Osteoclast Differentiation in Cocultures of Chondrogenic Cell Line RCJ 3.1C5.18 and Mouse or Rat Bone Marrow: Dependence on Culture Substrate and Association with Alkaline Phosphatase Positive Marrow Stromal Cells

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OSTEOCLAST DIFFERENTIATION IN COCULTURES OF CHONDROGENIC CELL LINE RCJ 3.1C5.18 AND MOUSE OR RAT BONE MARROW: DEPENDENCE ON CULTURE SUBSTRATE AND ASSOCIATION WITH ALKALINE PHOSPHATASE POSITIVE MARROW STROMAL CELLS

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Abstract

We investigated the formation of tartrate-resistant acid phosphatase positive (TRAP+) colonies and multinucleated cells (MNCs) in rat and mouse marrow cultures alone and cocultured with the chondrogenic cell line, RCJ3.1C5.18 on different substrata. In mouse marrow cultured in 35 mm dishes, few TRAP+ MNCs developed, while in rat marrow cultures, many TRAP+ MNCs, which possessed calcitonin receptors and resorbed bone, developed. In both rat and mouse cultures, TRAP+ MNC first appeared at Day 4 and grew in number up to Day 8. When both marrows were cocultured with RC3.1C5.18 cells, TRAP+ colony numbers increased relative to marrow alone. In marrow populations or C5.18 cocultures, TRAP+ colony and TRAP+ MNC formation differed in accordance to the substrates used, i.e., 35 mm Falcon tissue culture plastic dishes or insert baskets. Mouse marrow cultures alone developed similarly in dishes and inserts, but marrow cocultured with C5.18 in inserts formed fewer TRAP+ colonies than those in dishes. Rat marrow cultures and C5.18 cocultures developed differently on different substrata. Compared with cultures in dishes, TRAP+ cell formation in marrow cultures alone was greater on insert surfaces, while the number of TRAP+ colonies and MNC in marrow-C5.18 cocultures decreased on insert surfaces. The effects of soluble factors were investigated using conditioned media. In mouse and rat marrow cultures and C5.18 cocultures, strong colocalization of the TRAP+ cell colonies with alkaline phosphatase positive colonies was seen. X-ray photoelectron spectroscopy of the various substrates revealed differences in available surface oxygen.

Key Words: Osteoclast, coculture, rat marrow, mouse marrow, differentiation, substrate, alkaline phosphatase, tartrate-resistant acid phosphatase.

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Introduction

Osteoclast progenitors are haemopoietic in origin, reach bone via the circulation and proliferate and differentiate to become mononuclear osteoclasts within the bone microenvironment (Burstone, 1960; Gothlin and Ericsson, 1973, 1976; Walker, 1973; Ash et al., 1980). Multinucleated osteoclasts form by fusion of these mononuclear precursors and are characterized by their ability to resorb mineralised bone, the expression of calcitonin receptors and positive histochemical staining for tartrate-resistant acid phosphatase (TRAP) (Chambers and Magnus, 1982; Hattersley and Chambers, 1989; Taylor et al., 1993).

Osteoclast-like cells have been cultured from the marrow of several species including cats (Testa et al., 1981; Pharaoh and Heersche, 1986), baboons (Roodman et al., 1985), humans (MacDonald et al., 1987), rabbits (Fuller and Chambers, 1987), mice (Takahashi et al., 1988a; Hattersley et al., 1989; Shinar et al., 1990) and rats (Hata et al., 1992). Using feline marrow cultures Ibbotson et al. (1984) first noted the enhanced development of osteoclast-like multinucleated cells (MNCs) in cultures with added osteotropic factors such as 1,25 dihydroxy vitamin D3 [1,25(OH)2D3], parathyroid hormone (PTH) and prostaglandin E2 (PGE2).

Recently, the role of stromal cells in the proliferation and differentiation of osteoclast progenitors has been of great interest. Takahashi et al. (1988a) first noted that, in mouse bone marrow cultures, colonies of TRAP+ MNCs colocalized with alkaline phosphatase positive (AP+) cells. They also found that coculture of primary mouse calvarial osteoblast-like cell populations with mouse spleen cells in the presence of 1,25(OH)2D3 led to the formation of multiple TRAP+ mononuclear cells and MNCs. No TRAP+ cells formed in the absence of 1,25(OH)2D3 (Takahashi et al., 1988b). Cocultures of mouse spleen cells and two different marrow-derived stromal cell lines, MC 3T3-G2/PA6 and ST2, also resulted in the formation of TRAP+ MNCs (Udagawa et al., 1989) which, in presence of 1,25(OH)2D3, exhibited specific 125I sCT (synthetic salmon calcitonin)
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binding and formation of resorption pits on dentine slices (Takahashi et al., 1988b; Udagawa et al., 1989). Hata et al. (1992) found that rat bone marrow cultures also generated TRAP+ MNCs that express calcitonin receptors and resorb dentine when grown in medium supplemented with $10^{-8}$ M $1,25(\text{OH})_2\text{D}_3$. However, very low numbers of MNCs were seen in cultures grown without the addition of $1,25(\text{OH})_2\text{D}_3$.

