.05$) on glass coverslips in the same culture wells. Stippled bars represent cells on dentin slices. Gray bars represent cells on glass. The decrease in cell number in this model occurred later in the *in vitro* culture period than in the chick model. Significance was tested using Wilcoxon signed rank testing.

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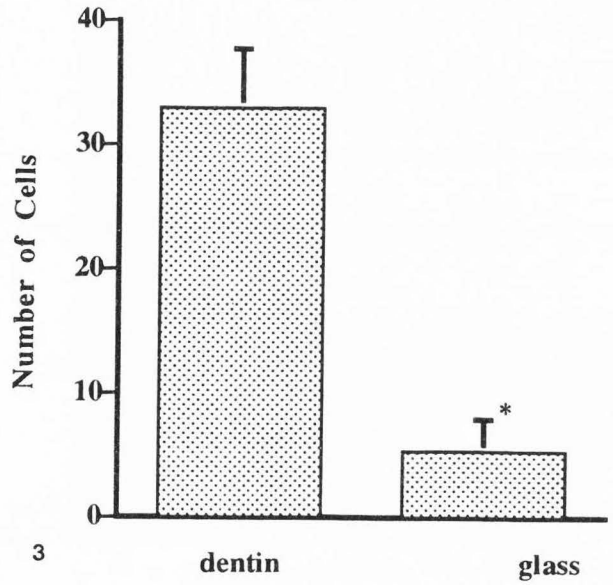
of cells was followed until all cells had lysed, another group was located and followed in the same way. After each experiment, the video recorder was rewound and the cell motion viewed. Time of lysis for each cell was recorded and then charted in relation to the time of osteoclast isolation. No time intervals were recorded under 16 hours after isolation, as the cells on dentin were allowed to adhere and recover *in vitro* for 16 hours before cinematography was carried out.

Nuclear dye staining of osteoclasts

Isolated avian osteoclasts were allowed to adhere to dentin and to glass coverslips in 1 ml of culture media (DMEM) in a 2 ml well. After 48 hours *in vitro*, cells were treated with either Hoechst dye (H1399) 1 μm/ml final concentration, or SYTO (S7578) 100 nM final concentration (Frey, 1995). Cells treated with SYTO were also exposed to propidium iodide (5 mM) for 1 minute before imaging. Cells on dentin and coverslips were imaged with a silicone intensified tube camera with ARGUS imaging software. A microscope with differential interference contrast was utilized. For Hoechst dye, multinucleate cells on dentin were imaged. For the SYTO and propidium iodide, mononuclear and multinuclear cells on dentin and glass coverslips were counted. The relative fraction of propidium iodide to SYTO positive cells was determined in 20-40 different fields.

Results

TRAP positive avian osteoclast populations on dentin, both mononuclear and multinuclear, decreased in number during *in vitro* culture, while TRAP positive avian cell populations on glass coverslips increased (Fig. 1). Acid phosphate positive rat osteoclast-like cell populations on dentin also decreased in number, while acid phosphatase positive cells on coverslips increased (Fig. 2). These experiments demonstrated that the decrease in cell number on dentin was not due to adverse culture conditions because coverslip cells were increasing in number while those on dentin were decreasing. We next tested the possibility that the decrease in cell number on



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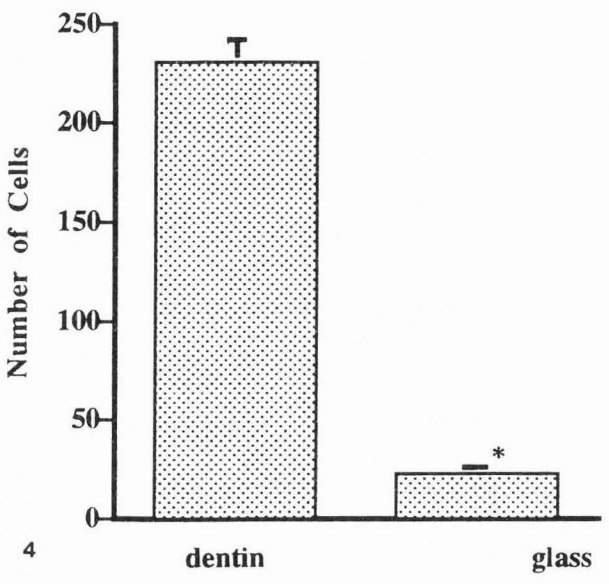

