Cells and Materials

Volume 3 | Number 3 Article 2

1993

Osteoclastic Resorption of Calcium Phosphate Ceramic Thin Films

J. E. Davies
University of Toronto

G. Shapiro
University of Toronto

B. F. Lowenberg
University of Toronto

Cells and Materials, Vol. 3, No. 3, 1993 (Pages 245-256) 1051-6794/93$5.00+.00
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

OSTEOCLASTIC RESORPTION OF CALCIUM PHOSPHATE CERAMIC THIN FILMS

J.E. Davies*, G. Shapiro and B.F. Lowenberg

Centre for Biomaterials, University of Toronto,
170 College Street, Toronto, Ontario, Canada, M5S 1A1

(Received for August 5, 1993, and in revised form September 30, 1993)

Abstract

Sub-micron calcium phosphate ceramic thin films were formed by vertically dipping transparent quartz plates in a particulate sol-gel suspension. Primary adult rat bone marrow cell populations were cultured on the ceramic thin films in conditions known to allow the differentiation of cells of the osteoclast lineage. Monitoring the cultures for periods of 11 to 28 days revealed the creation of resorption lacunae in the thin films by multinucleate cells. Some cultures were heated at 42°C overnight to remove adherent cells; using bright field light microscopy (LM), after staining with silver nitrate, the degree of resorption could be easily assessed. Other cultures were fixed and stained for tartrate-resistant acid phosphatase (TRAP) activity and prepared for LM and/or scanning electron microscopy (SEM). Examination of the cultures, following fixation, showed the multinucleate cells associated with resorption lacunae to be TRAP positive. The nuclearity of the cells varied considerably. SEM showed that the cells had resorbed the thin films to produce discrete resorption lacunae similar to those found in normal bone tissue. From their morphology, TRAP positive staining, and resorptive activity, the cells were considered to be osteoclasts. The size of individual or combined lacunae varied from $< 10\ \mu m$ to ~ 1 mm. These thin film culture substrata may be employed to investigate the function of individual resorbing cells or, especially after removal of the adherent cell layer, easily quantify resorption which is the major functional activity of osteoclasts. We conclude that these thin film ceramic culture substrata can be used as alternatives to bone slices in osteoclast resorption assays and thus could be employed to investigate both the functional and metabolic activities of osteoclasts.

Key Words: Ceramic thin films, calcium phosphate, osteoclasts, resorption, lacunae, *in vitro*, resorption assay.

*Address for correspondence:
J.E. Davies,
Centre for Biomaterials, University of Toronto,
170 College Street, Toronto, Ontario, Canada M5S 1A1
Phone no.: (416) 978-1471 / FAX no.: (416) 978-1462

Introduction

The cells which make bone, osteoblasts, and those which resorb bone, osteoclasts, have very precise functions and the balance between their activities is critical to the maintenance of the skeletal system. Osteoclasts are not only of central importance in modeling abnormalities, such as osteopetrosis, which is characterized by hypofunction, and Paget's disease where increased bone resorptive activity is seen, but also in some of the major metabolic bone diseases, which are generalized skeletal disorders. In these disease processes, the normal function of bone cells is modified. To assess the degree of perturbation of cell behaviour and how this may be further modified by the action of pharmaceutic agents is of central importance to both an understanding of the disease processes and the functions of the cells themselves.

Evidently, the activity of osteoclasts related to the overall bone remodeling process can only be measured *in vivo* but the activities of individual cells is difficult to assess. For this reason, several research groups have developed methods to directly observe the activity of isolated osteoclasts *in vitro*. Indeed, considerable success has been achieved when osteoclasts have been cultured on thin slices of either sperm whale dentine (Boyde *et al.*, 1984) or bone (Chambers *et al.*, 1984). These cells were shown to demonstrate resorptive activity which is not possessed by other cells of the mononuclear phagocyte series (Chambers and Horton, 1984). More recently, attempts to use other cell harvesting techniques to study osteoclast lineage have still had to rely on the use of cortical bone slices (Amano *et al.*, 1992, Kerby *et al.*, 1992) or dentine (Tamura *et al.*, 1993) as a resorption assay substratum. Using such translucent and relatively thick substrata, quantitation of the resorption activity relies upon either two dimensional analysis of resorption pits of variable depth, stereo mapping to compute the resorption volume (Boyde *et al.*, 1986) or, more recently, confocal microscopy (Jones and Boyde, 1993). The latter methods provide far greater accuracy when assessing resorption of thick substrata but, as has been pointed out such precise methods, while desirable, are not only expensive on time and apparatus (Jones and Boyde, 1993) but also require highly skilled personnel,

and, therefore, do not represent potentially routine or simple assays.

In order to take advantage of the ease with which osteoclasts may be cultured, but to avoid the complexities of using biological hard tissue as the culture substratum, we report herein our preliminary results using submicron thick calcium phosphate ceramic films as osteoclast resorption substrata.

Materials and Methods

Preparation of thin films

Fused quartz plates [half inch (1.27 cm) diameter, 1/8 inch (~ 3 mm) thick, G1 commercial grade, from Esco Products, Oak Ridge, NJ] were used as supports for the creation of calcium phosphate sol-gel thin films. The plates were cleaned by boiling in double distilled and deionized water (DDH_2O). Further cleaning with chromic-sulfuric acid cleaning solution (Fisher Scientific, Fair Lawn, NJ) was followed by two further periods of sonication, for one hour, and storage in DDH_2O.

Hydroxyapatite powder suspension was first created by adding, drop by drop, an ammonium dihydrogen phosphate solution (0.691 g $NH_3H_2PO_4$ in 30 ml DDH_2O at pH ~ 12) to a solution of calcium nitrate (2.361 g $Ca(NO_3)2.4H_2O$ in 20 ml DDH_2O at pH ~ 12) with constant stirring. In each case, the pH adjustments were made using concentrated 29.5% ammonium hydroxide. The mixture was further stirred at room temperature for 24 hours and the colloidal suspension aged, resulting in gelation, for a period of five days by standing at room temperature.

The thin films were made by vertically dipping the quartz plates into the particulate suspension following which they were fired, in air, at 1000°C for 1 hour. This sintering procedure resulted in the creation of a cell culture device (CCD) comprising an adherent calcium phosphate film, of approximately 0.5 μm thickness, on the underlying quartz support (now available from Millenium Biologix Inc., Kingston, ON). Cell culture devices, so prepared, were introduced into individual wells of 24-well culture plates. The latter were then individually sealed in plastic bags and sterilization was achieved by subjecting the packages to 2.5 Mrads ^{60}Co irradiation. This preparation procedure is illustrated in Figure 1.