We have shown previously that when mouse marrow cultures are cocultured with the chondrogenic cell line C5.18, colonies of TRAP+ cells containing osteoclasts are generated in cultures without added $1,25(\text{OH})_2\text{D}_3$ (Taylor et al., 1993). The TRAP+ colonies developed in close association with marrow-derived AP+ colonies.

In the present series of experiments, we compared the development of TRAP+ colonies and osteoclasts in marrow cultures from both mouse and rat and evaluated the effects of coculture with the rat-derived C5.18 chondrogenic cells. In the course of these investigations, we discovered that TRAP+ colony formation and osteoclast development were strongly dependent on the culture substrata. We also evaluated whether in both systems soluble factors released by C5.18 cells were responsible for the effects observed and explored, whether C5.18-induced TRAP+ colony formation was associated with the formation of AP+ colonies in both mouse and rat marrow-derived cultures.
Osteoclast development from mouse and rat marrow

Materials and Methods

Culture systems

Mouse marrow cells were obtained from the pooled tissues of eight-week-old female CD1 mice (Charles River Canada, Inc., Quebec, Canada). The femora were dissected free of attached soft tissues and placed into α-Minimal Essential Medium (α-MEM) (α-MEM + RNA, DNA, Flow Laboratories, Inc., McLean, VA) containing antibiotics (100 μg ml⁻¹ penicillin G, 50 μg ml⁻¹ gentamicin and 0.3 μg ml⁻¹ fungizone) (Sigma Chemical Co., Inc., St Louis, MO). Marrow was obtained by resecting the femoral metaphyses and vigorously flushing each marrow cavity four times with 1 ml of α-MEM supplemented with 15% heat inactivated fetal calf serum (FCS) and antibiotics. The rat marrow cells used were the pooled cell population obtained by irrigating the femoral and tibial intramedullary canals of male 125-150 g Wistar rats (Charles River, Ont., Canada).

Both mouse and rat marrow cells were plated at a density of 10⁶ cells per 35 mm dish in all cultures. In this study, rat and mouse marrow cells were cocultured with RCJ3.1CS.18 (C5.18), a chondrogenic clonal cell line derived from rat calvarial cells (Grigoriadis et al., 1988; Taylor et al., 1993). Cocultures of C5.18 and marrow cells were grown in Falcon (Falcon Labware, Becton Dickinson, NJ) tissue culture 35 mm dishes or Costar (High Wycombe, U.K.) 6 well trays. The C5.18 cells were plated at 10⁶ cells per 35 mm dish or well and cultured for 2 days in α-MEM supplemented with 15% FCS and antibiotics in a humidified atmosphere of 5% CO₂ in air at 37°C. After 2 days in culture, the marrow cells were added. This protocol was shown previously to be optimal for TRAP⁺ colony formation at the cell densities used. All cocultures were grown for 8 days after the addition of marrow cells (Taylor et al., 1993) (Fig. 1).

To determine if direct contact between the C5.18 cells and the marrow cells was needed for osteoclast-like cell formation, a culture system was employed in which the C5.18 cells and the marrow cells were physically separated (P/S) by Falcon tissue culture inserts that allow exchange of culture medium but do not allow physical contact of the two cell populations. The C5.18 cells were plated on the bottom of the 6 well trays at Day 0, and marrow cells were plated in the suspended inserts on Day 2. The medium bathing both cell populations was replaced every other day.

An alternative approach to determine whether soluble factor(s) produced by the C5.18 cells altered AP⁺ stromal cell and/or osteoclast-like cell formation from the marrow population was to take conditioned medium from the C5.18 cells and add this to marrow cultures in 35 mm dishes at concentrations of 5%, 10%, 15%, 25%, 33% and 50% by volume. The C5.18 cells used to generate the conditioned medium (CM) were plated at the same density and initiated in culture at the same time as the C5.18 cells used for coculture. Fresh CM was collected at each medium change and used fresh on the day of collection. Medium was changed every other day. The C5.18 populations from which the conditioned medium was taken thus matched the coculture groups in each experiment with respect to increasing cell density of C5.18 cells over the duration of the experiments.

To further investigate the association between the TRAP⁺ colonies, the marrow-derived AP⁺ colonies and AP⁺ C5.18 colonies, two experiments were performed using very low C5.18 plating densities such that after eleven days, separate C5.18 colonies were still apparent. C5.18 cells were plated at 150, 300 and 600 cells per well and grown for 3 days before the addition of 10⁶ mouse marrow cells. In a second experiment, C5.18 cells were plated at 100, 200 and 300 per well and grown for three days before the addition of 10⁶ rat bone marrow cells. In both series, AP and TRAP staining were performed after 8 days of coculture.

Cytotoxical staining

After culture, adherent cells were fixed with 10% neutral buffered formalin for 30 minutes at 4°C, washed twice in distilled water and stained for AP activity by incubating for 30 minutes in Tris buffer (0.2 M, pH 8.3) with AS-MX phosphate (Sigma) as a substrate and Fast Blue B salt as a stain (Burstone, 1960). After repeated washing with distilled water, the cultures were also stained for TRAP activity by incubating with AS-BI phosphate as a substrate in Michaelis-Veronal acetate buffer at pH 5.0 in the presence of 20 mM L-tartaric acid and with hexazonium pararosanilin as a coupling agent (30 minutes, 22°C). TRAP⁺ cells stained ruby red and AP⁺ cells stained blue/purple.