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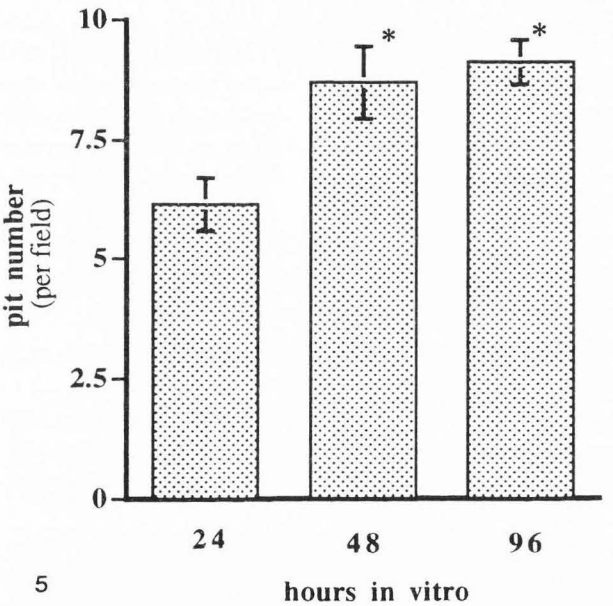

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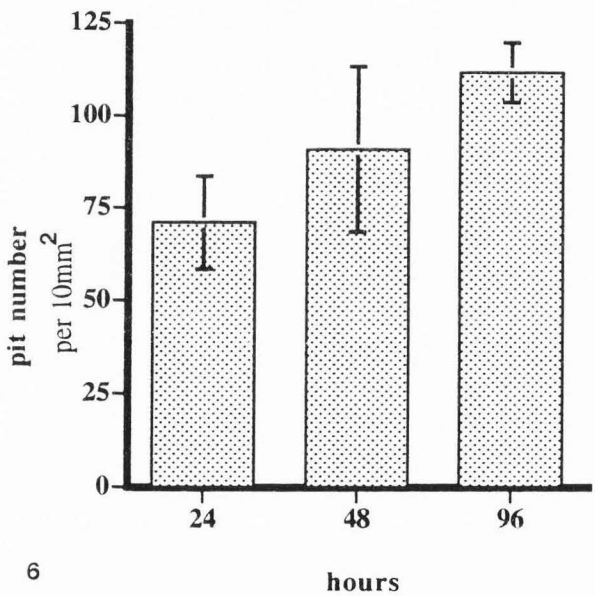

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Figures 3 and 4. Migration of avian (**Fig. 3**) and rat (**Fig. 4**) osteoclasts from dentin to glass over 24 hours. Few osteoclasts were found on glass 24 hours after osteoclast populations on dentin were placed in new culture wells. Osteoclast numbers on dentin were substantially greater than the numbers which migrated to glass. * = $p < 0.01$.

Figure 5. The number of resorption pits on dentin slices excavated by avian osteoclasts increased significantly from 24 to 48 hours *in vitro* (* = $p < 0.05$). There was no further significant increase in pit number over the next 48 hours.

Figure 6. The number of resorption pits on dentin slices excavated by rat osteoclasts increased significantly (* = $p < 0.05$) from 24 to 96 hours *in vitro*. The increase at 48 hours was not significant in this experiment.

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dentin was the result of cell migration from the dentin to the coverslip. Avian osteoclasts on dentin were placed in a new well with a new coverslip. Very few cells migrated back to the glass substrate over the next 24 hours *in vitro* (Fig. 3). We tested the same hypothesis with the rat osteoclast-like cells and found that little or no migration from the dentin to the coverslip occurred (Fig. 4). We confirmed that the increase in number on cover-

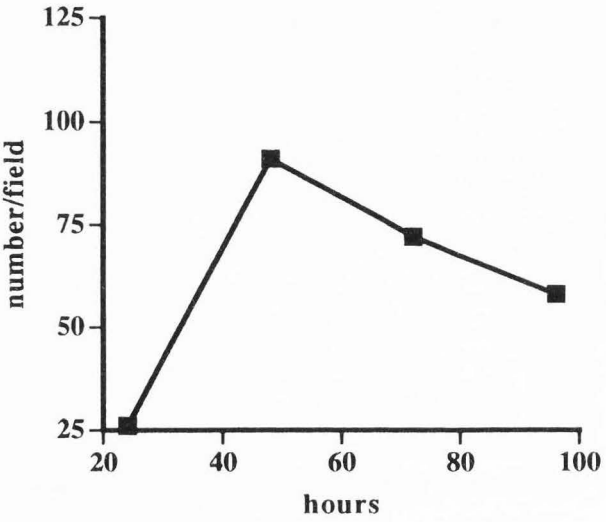

Figure 7. Using the rat model, a typical experiment is shown comparing the cell number on dentin at different times during the *in vitro* culture.