Rat marrow cell culture

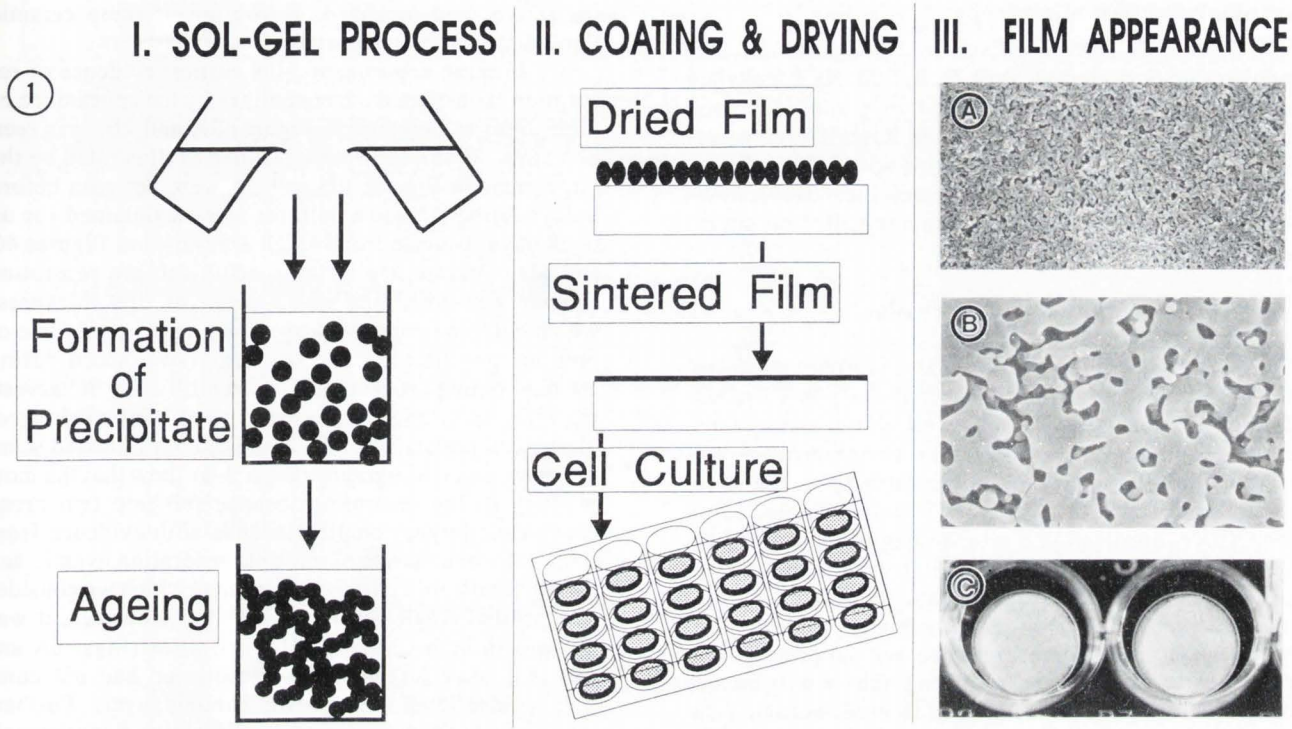
Bone marrow cells were obtained from femoral washouts of young adult male Wistar rats (approximately 120 gm). One of two culture media were employed. The first was that which we have described previously comprising α-Minimal Essential Medium (α-MEM) supplemented with 15% foetal bovine serum, 50 μg/ml of freshly-prepared ascorbic acid, 10 mM Na ß-glycerophosphate, 0.1 mg/ml penicillin G, 0.05 mg/ml gentamicin and 0.3 μg/ml fungizone (Davies *et al.*, 1991). The second was based on that used by Sato and Grasser (1990) comprising Medium 199 (Gibco, NY), 10% foetal bovine serum, and 10% of the antibiotics listed above. In every case, aliquots (1 ml) of the rat bone marrow (RBM) cell suspension were inoculated onto the CCD's. Due to the heterogeneity of the cell population, cell counting was not undertaken as this would not provide data pertinent to the osteoclast, or pre-osteoclast, populations alone. The medium was removed after the first 24 hours to remove non-adherent cells and replaced with 2 ml of the same medium. Subsequently, the medium was renewed at three day intervals up to two weeks and weekly thereafter and the cultures were maintained for up to 28 days in a humidified atmosphere of 95% air with 5% CO_2. Cultures were prepared for light (LM) and/or scanning electron microscopy (SEM) at this stage. In some cases, to remove adherent cells from the thin films prior to fixation, discs were incubated, in air, at 42°C overnight.

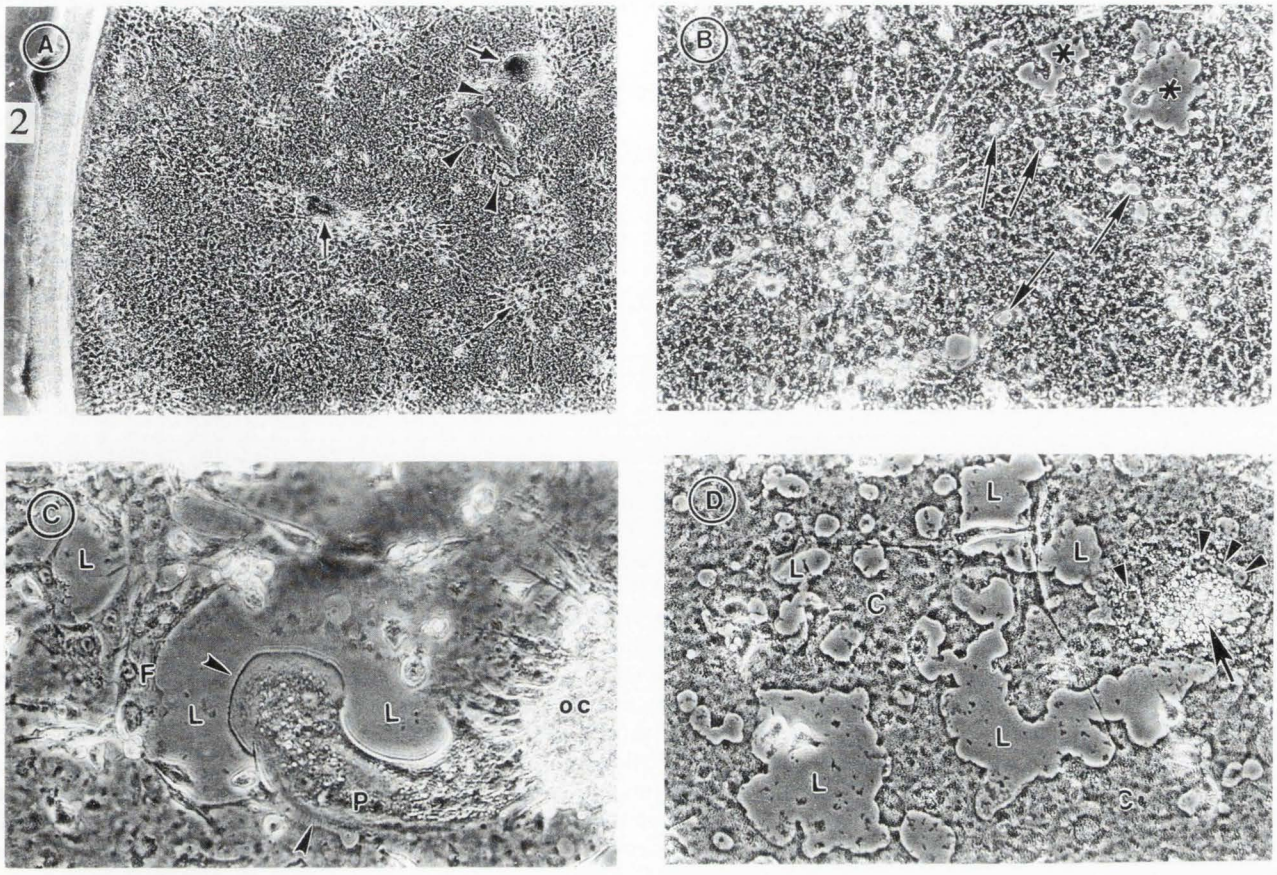
Figure 1. Schematic representation of the preparation of the cell culture devices. The insets show (**A**) the hydroxyapatite powder dipped layer before and (**B**) after sintering. (**C**) Shows two wells of a 24 well culture plate, each containing a CCD immediately prior to culture. Field Widths (FW): A & B: 3.9 μm; C: 42.4 mm.