Quantitation of TRAP⁺ colonies and multinucleated cells (MNCs) and AP⁺ colonies

A closely associated group of three or more TRAP⁺ cells was defined as a colony. Colonies of 3-14 TRAP⁺ cells were classified as small TRAP⁺ colonies, whereas colonies with greater than or equal to 15 cells were classified as large TRAP⁺ colonies. Cells containing three or more nuclei were designated as multinucleated TRAP⁺ cells (TRAP⁺ MNC). Groups of greater than five AP⁺ cells were designated as AP⁺ colonies. In some experiments, the percentage of TRAP⁺ colonies associated with AP⁺ colonies was determined. Statistical analysis was performed for each experiment using multiple analysis of variance to assess the differences between groups for each species.
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Figure 2. Micrograph of TRAP+ cells, after autoradiography and labelling with iodinated salmon calcitonin (125I sCT), shows TRAP+ mononuclear and multinucleated osteoclasts derived from rat marrow cultured for eight days in α-MEM with 15% fetal calf serum. Bar = 150 μm.

125Iodine sCT binding and autoradiography

Synthetic salmon calcitonin (sCT) (Bachem, Torrence, CA) was labelled with 125I using the chloramine T method as described previously (Taylor et al., 1989). The cultures were incubated for 1 hour at room temperature in α-MEM containing 3.1 μM 125I sCT. In control dishes, a 1500-fold excess of unlabelled sCT was added to assess the extent of nonspecific binding. Fixation and TRAP staining were performed as above. After TRAP staining, the dishes were coated with NTB2 photographic emulsion (Eastman Kodak Co., Toronto, Canada) and exposed for 7 days at 4°C. After developing, the autoradiographs were examined with light microscopy for the presence of black grains indicating positive CT binding.

cAMP assay after incubation with calcitonin

Cyclic adenylimidodiphosphate (cAMP) formation was measured as described previously (Grigoriadis et al., 1988; Takahashi et al., 1989). In brief, cultures were pre-incubated in α-MEM containing 3H-adenine at 1 μCi·ml⁻¹ for 3 hours. Cells were then washed twice and pre-incubated for 30 minutes with 2 ml phosphate buffer saline (PBS) supplemented with 0.2 mM isobutylmethylxanthine (IBMX), 1 mg·ml⁻¹ D-glucose and 1 mg·ml⁻¹ bovine serum albumin (BSA). 50 nM of sCT in supplemented PBS was added for 15 minutes which was then replaced with 1 ml 5% trichloroacetic acid (TCA)/5 mM adenine, adenosine, ATP (adenosine triphosphate), ADP (adenosine diphosphate), AMP and cAMP to stop the incubation and solubilize the cAMP. Using a two stage separation technique on alumina columns, cAMP was separated from other labelled nucleotide and then quantitated using liquid scintillation counting.

Substrate surface characterization

The surface characteristics of the 35 mm tissue culture plastic dishes, the 6 well tissue culture trays and the insert membranes were examined using X-ray photoelectron spectroscopy (XPS). The spectra were obtained using a Leybold (E. Syracuse, NY) MAX 200 XPS system utilizing an unmonochromatized MgKα X-ray source operating at an accelerating voltage of 12 kV and aperture of 35 mA. Atomic ratios of C, O, S and F were derived from spectra run in low energy mode (pass energy = 192 eV) that were normalized to unit transmission of the electron spectrometer. Binding energies and peak areas were obtained using the curve-fitting routines provided with the spectrometer (Callen et al., 1993).

Results

125Iodine sCT binding and bone resorption activity

125Iodine sCT binding to TRAP+ mononuclear cells and TRAP+ MNCs was seen in all four culture types, i.e., mouse marrow (MM) control, rat marrow (RM) control and C5.18 cocultures with mouse and rat marrow (Fig. 2). No labelling of TRAP+ cells was observed when excess unlabelled sCT was added to the incubation mixture. TRAP+ staining and CT labelling were first observed in both rat and mouse marrow cultures and in cocultures with C5.18 by Day 4 of culture, were maximal at Day 8 and had decreased by Day 13. Both the number of CT positive cells and the intensity of CT labelling of TRAP+ cells were lower on Day 4 than on Day 8 in both rat and mouse marrow cultures. By Day 13, very few TRAP+ cells remained in RM cultures, but those that remained showed intense CT labelling. In MM cultures, there were no TRAP+ cells nor any CT+ cells at Day 13. We have previously shown that TRAP+ MNC in cocultures of MM and C5.18 cells have been shown to form resorption lacunae when cultured on bovine bone slices (Taylor et al., 1993). Resorption lacunae were also observed at the sites of the TRAP+ colonies in RM cultures and in RM cocultured with C5.18 for 8 days on bovine bone slices (results not shown).