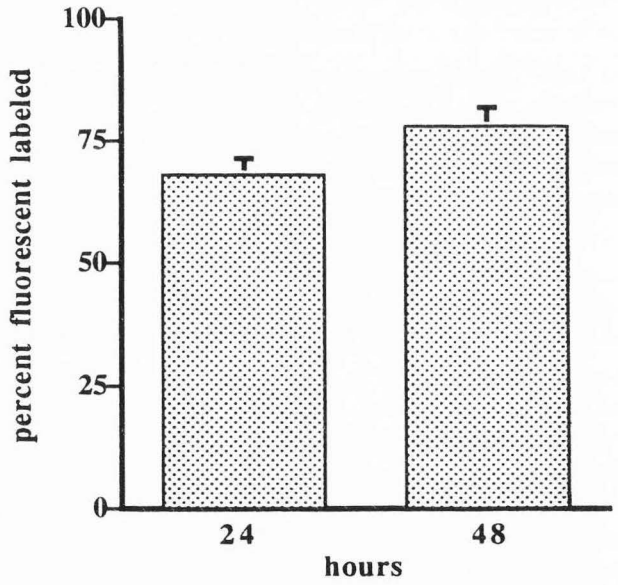

Figure 8. Fluorescent linker labeled cells were tagged on glass coverslips after 24 hours *in vitro* and allowed to migrate onto dentin slices. The first stippled bar shows the percent of cells on dentin which were labeled at 48 hours. The second stippled bar shows the percent of cells on dentin which were labeled at 72 hours. Cell numbers were decreasing on dentin by the second time interval (see Fig. 7).

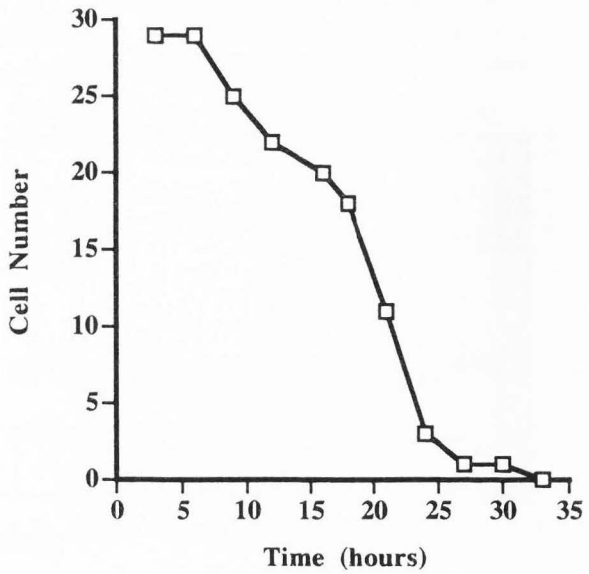

Figure 9. Analysis of time lapse cinematography documenting avian osteoclast cell death on dentin. Time zero, when cinematography was initiated, occurred at 16 hours after cell isolation. No further viable cells were seen after the 34 hour interval shown on the graph. The graph is formatted as a survival curve. The cell survival half life calculated from the time lapse cinematography is approximately 18 hours. Cell survival half life calculated from static experiments (see Fig. 1) was 14.5 hours.

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slip was due to cell proliferation and not to migration by using 5-bromo-2'-deoxyuridine staining as evidence of cell proliferation. When chick cells on coverslip were tested for proliferation during the first 24 hours of *in vitro* culture, 17.3 $\pm$ 1.1% of cells were labeled with 5-bromo-2'-deoxyuridine.