Figure 2. Appearance of CCD's photographed using phase optics, **A-C** during culture in 24 well dishes; **D** following fixation. (**A**) Shows a large field including the edge of the underlying half inch diameter quartz disc. Cells, which are predominantly arranged in clumps (arrows), appear refractile against the "ground glass" appearance created by the ceramic thin film. Even at this low magnification (viewed using a x 4 objective lens) resorption lacunae of varying size are evident (arrowheads). (**B**) Variation in size and shape of lacunae is evident, with the smaller lacunae being round or ovoid (arrows) while larger lacunae are multilobed (*). (**C**) Osteoclasts were sometimes observed to completely fill a lacuna or individual pseudopodia (p) would extend into a lacuna (L) from a neighbouring cell body (oc). Note the fimbriated border of this cell (arrowheads) and the fibroblast-like cells (F) which are bridging the lacuna. (**D**) Following fixation and transfer to a 35 mm dish, fields of multiple resorption lacunae (L) can be more clearly seen. In this field of view only a few small cells are seen within the lacunae, while a large foamy cell (arrow), with several distinct nuclei (arrowheads) is visible on the ceramic thin film (C). FW: A: 3.1 mm; B: 1.1 mm; C: 300 μm; D: 1 mm.

Light microscopy

Light microscopy was performed both during and after the culture period using inverted phase optics. Some fixed samples were stained for tartrate resistant acid phosphatase (TRAP) using a modification of the azo dye method of Barka (1960). Those samples, from which the cells had been removed, were stained with 5% silver nitrate for two hours, fixed in 5% sodium carbonate in 10% formalin, and examined by bright field optics.


I. SOL-GEL PROCESS
II. COATING & DRYING
III. FILM APPEARANCE
1
Formation of Precipitate
Ageing
Dried Film
Sintered Film
Cell Culture
A
B
C


2
A
B
C
D
L
F
P
OC
C

Scanning electron microscopy

Some CCD's were fixed in 2.0% paraformaldehyde in 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.3), then dehydrated in graded alcohols, critical-point dried from CO_2 (Ladd Research Industries Inc., Burlington VT), sputter-coated with gold (approximately 10 nm) (Polaron Instrument Inc., Doylestown PA) and examined in a Hitachi scanning electron microscope (models 2500 or 2700).

Results and Discussion

The body of these preliminary, morphological, results are provided in the figure captions. In all cases, cells settled on the CCD's as well as the areas of the culture wells not covered by discs. The granular ceramic thin film disrupted the optical pathway in the phase microscope and thus, while cells could be detected on the CCD's, details of cell morphology were not evident. This feature of the CCD's was important since, as resorption lacunae were produced, and the ceramic was removed, the underlying quartz support was exposed. Since the latter has almost identical optical properties to glass, cells which were resorbing the thin films, or others which occupied the created lacunae, became visualized in normal phase optics and were thus clearly discernible from those remaining on the film surface. The lacunae were clearly visualized at low magnifications, both during and after culture (Figs. 2-4). In unstained preparations, large, multinucleate osteoclasts could only be identified, employing phase optics, on the ceramic thin films by their foamy cytoplasmic character (Figs. 2D, 4A, 5A). However, during culture, once the ceramic film had been resorbed, osteoclasts or their pseudopodial extensions (Fig. 2C) were clearly visualized. In time-lapse video studies (to be reported elsewhere), we have monitored the movement of osteoclasts, of varying sizes, in and out of developing resorption lacunae. Furthermore, when similar ceramic thin films are employed for osteogenic cell cultures, no resorption lacunae were observed (Qiu *et al.*, 1993). These observations, together with both the histochemical and morphological data provided herein, would indicate that the cells responsible for active, and localized, resorption of these ceramic thin films can be designated as osteoclasts.

In these experiments, the earliest evidence of resorption, as assessed during culture by the appearance of clear areas as depicted in Figures 2A and 2B, was seen at 5 days. Frank resorption lacunae as illustrated by the large areas in Figures 2D and 3A were not seen before 8 days, although some cultures were maintained for up to 28 days, lacunae from which are shown in Figures 4C and 4D. Variability in the time to achieve resorption lacunae was noted and was a result of film thickness, which could be controlled by adjusting (a) either one or both the gel viscosity and the withdrawal speed during the dip-coating procedure, (b) variability in cell harvesting efficiency, and (c) the maturity of the plated osteoclastic cell population. Clearly, both our light and scanning electron micrographs (Figs. 2-6) show that the morphology of the resorption lacunae fell into two broad categories: large, complex lacunae with evidence from the lacuna boundaries of multiple resorption events, and small, almost round or ovoid, lacunae which we consider to represent single resorption events. However, it was obvious in both LM (Fig. 4D) and SEM (Figs. 6A and 6B) that some large areas of resorption had not completely penetrated the thin film ceramic layer. Furthermore, closer examination of the resorption floors revealed that the surface of the quartz had been changed as a result of the sintering procedure (not shown). In particular, a fine tessellated grain structure was observed although it is unclear at the present time whether this represented a unique interfacial phase or a reflection of the grain structure of the calcium phosphate layer apposed to the quartz surface.

Cellular resorption of solid (rather than phagocytosis of particulate) synthetic calcium phosphates *in vivo* was, until recently, an unknown phenomenon. Osteoclasts had not been definitively identified at either light or transmission electron microscope (TEM) level as being responsible for resorbing artificial substrata, in spite of numerous reports of multinucleate cell contact with ceramics. Such cells, in contact with artificial material only, have been called "ceramoclasts" by Osborn (1988). Recently however, we reported the results of experiments which have provided some optimism in being able

---

Figure 4. **A-D** show the appearance of CCD's following culture and fixation. **A** and **B** show the same lacuna area photographed before and after TRAP staining. The shape of the resorption lacuna (L), a complex multilobed structure which is almost devoid of cells, can be clearly seen in normal phase optics (A). Staining reveals a group of five large multinucleate TRAP-positive cells surrounding the resorption lacuna as well as other cells of fibroblast-like morphology on the ceramic surface. Due to the pH (5.1) of the TRAP staining reaction, the calcium phosphate thin film has been partially, but ubiquitously, dissolved to render the film thinner and more difficult to visualize, and thus the lacunae are less evident than seen before TRAP staining. To aid in comparison, the cells indicated (arrows) are easily distinguished in both images. **C** and **D** show the appearance of CCD's following 28 days of culture having removed the cells (as described in text). The remaining ceramic thin film has been stained using silver nitrate and photographed dry, using bright field optics. (**C**) Low magnification view showing a field of resorption lacunae of various sizes. Note that the smaller lacunae are punctate in appearance and do not possess the partially resorbed rim seen in the larger lacunae. (**D**) Higher magnification [rotated] of the lacuna (marked by an * in C) shows details of the resorptive border, and floor, of a large lacuna (maximum dimension from top to bottom left is 0.72 mm) in which smaller areas are apparently cleared through to the underlying quartz support (arrows). FW: A: 600 μm; B: 1 mm; C: 2.12 mm; D: 0.85 mm.