cAMP response to salmon calcitonin

To confirm the osteoclast-like nature of the TRAP+ cells in the rat marrow cultures, we evaluated whether these cultures responded to calcitonin with an increase in cAMP content. sCT addition at Day 4 to RM cultures
Osteoclast development from mouse and rat marrow

Figure 3. The number of TRAP+ colonies and TRAP+ MNC in mouse bone marrow cultures, mouse bone marrow-C5.18 cocultures and mouse bone marrow-C5.18 physically separated cocultures. Mouse marrow cells (M 35 mm) were cultured in 35 mm dishes either alone or with C5.18 cells (MCocult 35 mm), in insert baskets either alone (Marrow insert), or with C5.18 cells (MCocult Insert), or cocultured with but physically separated from C5.18 cells, with the C5.18 cells cultured on the bottom of the well and the marrow in the insert (Sep MCocult). Results represent means + standard error of 8-12 observations. Results marked * are statistically different from marrow control values (M 35 mm); # = statistically different from marrow insert controls, and δ = statistically different from cocultures in the inserts at p < 0.05.

or to RM cocultures had no effect (results not shown). At Day 4, the numbers of TRAP+ cells in both culture conditions were low. At Day 8, the addition of sCT induced a 1.4-fold increase in cAMP levels in RM cultures (322 ± 72 versus 435 ± 126, n = 8, p < 0.05) and a 1.5-fold increase in RM-C5.18 cocultures (545 ± 70 versus 808 ± 132, n = 8, p < 0.001). We did not measure cAMP levels in response to addition of CT to osteoclast-containing mouse marrow cultures since such cultures have been shown to respond to CT previously (Takahashi et al., 1988b).

Osteoclast and TRAP+ colony formation in mouse marrow cultures and C5.18 mouse marrow cocultures

Under the present culture conditions, MM control cultures in 35 mm dishes, contained 7.8 ± 2.5 small TRAP+ colonies, 2.2 ± 1.3 large colonies and 1.8 ± 0.4 TRAP+ MNC (Fig. 3). This agrees with previously reported results (Taylor et al., 1993). Coculture of MM cells with C5.18 cells increased the number of small TRAP+ colonies by 4 times, the number of large TRAP+ colonies by 7.5 times and the number of TRAP+ MNC by 7 times when compared to the control cultures (p < 0.05).

When mouse marrow cells were cultured in insert baskets, the number of small TRAP+ colonies, large TRAP+ colonies and TRAP+ MNC was somewhat higher than the numbers seen in 35 mm dishes, but this difference did not reach statistical significance. In MM-C5.18 cocultures in the inserts, the number of small TRAP+ colonies was increased significantly when compared to the insert control marrow cultures, but no significant increase was observed in the number of large TRAP+ colonies or MNC. Thus, the behaviour of the cultures on the insert surface appeared to be different from that on the 35 mm dish surfaces. Most strikingly, the total number of TRAP+ colonies (large and small together) in cocultures in 35 mm dishes (53 ± 8) was significantly greater than that in cocultures grown on the insert membranes (26 ± 1, p < 0.05). In the cocultures, the number of TRAP+ MNC was also less on the inserts relative to the cocultures on 35 mm dish tissue.
Table 1. The effect of culture substratum and type of coculture on the number of TRAP+ cells per small or large colony. Mouse and rat marrow cells were cultured alone or in contacting coculture with C5.18 cells in 35 mm dishes or in insert baskets. Marrow cells were also plated on the inserts and suspended above C5.18 cells that were plated on the bottom of the dish (physically separated, P/S). Results are expressed as the mean number of cells per colony ± standard error of mean for five observations.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Substrate</th>
<th>Mouse Small colony</th>
<th>Mouse Large colony</th>
<th>Rat Small colony</th>
<th>Rat Large colony</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>7.3 ± 1.8</td>
<td>27.0 ± 4.0</td>
<td>6.7 ± 0.7</td>
<td>36.4 ± 2.8</td>
</tr>
<tr>
<td>Marrow</td>
<td>35 mm dish</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Insert</td>
<td>8.6 ± 1.7</td>
<td>34.1 ± 4.0</td>
<td>6.3 ± 0.6</td>
<td>61.9 ± 12*</td>
</tr>
<tr>
<td></td>
<td>P/S insert</td>
<td>10.0 ± 1.6</td>
<td>88.9 ± 28.5*</td>
<td>9.3 ± 1.3</td>
<td>47.1 ± 8.4</td>
</tr>
<tr>
<td>Coculture</td>
<td>35 mm dish</td>
<td>8.6 ± 0.9</td>
<td>34.8 ± 4.7</td>
<td>8.0 ± 2.8</td>
<td>61.1 ± 17.6</td>
</tr>
<tr>
<td></td>
<td>Inserts</td>
<td>9.1 ± 3.2</td>
<td>59.0 ± 49.0</td>
<td>10.0 ± 1.4</td>
<td>43.5 ± 12.9</td>
</tr>
</tbody>
</table>

*Values are significantly different from control marrow values (p < 0.05).

culture plastic (1 ± 0.8 versus 7 ± 5). When MM cells were cultured in inserts suspended above wells containing C5.18 cells (P/S), the number of small and large TRAP+ colonies was the same as that in the cocultures in the inserts. However, the number of MNC was increased relative to marrow insert controls and to cocultures in the inserts (Fig. 3).