We next tested the intuitive hypothesis that resorption pit number increases over time in culture. Resorption pits are excavated by active osteoclasts and are permanent markers of previous osteoclast activity. As expected resorption pit numbers on dentin increased over time in both the avian and rat osteoclast models (Figs. 5 and 6). Figure 7 shows the changes in rat osteoclast cell number from a representative *in vitro* experiment. The decrease in cell number on dentin typically occurs after 48 hours *in vitro*. Although the initial osteoclast-like cell numbers on dentin in the rat osteoclast model suggested that migration to dentin occurred over 24 hours *in vitro*, we also used a fluorescent linker to directly label cells on the glass coverslip. 24 hours later, the percent of fluorescent cells which had migrated to dentin was counted. Figure 8 shows that fluorescent labeled cells which were originally on the glass coverslip

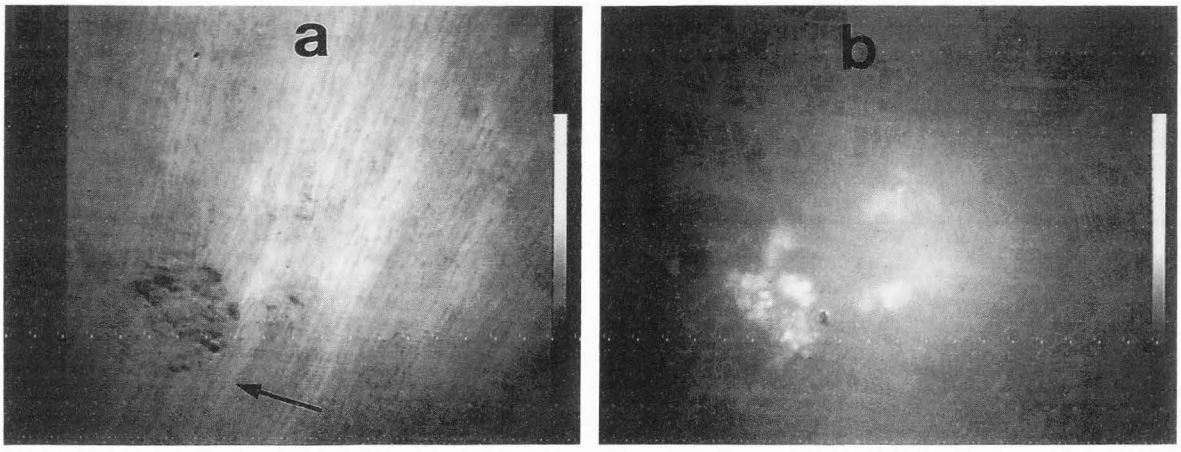

Figure 10. Apoptosis of avian osteoclasts on dentin was demonstrated using the nuclear dye Hoechst (H1399). (a) and (b) show differential interference contrast and fluorescence, respectively, of the same dentin field with a large multinucleate osteoclast (arrow).

migrated to the dentin over the 24 hour time period. However, the total cell number on dentin was decreased over 24 hours (Figs. 1 and 2). This decrease in the total cell number on dentin occurred even though new cells continued to migrate onto dentin. To investigate the disappearance of the cells on dentin, we employed time lapse cinematography of chick osteoclasts. Cell motion and then cell lysis were observed. These changes are indicative of apoptosis in other cells (Pittman *et al.*, 1993). Figure 9 shows the summary of the recordings from this cinematography. Groups of multinucleate avian osteoclasts were observed until all cells had lysed. Avian osteoclast half life was calculated as the number of hours elapsed which resulted in a loss of 50% of the observed population. The half life, which we had calculated from previous static avian osteoclast population studies, was 14.5 hours (Fig. 1). From the cinematography studies, avian osteoclast half-life on dentin was calculated at 18 hours. Similar studies were not performed on coverslip cells as these populations were increasing in number throughout the observation period. To further characterize the type of cell death which osteoclasts on dentin undergo, two nuclear dyes were employed. Hoechst bright nuclear staining was seen in approximately 50% of multinuclear osteoclasts on dentin (Fig. 10), but faded rapidly. Nuclear staining with SYTO was used to visualize all osteoclasts and the proportion of propidium iodide positive cells, indicating membrane permeability associated with apoptosis, was analyzed. Propidium iodide staining was present in 40% of multinucleate osteoclasts on dentin and in 35% of mononuclear cells on dentin. Propidium iodide was present in 14% of multinucleate osteoclast-like cells on glass and 22% of mononuclear cells on glass.