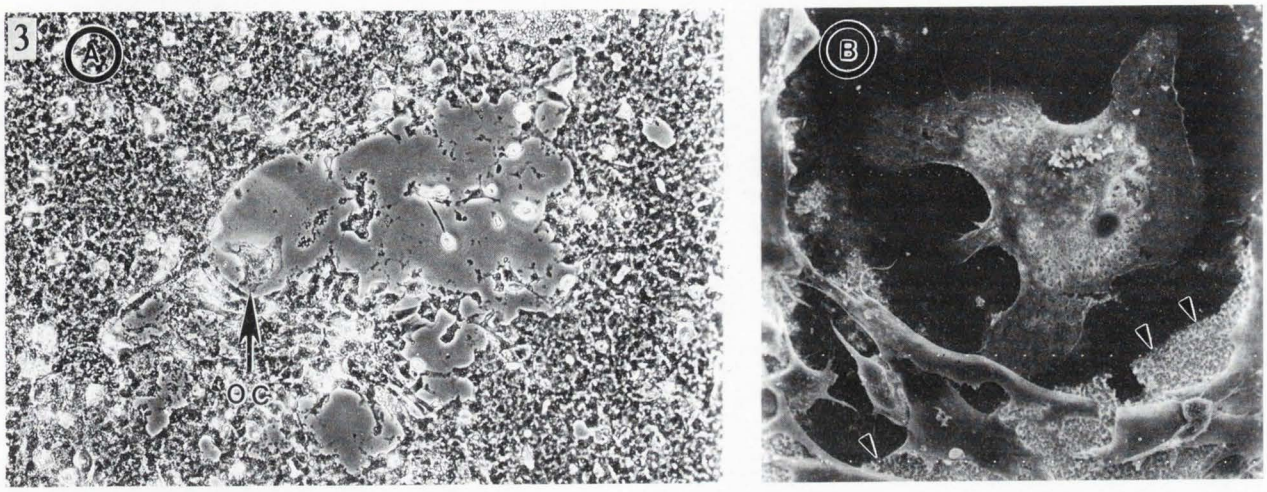
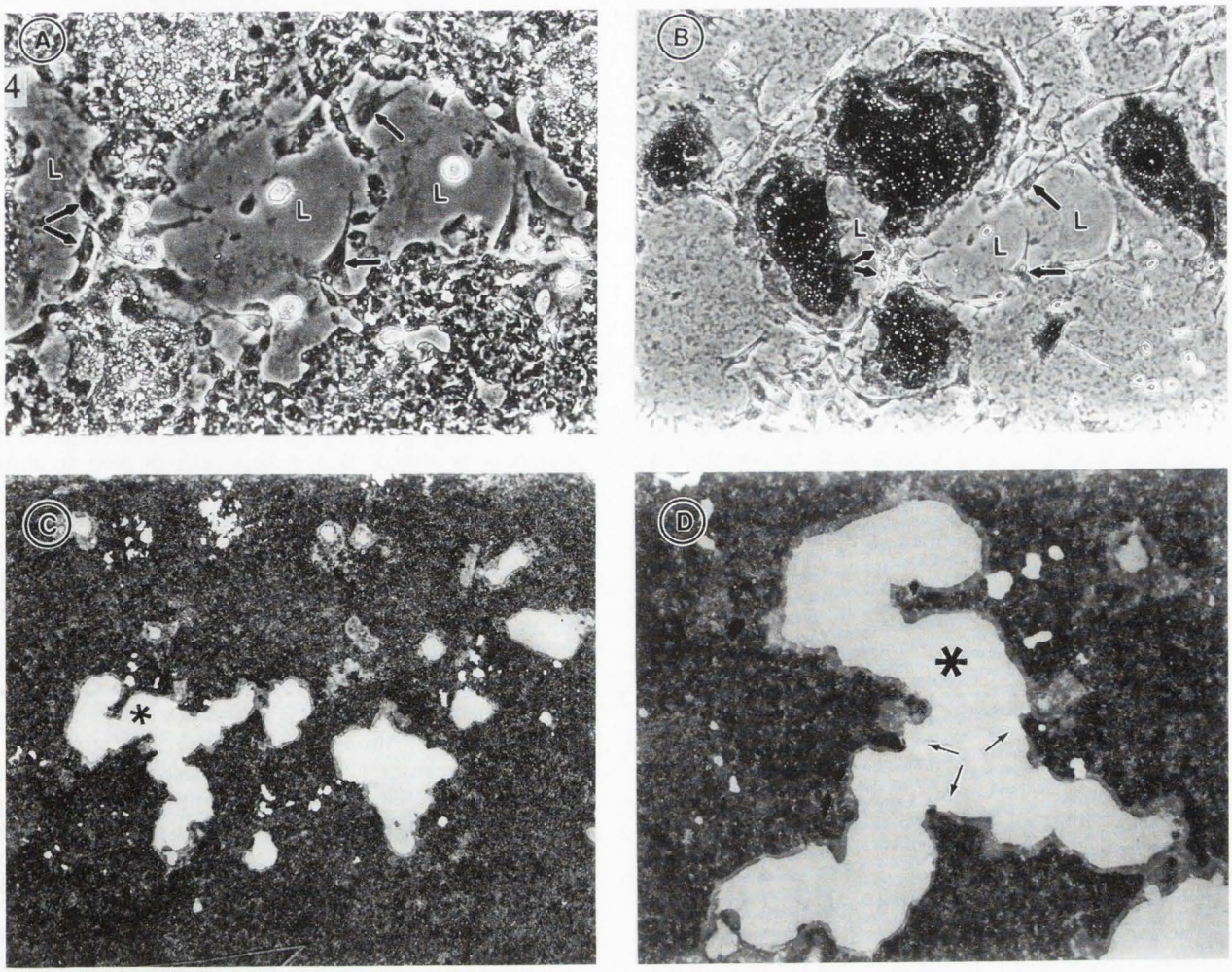


Figure 3. **(A)** Shows a single large (maximum dimension = 1 mm) multilobed lacuna, following 11 days culture and fixation, photographed using phase optics. The osteoclast (OC) is shown at higher magnification by SEM in **(B)** where the rim of the resorption lacuna is also clearly visible (arrowheads). FW: A: 1.1 mm; B: 150 μm.



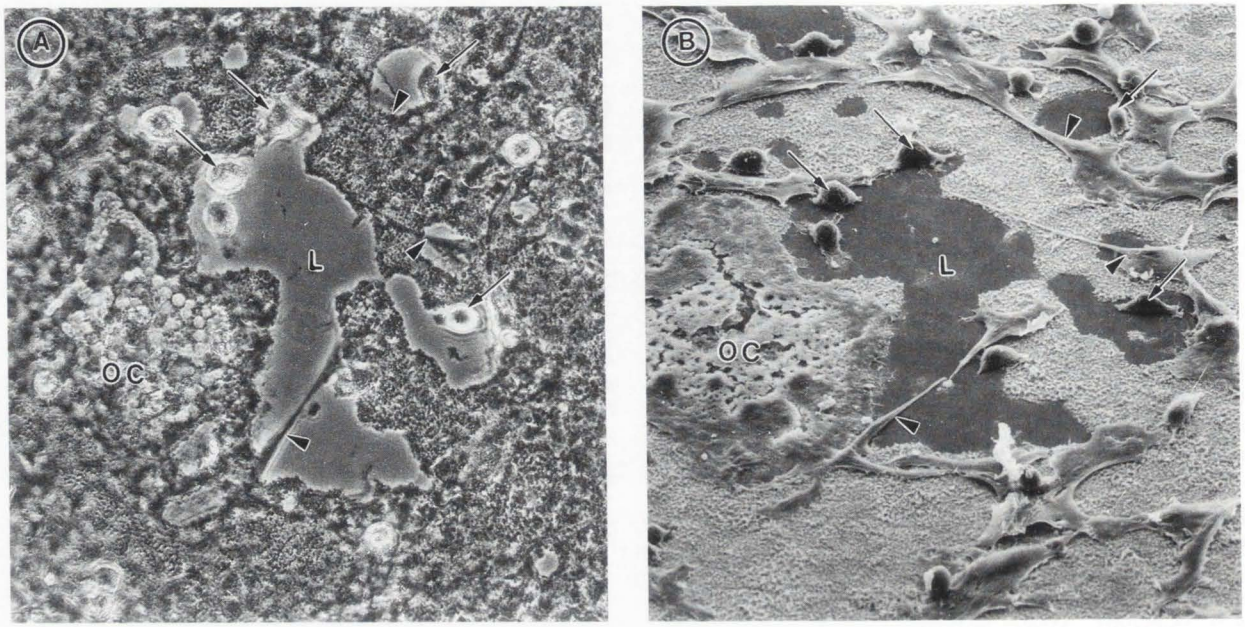


Figure 5. (**A**) A similar giant cell (OC) to those shown in Figures 2D, 4A and 4B can be seen, in phase optics during culture, to the left of the large central lacuna (L). Other smaller cells, exhibiting a refractile skirt, are seen within the resorption lacunae (arrows) as well as cells exhibiting fibroblast morphology bridging the lacunae (arrowheads). (**B**) The same field of view visualized by SEM [45° tilt and dynamic focus]. The osteoclast (OC), lying on the ceramic thin film, exhibits a vacuolar appearance. The small cells (arrows) and fibroblast-like cells (arrowheads) are the same as those in (A). This preparation was TRAP stained before examination by SEM. This additional aqueous processing has resulted in the poor condition of the osteoclast in (B). FW: A: 240 μm; B: 223 μm.