When comparing MM control cultures and MM-C5.18 cocultures grown on the insert basket surface with those cultured in 35 mm dishes, differences were also apparent in the morphology of the cultures. In marrow cultures in inserts, the mononuclear TRAP+ cells and TRAP negative cells were oriented in rows in the peripheral areas of the insert where cell density was lower. This may reflect stereotaxis along fibres of the insert surface.

Colony size is also a contributing factor in evaluating the effects of coculture and substratum on TRAP+ cell formation. In the MM cultures and cocultures (seen in Table 1), the size of the small TRAP+ colonies was similar on 35 mm dish plastic and the insert surface. While the large TRAP+ colonies increased significantly in size in the P/S MM cultures (Table 1).

When conditioned medium from C5.18 cultures was added to MM cultures, there was a significant increase in the number of small TRAP+ colonies with the addition of 15% and 25% CM (Fig. 4). The addition of C5.18 CM to MM did not increase the numbers of large TRAP+ colonies or TRAP+ MNC at any of the above concentrations. Small and large TRAP+ colony numbers and numbers of MNC in the physically contacting cocultures were consistently higher than in any of the conditioned medium conditions (Fig. 4).

Rat marrow and C5.18 coculture system

With respect to development of TRAP+ colonies and TRAP+ MNCs, the major difference between rat and mouse cultures is that in the RM cultures alone, many more TRAP+ MNC develop relative to the numbers of small and large TRAP+ colonies seen in the MM cultures in both 35 mm dishes and tissue culture insert baskets (Figs. 3 and 5).

Similar to the observation in MM-C5.18 cocultures, the RM-C5.18 cocultures in the 35 mm dishes showed increased numbers of large TRAP+ colonies and TRAP+ MNC relative to the marrow cultures alone. When RM and RM-C5.18 cocultures were grown in the tissue culture insert baskets, we again observed significant differences between the development of the cultures in the inserts and in the 35 mm dishes. The RM-insert group developed similar numbers of small TRAP+ colonies as the RM controls in 35 mm dishes, but the number of large TRAP+ colonies and TRAP+ MNC in cultures grown in inserts was greater than that in cultures on 35 mm tissue culture plastic dishes (p < 0.05). However, significantly fewer small and large TRAP+ colonies and MNC were seen in the insert cocultures than in the cocultures in 35 mm dishes (Fig. 5). Small colony size showed a trend to increase in the RM-C5.18 cocultures in the inserts when compared to small colony size in the 35 mm dishes. In the 35 mm dishes, the large TRAP+ colonies were significantly larger in the cocultures than in the cultures of marrow alone (p < 0.05) (Table 1). In the P/S cocultures in the inserts, numbers of small and large TRAP+ colonies and of TRAP+ MNC were essentially similar to those seen in the RM cocultures in inserts. It is noteworthy that the
number of TRAP+ MNC in both types of cocultures was significantly lower than that in RM cultures in the inserts. In agreement with this, conditioned medium from C5.18 cells added to RM cultures either reduced numbers of TRAP+ colonies and MNC (15% or 33% CM) or had no effect (25% CM) (Fig. 6).

Substrate surface characterization

It is of interest to note that osteoclast formation was increased in rat marrow cultures grown in inserts when compared to cultures in 35 mm dishes. This strongly indicates that the culture surface characteristics and/or the conditions associated with culturing cells in inserts has significant consequences for osteoclast formation. This was confirmed by the observation that in cocultures of RM and C5.18 cells osteoclast formation was increased in 35 mm dishes but decreased in cocultures grown on insert membranes (p < 0.05) when compared with the corresponding control cultures (Fig. 5). Also, RM-C5.18 coculture increased TRAP+ colony formation in 35 mm dishes but had no effect on TRAP+ colony formation on insert surfaces (Fig. 5) (in contrast to MM-C5.18 cocultures) (Fig. 3).

We therefore analysed the surfaces by X-ray photo-
Figure 5. The effect of coculture with C5.18 on the number of TRAP+ colonies and TRAP+ MNC formed from rat marrow cultures. Rat marrow (RM) and C5.18 cocultures (RMCocult) in 35 mm dishes, RM in insert baskets (RMarrow Insert), RMarrow cocultures in inserts (RCocult Insert) and under physically separated (Sep RCocult) conditions. Means and standard errors of two experiments are shown with values that are statistically different from rat marrow in 35 mm dishes marked with an asterisk. (p < 0.08; minimum n = 8).

electron spectroscopy to further characterize the substrata upon which the cells were grown and investigated cell proliferation of both marrow cells and C5.18 cells on these different surfaces. XPS analysis indicated that the three surfaces (Falcon 35 mm dishes, Costar 6 well trays and the Falcon tissue culture insert membranes) differed with regard to the available surface oxygen. The 6 well trays had the least available surface oxygen followed by the 35 mm dishes, and the highest surface oxygen was seen in the insert membranes. The insert membranes also showed traces of fluoride and sulphur, which were not seen in the polystyrene tissue culture dishes or wells. Rat marrow cultures seeded at 10^6 cells per vessel and grown for 8 days, proliferated least in the 6 well trays, followed by the insert membranes and then the 35 mm dishes. In contrast, C5.18 cells, seeded at 10^4 cells per vessel, yielded the greatest number of cells after 8 days of culture in the 6 well trays, intermediate numbers on the insert membranes and the least cells in the 35 mm dishes (Table 2).