Discussion

Osteoclast mediated calcified tissue destruction has often been assessed by evaluating the number of cells and number of resorption pits on a calcified substrate which has been maintained *in vitro*. Some authors also report the ratio of cells to pits as an additional measure of osteoclast efficacy. Our studies of the kinetics of the osteoclast demonstrate a complex process of osteoclast resorption *in vitro*, starting with initial migration, followed by resorptive work and subsequent cell death. Osteoclast apoptosis appears to be a characteristic of the osteoclast lineage (Boyce *et al.*, 1995). Similar population changes have been noted before in the mouse osteoclast model; those studies also noted discrepancies in cell and pit numbers, but the mechanism of these changes were not explored (Notoya *et al.*, 1993; Tamura *et al.*, 1993). The rapid cell loss on dentin *in vitro* should be considered in concert with the ongoing recruitment of developing osteoclasts. In our studies, fluorescent markers were utilized to follow the recruitment process.

Fluorescent linkers have been utilized to follow cell migration both *in vivo* and *in vitro*. The fluorescent marker stays within the cell which has been labeled until cell death, although cell proliferation may diminish the intensity of the fluorescent signal (Greenwood and Croy, 1993; Lansdorp, 1993; Van de Langerijt *et al.*, 1994). Thus, fluorescent cells labeled on the culture dish and found 24 hours later on the dentin substrate were previously resident on the coverslip allowing us to observe the flux of cells toward the calcified substrate. As far as rationalizing the decrease in cell number on dentin, either cell death or migration from the dentin must occur. When migration from dentin was analyzed, the

small number of cells found could not explain the large decrease in population numbers. Thus, cell death was postulated to be the predominant mechanism of the loss of cells on dentin, *in vitro*. Indeed, time lapse cinematography supported previous static population studies showing a substantial decrease of cell numbers on dentin *in vitro* due to cell death. In cell death by apoptosis, many characteristic changes occur in the cell. Many of these changes are difficult to demonstrate in osteoclast populations on dentin. Analysis of DNA laddering was not possible to perform under these conditions. Detection of terminal transferases was attempted, but the dentin surface resulted in positive artifacts. Findings consistent with apoptosis were the cytoskeletal changes seen during video-cinematography, the high percentage of multinucleate cells with Hoechst bright nuclear dye staining and the higher proportion of propidium iodide positive cells seen on dentin in comparison to glass from the same culture wells.

In conclusion, it appears that osteoclast resorption of dentin *in vitro* is a dynamic process. Osteoclasts actively resorb calcified substrate while new precursors are migrating to dentin and replacing the rapidly dying osteoclasts. While there is no direct connection between the cell number and the pit number at a specific time point *in vitro*, osteoclasts which have been present on the dentin have left pits as a record of cellular activity. Cell number and pit number at a specific time point each give some information on the *in vitro* resorptive history of the population originally plated. New waves of competent osteoclasts produce resorption pits before lysing. Thus, the resorption pit number reflects the total *in vitro* history of the populations of osteoclasts which have been recruited to the calcified substrate, have resorbed calcified tissue and then died. The cell number on dentin at any time reflects the dual processes of recruitment and lysis which are present at the time of fixation. Osteoclast cell number on dentin is a summation of the complex relationship between the rate of precursor recruitment and the rate of cell death.

Acknowledgments

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Discussion with Reviewers

S.C. Miller: It would be of interest to the *in vivo* biologist if the authors would consider some of the older literature and compare their kinetics of osteoclast formation and turnover with the kinetic studies (using thymidine) of Tonna, Kember, Young, Jee and others.

Authors: We have reviewed some of the relevant literature using tritiated thymidine (see additional references) and have found the reference to mouse osteoclast half-life. In one of these studies, ^{3}H-thymidine labeling first appeared in osteoclasts at 9-36 hours after the label was injected and labeling could be detected for up to 2 weeks. However, the authors question using this data to calculate a lifetime for osteoclasts because "it is difficult to speak of a duration time for osteoclasts since no convincing experimental evidence is yet available which determines whether fusion of precursors is a singular act or a continuous phenomenon" (Tonna, 1966). Additionally, it is now appreciated that ^{3}H-thymidine is recycled in many tissues and may appear in cells which were not present at the time of injection. In a second study, the first time point where labeled osteoclast nuclei are seen is at 41 hours and there are still labeled nuclei at the end of the experiment at 377 hours. These are studies from rats weighing 100 grams (Miller *et al.*, 1977). In a third *in vivo* experiment, osteoclast nuclei from 100 gram rats were first labeled at about the same time interval, but the label had disappeared by 5 days post injection (Kimmel and Jee, 1980). These studies suggest that *in vivo* osteoclasts may have a longer half-life than *in vitro*.