to both investigate and monitor cellular resorption of calcium phosphates by osteoclasts *in vivo*. One experimental technique described a novel method of enabling bioactive ceramics, implanted in the feline post-canine diastema, to be challenged by osteoclastic resorption fronts generated by orthodontic tooth movement (Davies and Brady, 1990). Another report described several of the classical morphological features of osteoclasts (i.e., multinucleate cell, brush border, clear zone, cytoplasmic inclusions) in cells sited simultaneously in resorption lacunae in the host bone of the implant site, and the artificial calcium phosphate substratum (Davies *et al*, 1989). We have since conducted several *in vitro* experiments to study osteoclast reactions to various calcium phosphate materials. In the latter, we have shown that, while multinucleate TRAP positive cells will colonize almost all substrata, only certain preparations of calcium hydroxyapatite will be actively resorbed by osteoclasts (Gomi *et al.*, 1993a, b). Our cell culture method, employing mixed rat bone marrow primary cell populations, is adapted from a method developed in our laboratories by Maniatopoulos *et al.* (1988) and has also been successfully employed to demonstrate the resorption of living bone tissue in culture (Lowenberg *et al.*, 1990; Davies *et al.*, 1991).

As mentioned above, the examination of osteoclast behaviour *in vitro* is not new. However, the use of artificial calcium phosphate preparations as substrata for osteoclast culture, which would be a logical alternative to a biological substratum, has met with little success. Jones *et al.* (1984) reported that osteoclasts will resorb synthetic apatites *in vitro* although these results were not illustrated. More recently, Shimizu *et al.* (1989) have reported that isolated osteoclasts will resorb only devitalized bone surfaces and not synthetic hydroxyapatite.

Our present results clearly demonstrate that osteoclasts of various sizes were involved in resorption of the ceramic thin films. Since these cells showed no ability to resorb the underlying quartz, we assume that continued resorptive function led to lateral expansion of the lacunae resulting in removal of larger surface areas than normally reported with the resorption of biological hard tissue. Even when thin, 10 μm, bone slices are prepared, itself a time-consuming process, the depth of the slice exceeds that of the resorption pits created (Kanehisa and Heersche, 1988), although in thicker substrata pits may exceed 10 μm in depth (S.J. Jones, personal communication). Thus, while resorption pits in bone slices can be visualized by phase microscopy during culture, computation of resorption has to rely on SEM stereophotomicrography or optical slicing by confocal microscopy. As the ceramic thin films were essentially plano-parallel, and orders of magnitude thinner than prepared slices of bone or dentine, the depth to which the cells resorb to create a lacuna can be both controlled by thin film processing and easily factored

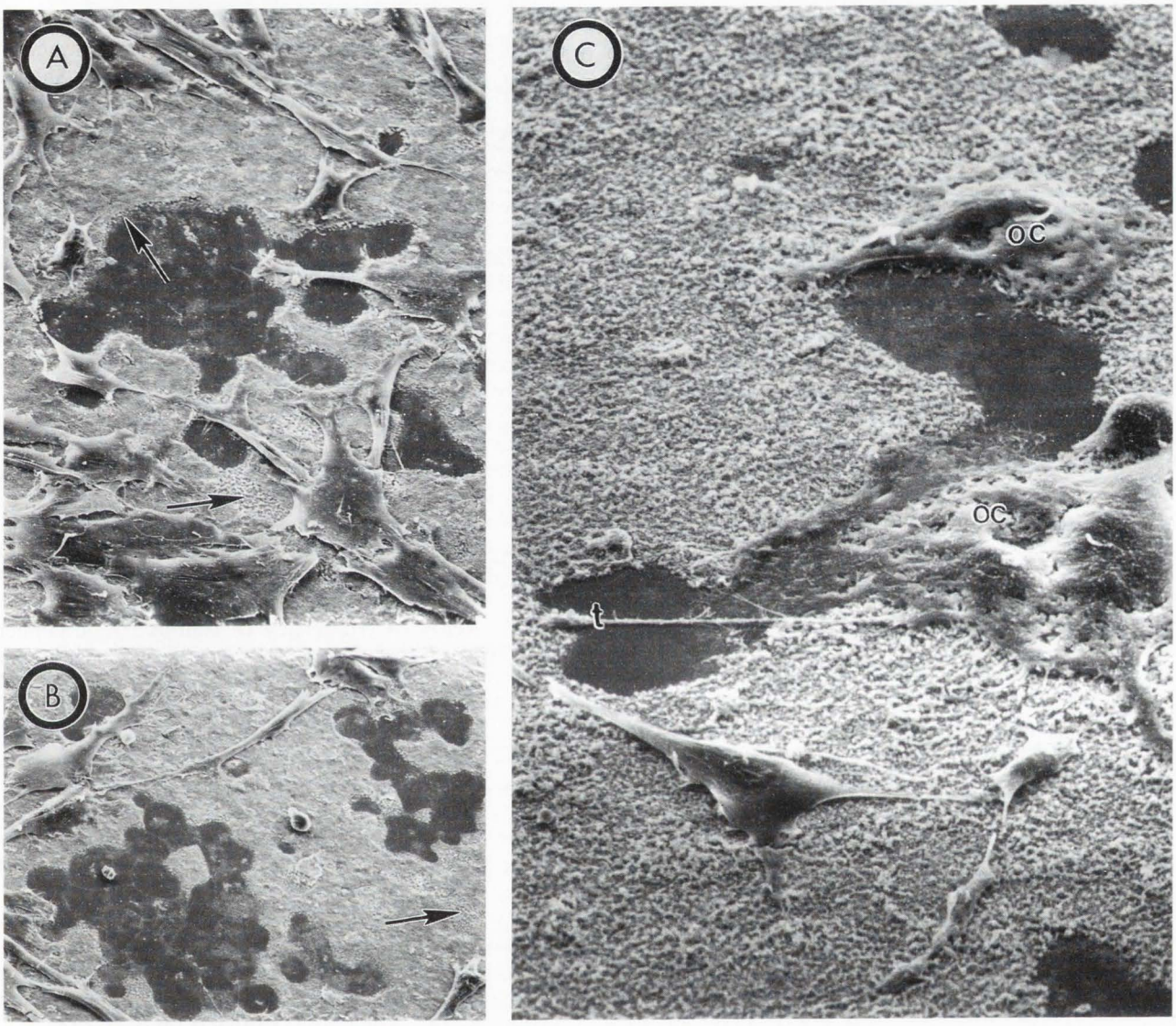


Figure 6. In some thicker films (A, B) the margins of the lacunae, and the relative depth of penetration of the osteoclasts through the films, could be easily visualized [45° tilt and dynamic focus]. (**A**) A multilobed lacuna, evidence of multiple resorption events, is radiating form the centre of the field of view. Some residual ceramic remains in the floor of the lacuna, while around the margins, partial thickness resorption is evident (arrows). (**B**) These lacunae exhibit clearly demarcated resorption events, while some smaller lacunae have not completely penetrated the thin film. The serpiginous appearance of the lacuna on the right is similar to those reported in bone slices. In some areas partial resorption is evident (arrow). (**C**) Two osteoclasts (OC) are associated with this single lacuna. Each osteoclast clearly exhibits a thicker, central area partially surrounded by a broad zone where the dorsal cytoplasm has collapsed in punctate fashion corresponding to the vacuolar regions seen in light microscopy [compare with Fig. 5A], peripheral to which there is a flat cytoplasmic skirt. Several areas of the cell periphery exhibit fimbriated borders. FW: A: 173 μm; B: 285 μm; C: 104 μm.