Colocalization of TRAP+ colonies and AP+ colonies

We observed previously in MM-C5.18 cocultures
Osteoclast development from mouse and rat marrow

![Graph showing TRAP+ colony numbers and MNC numbers in rat marrow alone, in marrow with C5.18 conditioned medium (CM) added at 10-33% by volume and in contacting coculture. Values shown are the means of five dishes + standard error of the means. Values marked with an asterisk are statistically different from rat marrow alone.](image)

**Figure 6.** TRAP+ colony numbers and MNC numbers in rat marrow alone, in marrow with C5.18 conditioned medium (CM) added at 10-33% by volume and in contacting coculture. Values shown are the means of five dishes + standard error of the means. Values marked with an asterisk are statistically different from rat marrow alone.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rat Marrow cell</th>
<th>C5.18 cell</th>
<th>Surface $O_2$ (eV)</th>
<th>Rel. %$O_2$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35 mm Dishes</td>
<td>$7.9 \pm 0.1 \times 10^5$</td>
<td>$4.53 \pm 0.1 \times 10^5$</td>
<td>$1.97 \times 10^5$</td>
<td>129</td>
</tr>
<tr>
<td>6 Well Trays</td>
<td>$4.3 \pm 0.4 \times 10^5$</td>
<td>$7.30 \pm 0.6 \times 10^5$</td>
<td>$1.56 \times 10^5$</td>
<td>100</td>
</tr>
<tr>
<td>Insert Membrane</td>
<td>$6.4 \pm 0.4 \times 10^5$</td>
<td>$5.70 \pm 0.6 \times 10^5$</td>
<td>$3.30 \times 10^5$</td>
<td>217</td>
</tr>
</tbody>
</table>

Table 2. The effect of culture substratum on cell proliferation. Surface oxygenation of and cell growth on different substrata. $10^5$ marrow cells or $10^4$ C5.18 cells were cultured for eight days on each of the substrates and counted on Day 8. Cultures were kept at 37°C in humidified 5% CO$_2$ with medium changes performed every other day. Numbers represent the mean (+ standard error of mean) cell number at Day 8 of a minimum of four dishes of each of the surfaces being examined. Available surface oxygen was determined for each surface type using X-ray photoelectron spectroscopy (XPS) and expressed in eV and as a percentage of the substrate with the lowest available surface $O_2$; i.e., the polystyrene of the 6 well trays which was designated as 100%.

(Taylor et al., 1993) that the majority of the TRAP+ colonies formed in close association with marrow-derived AP+ colonies. The AP+ colonies with which the TRAP+ colonies colocalize in either marrow alone or in the coculture system stain dark blue. These darker staining AP+ colonies are comprised of more fusiform and less cuboidal cell populations than the lighter staining C5.18-derived AP+ cells. Taylor et al. (1993) determined these darker AP+ colonies in the MM-C5.18 coculture system to be of mouse marrow origin and not from the rat-derived C5.18 cell line. We wanted to evaluate whether TRAP+ colonies and AP+ marrow-
Figure 7. The colocalization of TRAP+ colonies and alkaline phosphatase positive colonies in contacting cocultures. TRAP+ colonies (brighter central colonies) were seen to colocalize strongly with the underlying mouse marrow-derived alkaline phosphatase positive colonies (darker tones). These in tum were superimposed on pale alkaline phosphatase positive CS.18-derived colonies (lighter tones). Bar = 150 μm.

derived colonies also colocalized in RM cultures. In MM cultures alone, 100% of the TRAP+ colonies colocalized with AP+ colonies as seen in Figure 7. In RM cultures, both in RM alone and in contacting RM-CS.18 cocultures, we also found very strong colocalization of the TRAP+ colonies and AP+ colonies (95-98% of TRAP+ colonies were in close association with AP+ colonies) similar to the observations in mouse marrow culture (Taylor et al., 1993). We observed, however, that the dark marrow-derived AP+ colonies, with which the TRAP+ cells were closely associated, showed a strong tendency to colocalize with the paler AP+ staining areas in the C5.18 population. This was confirmed in an experiment where C5.18 cells were plated at colony densities to allow single colony identification. In this experiment, we observed that both in rat and mouse marrow cocultures, marrow-derived AP+ cells and TRAP+ colonies were associated with C5.18 colonies that were slightly AP+. It was difficult to quantitate colocalization of these two AP+ colony types, as the intensity of staining of the dark marrow-derived AP+ cells obscured full visualization of the lighter underlying C5.18-derived AP+ colonies when colony density was high (results are not shown.)