K.M. Kim: "Osteoclast recruitment" should be clearly defined. Is it from the beginning of the precursor migration to the point of beginning or ending of multinucleation?

Authors: We have used the neonatal rat model to explore the beginning of osteoclast recruitment as defined by the initial migration of a mixture of TRAP negative and TRAP positive mononuclear cells to the calcified substrate. In addition, we have documented that this migration event occurs repetitively over time from the initial plating until at least 72 hours *in vitro*. At later time points, almost all osteoclast precursors on dentin slices fuse and become multinucleate and TRAP positive, but these time-points are nearer to 96 hours after initial plating in this model. There are obviously some multinucleate cells and there are pits being formed, but the majority of cells at early time points are mononuclear.

K.M. Kim: What cells were counted to determine the half-life of osteoclasts? Was it at the beginning of multinucleation? If not, how was the heterogeneity of cells and their stages of maturation handled? Did only precursor but no preformed osteoclasts migrate to dentin?

Authors: To determine the half-life of avian osteoclasts, TRAP positive multinuclear cells on dentin slices were counted at 24, 48 and 96 hours after initial plating. Results were graphed versus time and a half-life calculated. Half life was defined as the elapsed time required to halve the observed population, assuming an exponential decay of the population initially present.

$$t_{1/2} = t2 - t1,$$

where $N_{t2} = [N_{t1}/2]$; $t_{1/2}$ = osteoclast half-life; t1 = first observation interval, t2 = second observation interval, N_{t1} = number of osteoclasts observed at time t1, N_{t2} = number of osteoclasts observed at time t2.

K.M. Kim: What benefit did the stain (fluorescent linker) provide for tracking of the cells?

Authors: At the first sampled time, 48 hours, the cells labeled by the fluorescent marker and counted on the dentin slice were previously resident on glass. The cells on dentin which were not labeled were resident on dentin but had migrated there since the time of initial plating, but before the labeling. After labeling, two consecutive 24 hour periods were sampled for the ratio of unlabeled to labeled cells on dentin. If no cells had migrated, the percentage of labeled cells on the dentin slice would be zero. If all cells on dentin had disappeared, and other cells had also migrated from the glass, the percentage of labeled cells on dentin would be 100%. For the first 24 hour period sampled, the percentage of labeled cells was approximately 75%. We interpreted this as evidence that three quarters of the cells on dentin had migrated there in the last 24 hours. For the second 24 hour period, the percentage of cells labeled was slightly larger, near 85% labeled. This suggested that, assuming that the rate of migration onto the dentin is constant, more unlabeled cells were disappearing from the dentin. However, if the total number of cells on dentin is counted during this interval, the total number of cells has decreased (see Fig. 7). We consider that these two parts of the experiment taken together indicate a loss of cells predominately from the earliest unlabeled cohort, with a less severe loss of cells from the next cohort of cells which arrived on the dentin soon after labeling.

K.M. Kim: Apoptotic cells display characteristic morphological changes of cell shrinkage, nuclear chromatin condensation and blebbing, or DNA fragmentation giving the ladder pattern on agar gel. The references cited indeed used these criteria for identification of apoptosis in their studies. At least, a detailed description of morphological changes characteristic of apoptosis should be included. Loss of cells and the lack of cell migration from dentin to coverslips are hardly sufficient for the claimed occurrence of apoptosis. In fact, macrophages have been shown to ingest apatite and may eventually be killed by crystals (van der Meulen *et al.*, 1993). Please elaborate the sequence of changes observed at the time of cell death.

Reviewer III: It is generally accepted that the bone and marrow cells (osteoclast precursors) migrate, mitose, fuse and die *in vitro*, and that the numbers of pits will increase as long as osteoclasts remain alive or are generated from precursors.

Authors: Blebbing and cell shrinkage were seen before cell lysis on video cinematography of avian osteoclasts on dentin. Osteoclast nuclei on dentin were stained with Hoechst dye and showed bright staining typical of apoptosis (Fig. 10) SYTO, a nuclear staining dye which images all nuclei and is replaced by propidium iodide if cells are permeable, as in early apoptosis, was used to quantify the number of apoptotic osteoclasts on dentin versus glass. The results show that 40% of osteoclasts on dentin are apoptotic compared to 14% on glass which is consistent with osteoclast apoptosis on the dentin substrate.

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