---

into a calculation of resorption volume. The potential to easily eradicate the inaccuracies associated with computation of resorption volume in slices of biological hard tissue offered by the use of ceramic thin films, such as those shown here, is therefore evident. The staining of the films after removal of cells to rapidly assess the degree of resorption is a particular advantage offered by these culture substrata.

The preliminary work reported herein has also allowed us to examine the morphology of the resorption lacunae created by the osteoclasts and compare these to similar lacunae produced in biological hard tissue

substrata (Jones *et al.*, 1984). Of particular interest is that the variety of resorption configurations discussed by Taylor *et al.* (1989) as typical of biological matrices, small pits spreading radially, or shallow resorption below large ruffled border zones, seem to be reproduced in these artificial substrata.

Conclusions

Thin film calcium phosphate ceramics may be used as culture substrata for resorptively active osteoclasts. The resorption lacunae created are similar in morphology, although larger, than their equivalents on biological hard tissue slices. Visualization of the creation of lacunae during culture is straightforward in normal inverted phase optics and imaging the resultant lacunae following culture lends itself to simple quantification methods.

Acknowledgments

We are grateful to several colleagues who have provided important contributions to this work. First, we should like to acknowledge Mike Sayer for so enthusiastically introducing us to sol-gel thin film technology. Our thanks to Raisa Yakubovich for help with film production and Bob Chernecky and Amy Shiga for the photomicrography. Financial support was received from the Medical Research Council (Canada) programme grant #11439.

References

Amano S, Hanazawa S, Kawata Y, Ohta K, Kitami H, Kitano S (1992). An assay system utilizing devitalized bone for assessment of differentiation of osteoclast progenitors. J. Bone Min. Res. **7**, 321-328.

Barka T (1960). A simple azo dye method for histochemical demonstration of acid phosphatase. Nature **187**, 248-249.

Boyde A, Ali NN, Jones SJ (1984). Resorption of dentine by isolated osteoclasts in vitro. Br. Dent. J. **156**, 210-216.

Boyde A, Howell PGT, Frank F (1986). Simple SEM stereophotogrammetric method for three dimensional evaluation of features on flat substrates. J. Microsc. **143**, 257-264.

Chambers TJ, Horton MA (1984). Failure of cells of the mononuclear phagocyte series to resorb bone. Calcif. Tissue Int. **36**, 556-558.

Chambers TJ, Revell PA, Fuller K, Athanasou NA (1984). Resorption of bone by isolated rabbit osteoclasts. J. Cell Sci. **66**, 383-399.

Davies JE, Brady J (1990). A method to assess the response of a bone substitute material to osteoclasis. In: Handbook of Bioactive Ceramics, Vol. 2. CRC Press, Boca Raton, FL. pp. 283-294

Davies JE, Nagai N, Takeshita N (1989). Osteogenesis and osteoclasis at the interface with a bioactive bone-substitute. In: Transactions, 15th Ann. Meet. Soc. for Biomaterials (Minneapolis, MN). p. 5 (abstract).

Davies JE, Chernecky R, Lowenberg B, Shiga A (1991). Deposition and resorption of bone by rat marrow cells *in vitro*. Cells and Materials **1**, 3-15.

Gomi K, de Bruijn JD, Ogura M, Davies JE (1993a). The effect of substratum roughness on osteoclast-like cells *in vitro*. Cells and Materials **3**, 151-160.

Gomi K, Lowenberg B, Shapiro G, Davies JE (1993b). Resorption of sintered synthetic hydroxyapatite by osteoclasts *in vitro*. Biomaterials **14**, 91-96.

Jones SJ, Boyde A (1993). Histomorphometry of Howship's lacunae formed *in vivo* and *in vitro*: Depths and volumes measured by scanning electron and confocal microscopy. Bone **14**, 455-460.

Jones SJ, Boyde A, Ali NN (1984). The resorption of biological and non-biological substrates by cultured avian and mammalian osteoclasts. Anat. Embryol **170**, 247-256.

Kanehisa J, Heersche JNM (1988). Osteoclastic bone resorption: *in vitro* analysis of the rate of resorption and migration of individual osteoclasts. Bone **9**, 73-79.

Kerby JA, Hattersley G, Collins DA, Chambers TJ (1992). Derivation of osteoclasts from hematopoietic colony-forming cells in culture. J. Bone Min. Res. **7**, 353-362.

Lowenberg B, Chernecky R, Davies JE (1990). Cell mediated resorption of bone tissue formed *in vitro*. J. Bone Min. Res. **5** (S2), #34 (abstract).

Maniatopoulos C, Sodek J, Melcher AH (1988). Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. Cell Tissue Res. **254**, 317-330.

Osborn JF (1988). Histologic evaluation of ceramo-osseous regeneration complexes in human bone following hydroxyapatite ceramic implantation. In: Implant Materials in Biofunction. de Putter C, de Lange GL, de Groot K, Lee AJC (eds.). Elsevier. pp. 227-232.

Qiu Q, Vincent P, Lowenberg B, Sayer M, Davies JE (1993) Bone growth on sol-gel calcium phosphate thin films *in vitro*. Cells and Materials. **3**(4), in press.

Sato M, Grasser W (1990). Effects of bisphosphonates on isolated rat osteoclasts as examined by reflected light microscopy. J. Bone Min. Res. **5**, 31-40.

Shimizu H, Sakamoto M, Sakamoto S (1989). The effect of substrate composition and condition on resorption by isolated osteoclasts. Bone and Mineral **6**, 261-275.

Tamura T, Takahashi N, Akatsu T, Sasaki T, Udagawa N, Tanaka S, Suda T (1993) New resorption assay with mouse osteoclast-like multinucleated cells formed *in vitro*. J. Bone Min. Res. **8**, 953-960.

Taylor ML, Boyde A, Jones SJ (1989). The effect of fluoride on the patterns of adherence of osteoclasts cultured on and resorbing dentine: a 3-D assessment of vinculin-labelled cells using confocal optical microscopy. Anat Embryol **180**, 427-435.

Discussion with Reviewers

G. Rodan: This is an interesting study, which combines expertise in material science with cell culture technology and which has generated the significant observation that matrix molecules are not required for osteoclast activation. This is a very important conclusion, which raises many questions: Does calcitonin inhibit the dissolution of the calcium phosphate?

S.C. Miller: Are any quantitative data yet available comparing the response to known calciotrophic substances in this assay versus the bone slice assay?

Authors: These are clearly important assays to perform and, while we would emphasize that our work, to date, has been focused on developing the substrata, we have carried out some preliminary cultures in the presence of calcitonin. However, our results were equivocal when we employed long administration times with our adult cell populations. Thus, we are now repeating these experiments using neonate culture systems which facilitate observation of the effects of this, and other, osteotrophic agents in culture (Heersche, 1992).

G. Rodan: Does this resorption also require clear zone and ruffled border formation?

Authors: We do not believe the concept of a complete sealing zone surrounding a single ruffled border represents the only possible osteoclast morphology either *in vivo* at the bone surface (Zhou *et al.*, 1993) or *in vitro* on artificial materials (Gomi *et al.*, 1993a, b; text references). It is more likely that an individual osteoclast exhibits several transient resorptive "organs" which will each demonstrate areas where the cell membrane is closely attached to the underlying substratum with an associated cytoplasm relatively devoid of organelles, and an area of ventral cytoplasmic ruffling. This view would seem to agree with the results of Taylor *et al.* (1989; text reference) who reported similar morphologies.