Discussion

We have observed that both mouse and rat marrow populations in coculture with C5.18 develop TRAP+ colonies and TRAP+ MNCs which possess calcitonin receptors when cultured in α-MEM without 1,25(OH)2 vitamin D3 hormone supplementation but with the addition of 15% fetal calf serum. In both species, the development of TRAP+ colonies and TRAP+ MNC increased from Day 4 to Day 8 with the number of TRAP+ MNC greatest at Day 8 and decreasing thereafter. Both MM and RM populations generated greater numbers of TRAP+ colonies when cocultured with C5.18 cells in 35 mm dishes. However, when comparing cultures in the Falcon insert baskets or in Costar 6 well trays, it became clear that TRAP+ colony formation and TRAP+ MNC formation depended strongly on the culture substratum. When small TRAP+ colony numbers or large TRAP+ colony numbers were considered separately, mouse marrow cultures and C5.18 cocultures in insert baskets developed similarly to development observed on 35 mm culture dish surfaces. RM and RM-C5.18 cocultures, however, behaved quite differently in that TRAP+ cell formation was increased on the insert surface in marrow cultures alone, but was dramatically decreased in the RM-C5.18 cocultures on the insert surface. When rat and mouse marrow cells were cultured in the tissue culture inserts and physically separated from the C5.18 cells, this type of coculture increased TRAP+ MNC formation in the mouse marrow system but inhibited MNC formation in the rat system when compared to the corresponding C5.18 cocultures in the inserts.

The addition of 15-25% C5.18 conditioned medium to mouse marrow cultures increased small TRAP+ colony numbers and TRAP+ MNC formation. In the mouse system, enhancement of TRAP+ colony development, with 15% and 25% C5.18 conditioned medium, and in the physically separated cocultures, suggests the presence of stimulatory soluble factors from the C5.18 population. The stronger stimulation of osteoclast development seen in the contacting cocultures suggests that cell-membrane associated stimulatory factors play an important role in the system. It is also possible however, that the greater effect is caused by the requirement for high local concentrations of the stimulatory factor(s). The lack of enhancement of TRAP+ colonies with the addition of either 33% or 50% CM, however, supports the presence of a cell-membrane associated stimulatory factor in the system. Huira et al. (1991) found that CM from MC 3T3-E1 cells had variable effects on the formation of MNC osteoclast-like cells from spleen colony blast cells at various concentrations, indicating that CM from these cells contains both stimulatory and inhibitory
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factors. This suggests that the differences seen with higher and lower concentrations of conditioned medium could also reflect the interplay between concentrations of stimulatory and inhibitory factors in the system.

We observed marked differences in the formation of TRAP + colonies and TRAP + MNC with rat marrow cells cultured on the insert membranes compared with cultures in the 35 mm dishes. The effects of coculture with C5.18 cells and RM cells cultured on these different surfaces also differed, possibly in response to the available oxygen concentration and its resultant effect on cell proliferation. With regard to the difference observed in cultures on 35 mm Falcon tissue culture plastic dishes and those on the Falcon insert membranes, we established that these surfaces were different with respect to the amount of available surface oxygen. This observation is interesting in view of the findings of Callen et al. (1993), who observed that primary bone cells from neonatal rat calvariae proliferate more on surfaces with higher available oxygen. In our experiments, the C5.18 cells attained lower cell numbers on the 35 mm dish polystyrene than on the less well oxygenated surface of the inserts. It is tempting to speculate that the chondrogenic cells "prefer" a less oxygen rich environment, more like the local milieu that is experienced by their chondrocyte phenotype of origin.

Differences in cell proliferation on the different surfaces are likely to have resulted in differences in ultimate concentrations of soluble cell products and cell surface associated factors associated with TRAP + cell differentiation. We have previously demonstrated that changes in the cell density at which C5.18 cells are plated have significant effects on osteoclast differentiation in marrow populations in coculture (Taylor et al., 1993). Differences in surface rugosity and the consequent tendency for stereotaxis of the cells along the mesh fibres of the inserts could have additive effects. Our results indicate that differences due to cell-cell interactions in these more complex systems have to be evaluated as being influenced by the very different surfaces upon which the various cell populations have been cultured.

One result of the differential growth rates on different surfaces of the cell populations in our coculture system could be differential production of growth factors. In addition, species specific responses to these growth factors may play a role. It has been observed, for example, that transforming growth factor beta (TGF-β) inhibits bone resorption and formation of osteoclasts from rat bone marrow progenitors in vitro in long-term marrow cultures (Chenu et al., 1988; Pfeilschifter et al., 1988). In contrast, in the mouse calvarial system, TGF-β stimulated prostaglandin generation leading to increased osteoclast formation and bone resorption (Tashjian et al., 1985; Pfeilschifter et al., 1988). Moreover, there is evidence that the effect of TGF-β may depend on other culture-derived or serum-derived factors (Oreffo et al., 1989; Marcelli et al., 1990). It is also possible that developing rat and mouse osteoclasts are sensitive at different degrees to osteoclast-derived factors such as prostaglandins (Desimone et al., 1993). Different species sensitivity to other cytokines such as IL (interleukin)-1, IL-4, or variation in IL-1 ra concentration may also play a role in the inhibition of MNC formation in the rat system relative to that seen in the mouse (Riancho et al., 1993). It has been recently suggested that changes in IL-4 concentrations may modulate the fate of osteoclast and macrophage precursors in that precursors contacting low doses of IL-4 may go on to become macrophages and then, if subjected to higher levels of IL-4, may become macrophage polykaryons. In contrast, precursors contacting high local IL-4 levels early in their development would become preosteoclasts and then MNC osteoclasts. Thus, it is proposed that pluripotential precursors are directed along different differentiation pathways depending on their early exposure to IL-4 (Riancho et al., 1993). It seems reasonable to suggest that the different substrates, by leading to different proliferative rates, result in the formation of markedly different ultimate concentrations of the stimulatory, or inhibitory, factors regulating osteoclast differentiation in this system. The interpretation of coculture systems using different culture substrates is thus complicated by potential variability in cell population behaviours due to the inherent differences in the substrata used.