G. Rodan: What is the action of macrophages in this system?

S.C. Miller: Bone resorption involves the degradation of matrix and the dissolution of mineral. It seems that one disadvantage of this system, compared with the bone slice assay, is that matrix degrading properties of the cells cannot be assessed. Also some cells other than osteoclasts may be able to solubilize the mineral by the secretion of acids, such as macrophages and macrophage polykaryons. Thus, it seems that this system may be a useful adjunct, rather than a replacement for the established bone slice assays.

E.F. Nemeth: One possible limitation of this assay is that it might not discriminate matrix dissolution mediated by osteoclasts from that potentially mediated by macrophages. The latter can dissolve bone particles but fail to form pits on thin slices of cortical bone. It would be important to know if macrophages can dissolve the calcium phosphate matrix prepared by the authors method.

P. Osdoby: It is not completely clear that the observed removal of the films represents true extracellular resorptive activity as compared to phagocytic removal which could be carried out by macrophages as well as osteoclasts.

Authors: We have cultured primary macrophages, harvested by peritoneal lavage, on these calcium phosphate thin films for periods up to 18 days. None of these "macrophage" cultures showed any evidence of resorption. Thus, we feel that this system does discriminate between osteoclastic and macrophagic resorptive activity. However, we would point out that while these observations lead us to believe that macrophages are not capable of producing the resorption lacunae which our results so clearly demonstrate, they do not negate the possibility that once the ceramic thin film is resorbed, individual ceramic grains may be phagocytosed by macrophages. Indeed, it is commonly understood that grain boundary dissolution will be the most rapid form of ceramic degradation and thus, the acidic environment created by osteoclasts could well cause an initial grain boundary dissolution which would lead to separation of ceramic grains from the bulk. These would be available for phagocytosis by macrophages or indeed other cells.

Regarding the question of organic matrix, it is clear that the resorptive activity which we report is independent of the presence of bone matrix proteins and thus cannot be used to assess this function of osteoclasts. Nevertheless, we would agree with Dr. Rodan (above) that these results clearly demonstrate that osteoclasts are not dependent on the presence of an organic matrix for their resorptive function.

S.C. Miller: Some suggestions for future characterization of this system would be a demonstration that these could be used for quantitative purposes and more direct comparisons (quantitative) between the lacunae observed in this system and those observed on bone slices.

G. Rodan: What is the efficiency of osteoclasts in this system relative to pit formation in dentin and bone (μm^3/osteoclast)?

Authors: We agree that such quantitative comparisons are not only important steps to undertake in the future, (see also response to Dr. Suva, below, and Figure S2 discussed there) but also that they could render important information concerning the basic resorptive activities of inorganic matrices by osteoclasts without the complication of having to take an organic matrix phase into consideration (see also discussion above). It would also be necessary to undertake such studies with various culture models to include variations in species, maturity of donor (i.e., foetal, neonate, or adult), and state of differentiation of the osteoclastic population in question (i.e., harvested precursors compared to harvested mature multinucleate cells), all of which will affect the final state of resorption of the substratum at the end of the culture period. Since we have not yet carried out such extensive comparisons, we are unable to specify levels of efficiency.

E.F. Nemeth: It is unfortunate that the earliest evidence of resorption was seen at 5 days. Using isolated rat osteoclasts on thin slices of cortical bone, significant pit formation is seen after only 18 hours in culture. What might be some of the reasons for this temporal difference?

Authors: The major difference is due to the state of differentiation of the cells at the time of plating onto the culture substratum. Rapid pit formation, within the first 24 hours, is only achieved when the harvested cells are already differentiated. This is the case in culture from chicks (Piper *et al.*, 1992) or calcium deficient hens (Alvarez *et al.*, 1991), or neonate systems of which the work by Sato and Grasser (1990; text reference) with rats and that of Kanehisa and Heersche (1988; text reference) with rabbits would be examples. In the culture system we describe herein, we used marrow washouts from adult animals. In this case, osteoclast precursors are plated, which have to differentiate during the first days of culture. The lag of 5 days in this system is therefore to be expected, and is in accord with other studies such as that of Takahashi *et al.* (1988) who reported that multinucleate osteoclasts formed after 8 days of mouse bone marrow culture. Recently, in conjunction with Dr. J. Heersche's group, we have cultured neonate rabbit osteoclasts on these ceramic thin films and found evident resorption after 24 hours (unpublished). Similarly, in conjunction with Dr. H. Tenenbaum's group, M. Lue has used marrow from calcium-deficient egg-laying hens [Lue M, Davies JE, Tupy K, Cheng P-T, Tenenbaum HC (1994) Resorption of a commercially available substrate by avian osteoclasts *in vitro*. Int. Assoc. Dental Res., March 9-13, 1994 meeting at Seattle, WA. Submitted (abstract)]. In this case, significant resorption was seen at 24 hours (the earliest time point examined in both of these preliminary studies) as illustrated by Figure S1 discussed below.

M. Sato: How does film thickness vary with viscosity and dipping speed? Are there edge effects? How was the thickness measured?

S.J. Jones: The authors state that the thickness of the calcium phosphate film can be varied. Can it also be made to a constant, reproducible thickness?

Authors: For a detailed discussion of film thickness parameters the reader is referred to the standard text by Brinker and Scherer (1990). Yes, there are edge effects which result from both physical and physico-chemical effects and can be seen in Figure S1. However, as these are small, it is straightforward to mask the rim of the disc when computing total resorption. We have measured film thickness by a number of different methods including micrometry, profilometry and SEM. By controlling processing parameters and, if necessary, undertaking multiple dip coatings, we can produce films of reproducible thickness. Further quantitative information will be published in Qiu *et al.* (1993; text reference).

S.J. Jones: In Figure 4D, the rim of the lacuna has been thinned. It is not clear whether the authors believe that this happened solely as a result of the TRAP staining, or was due in part to osteoclast activity?

Authors: The preparation of this sample did not include TRAP staining and thus we believe that the partial resorption at the rim of the lacuna was osteoclast mediated.

S.J. Jones: It is difficult to see why the appearance of the calcium phosphate sintered film should have been altered so in areas not covered by cells. What is the appearance of the film after culturing in medium but without cells for a comparable time? How consistent is the sintering process?

Authors: There are several reasons for changes in the appearance of the film during the culture period. First, while the sol was prepared using stoichiometric quantities of starting materials to achieve hydroxyapatite, it is commonly known that sintering results in a non-monophasic system. It is for this reason that we refer to our films as calcium phosphate, rather than hydroxyapatite. Thus small changes will inevitably occur due to partial dissolution which will be predominantly intergranular in nature. Second, the adsorption of proteins from the culture medium will change the morphology of the surface. Third, proteins secreted by the cells of this heterogeneous culture system will also affect the appearance of the surface. It should also be stressed that, since cells are motile, the areas of the thin films devoid of cells at the time of preparing the photomicrographs were not necessarily devoid of cells for the whole culture period.

The sintering process is essentially governed by the reproducibility of the temperature conditions in the furnace and the cooling rates employed. In our case, the reproducibility of the furnace temperature, at 1000°C was $\pm$ 2°C and cooling was always carried out overnight, to room temperature, in the closed furnace.

L.J. Suva: In the rat bone marrow cultures, the plating density/ml of marrow suspension should be given. In this system, why were the non-adherent cells removed after the first 24 hours after plating? Why were the cells removed by overnight incubation at 40°C and not by sonication?

M. Sato: Please provide the plating cell density (cells/cm^2) that these phenomena were observed for?

Authors: The bone marrow cell population obtained in the manner described is heterogeneous and thus counting cells at the time of plating would be meaningless with regard to the osteoclast, or osteoclast precursor, populations. The plating of the marrow population is therefore measured by volume of suspension rather than total cell number. We make no attempt to remove unwanted cells, or produce an "enriched" osteoclast population before plating. The non-adherent cells, therefore, include large numbers of red blood cells, and others, which due to their density, obscure the culture substratum. Since, in developing this method, we were particularly interested

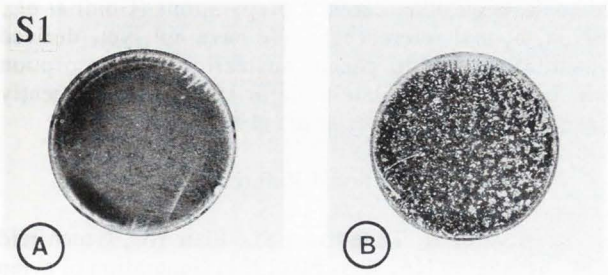


Figure S1. Photomicrographs of whole half inch (1.27 cm) diameter discs following culture for a period of 24 hours, and processing with silver nitrate as described in the text. (**A**) Control disc cultured without cells. (**B**) Cultured in the presence of osteoclasts derived from calcium deficient egg-laying hens. Significant resorption is evident in (B). Figure curtsey of Lue *et al.* (submitted to IADR, with permission).

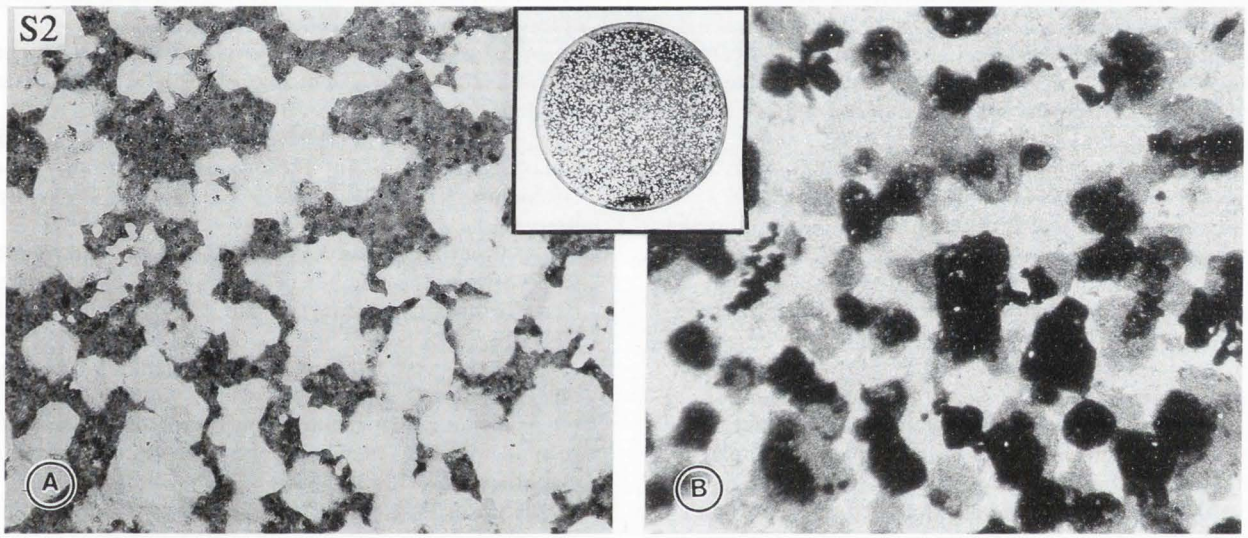


Figure S2. Using bright field optics, the inset shows a disc following culture, in the presence of osteoclasts derived from calcium deficient egg-laying hens, for a period of 96 hours. The disc was processed with silver nitrate as described in the text. Rapid quantification of resorption, measured as the light areas, using a Bioquant 4 system, showed that 53% of the ceramic layer had been removed during this culture period. (**A**) Bright field and (**B**) in dark field, show the same magnified field of view of this disc (FW = 1.8 mm). (B) clearly demonstrates that many areas of lacunae visible in (A) are only "partial thickness" which is judged by the varying shades of gray in this image.

---

in monitoring the progress of resorption during the culture period, we removed as many non-adherent cells as possible to facilitate our observations. However, if simply an end-point measurement is required (see response to the next question), removal of cells after 24 hours is not obligatory.

Sonication will partially remove the ceramic thin films from the underlying quartz support (Qiu *et al.*, 1993; text reference), in addition to the cells, and thus is not a suitable method to employ.

L.J. Suva: The authors should give the percentage of non-full thickness (shallow) pits after the number of total holes. In addition, is there a thickness at which these shallow pits are not present? Is this number a function of the film thickness? This point is very important because it provides the reliability of a system which has the potential to become routine in bone laboratories around the world.

Authors: Since the films are of a finite thickness, and the osteoclasts resorb from the surface to the base of the film, theoretically, there can be no thickness at which shallow pits do not exist, and, for any given culture protocol, the number of partial resorption pits will be a function of film thickness. Even with an hypothetical film which was only one ceramic grain thick, that is approximately 150-200 nm, we would still expect resorption to occur through the grain structure. However, the real issue would be if such shallow resorption events were visible using the examination method chosen by the investigator. An example of how depth information can be gained, even from within the thickness of the films we have employed to date, is shown in Figure S2. From this illustration it is evident that a rapid assay of resorption could be gained (in fractions of a second) by analyzing the bright field image. However, taking a little more time to produce a dark field image, and comparing it with the image obtained in bright field, provides

considerably more information. In the first case, the "depth" information that is lost, by definition, is less than the thickness of the film (0.5 μm). In the second case, even if only three gray scales are used (with the intermediate level assumed to represent half the film thickness), resorption volume could be computed, equally quickly, from two 250 nm thick images. To put this in context, routine optical slicing with confocal microscopy is carried out in 1 μm steps (although smaller increments are possible) which is double the thickness of the films we have employed herein.

M. Sato: The authors state that "resorption was carried out by small cells". What proportion of the resorption activity was carried out by mononuclear cells or multinuclear cells?

S.J. Jones: How many nuclei do the small cells have (e.g., when stained with toluidine blue)?

Authors: This observation is based on our monitoring of the cultures by video-microscopy and also observations that the multinuclear cells, in this culture system, generally have less than 10 nuclei. Toluidine blue stained preparations show multinucleate cells with between 2 and 15 nuclei, with the majority between 2 and 6 nuclei. It would appear that cells with the larger numbers of nuclei [which in these larger cells are often located in the peripheral margins of the cell, and display an annular arrangement, similar to that demonstrated in avian cells by Alvarez *et al.* (1991)] are not involved in resorption although they may still be TRAP-positive. This would also accord with similar observations we have made of osteoclasts on other ceramic preparations (Gomi *et al.*, 1993a, b; text references). We have not, yet, derived quantitative data to equate nuclearity with resorption which would necessitate a major study as has recently been published by Piper *et al.* (1992).

Additional References

Alvarez JI, Teitelbaum SL, Blair HC, Greenfield EM, Athanasou NA, Ross FP (1991) Generation of avian cell resembling osteoclasts from mononuclear phagocytes. Endocrinology **128**, 2324-2335.

Brinker CJ, Scherer GW (1990) Sol-Gel Science: The Physics and Chemistry of Sol-Gel Processing. Academic Press. pp 235-301.

Heersche JNM (1992). Systemic factors regulating osteoclast function. In: Biology and Physiology of the Osteoclast. Rifkin BR, Gay CV (eds.). CRC Press, Boca Raton, FL. pp. 151-169.

Piper K, Boyde A, Jones SJ (1992). The relationship between the number of nuclei of an osteoclast and its resorptive capability *in vitro*. Anat. Embryol. **186**, 291-299.

Takahashi N, Yamana H, Yoshiki S, Roodman GD, Mundy GR, Jones SJ, Boyde A, Suda T (1988). Osteoclast-like cell formation and its regulation by osteotrophic hormones in mouse bone marrow cultures. Endocrinology **122**, 1373-1382.

Zhou H, Chernecky R, Davies JE (1993). Scanning electron microscopy of the osteoclast-bone interface *in vivo*. Cells and Materials **3**, 141-150.