TRAP + colonies, both large and small, were located in close association with alkaline phosphatase positive colonies. This colocalization was observed in both rat and mouse marrow cocultures with C5.18 and in marrow populations grown alone. In the physically-contacting cocultures, the percentage colocalization was the same for both species, i.e., TRAP + colony formation was associated with AP + stromal cell colonies derived from the marrow. Taylor et al. (1993) have also described this phenomenon in the mouse marrow coculture system and identified the darker staining AP + colonies with which the TRAP + colonies colocalize as being of mouse marrow origin. We observed here that the darker AP + colonies derived from the marrow populations were forming preferentially in association with a C5.18 subpopulation of cells. It was difficult to quantitate colocalization of these two AP + colony types as the intensity of staining of the dark marrow-derived AP + cells obscured full visualization of the lighter underlying C5.18-derived AP + colonies when colony density was high. However, in the low density plating experiments, C5.18-derived AP + colonies could be seen to be always
associated with the dark AP+ colonies on which the TRAP+ colonies develop. The consistency of this observation indicates that a two tier system exists in which C5.18-derived AP+ colonies create a local micro-environment favourable to the development of the marrow-derived darker AP+ colonies, which in turn locally enhance the development of TRAP+ colonies.

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Discussion with Reviewers

D.B. Jones: Cell culture plastic (polystyrene or polystyrol) is treated by manufacturers to promote cell attachment. This is done in some cases by concentrated sulfuric acid, which adds both O₂ and S to the surface. Sometimes nitric acid is also added. More modern treatments are kept secret by manufacturers, but add O₂, N and C groups to the culture plastic surface. In the case of Falcon, there is treatment used on the Primaria cell culture ware line which does this. The inserts are derived from a material called Cyclopore from I.B.A.

Different surfaces can bind proteins with different kinetics and affinities (Leonard and Vroman, 1991); some cell behaviour can be attributable to this, and this appears to be the case with different cell culture plastics.

Therefore, surface O₂ is but one of numerous parameters in the interaction of cells with surfaces, which include hydrophobicity, zeta potential (fixed charges), the chemical nature of the fixed charges and regions of hydrophilicity. The surface O₂ measured by the authors with XPS probably reflects the amount and type of surface treatment; the finding of S groups also is indicative of the different surface treatments. Therefore, the correlation between surface O₂ and the development of colonies is probably spurious. However, the fact that different surfaces theoretically absorb different populations of proteins and bind these proteins at different interfacial strengths might account for the authors' correlation. Please comment.

Authors: The dishes used in these experiments were not the specially treated Primaria variety but regular tissue culture polystyrene. The inserts were Cyclopore which is a mesh form of tetraphthallate. Surface chemistry of substrates will obviously alter protein adsorption and subsequent cell adhesion and behaviours markedly. Hydroxyl groups seem to be an advantage in this respect as is the adsorption of vitronectin (Thomas and McFarland, 1996; McFarland et al., 1996). I agree that the different surface chemistries will modify the protein turnover and it is this that will modify the cell behaviours.

D.B. Jones: In the Discussion, the authors imply that surface oxygen, as measured by XPS, is somehow related to metabolic oxygen. This is not the case, nor is it probable that surface oxygen is available to the cells as gaseous or molecular oxygen. Please comment.

Authors: The surface oxygen, we think, is more related to the extent of hydroxylation which, in turn, alters the protein adsorption and then, the subsequent cell adhesion rather than having any effect directly on the cells.

D.B. Jones: Different surfaces have different surface structures which can affect cells. Structures of 500 nm might well have significant effects. Have the authors investigated this aspect?

R. Sawtell: Are there any differences between the plastics used, in the composition and/or methods of preparation?

B.D. Boyan: Do you think that topology of the culture surface played a more significant role in modulating colony phenotype, or did the difference in surface chemistry? Why?

Authors: As mentioned in the Discussion, the rugosity of the cell membranes was different from that of plastic materials, but was not investigated specifically in this paper. Bone cells are known to follow ridges, especially cells of macrophage/osteoclast type as has been well illustrated by Gomi et al. (1993). However, the effect of rugosity on cell numbers is less well-defined although Gomi et al. (1993) have suggested that the fusion of TRAP+ cells is modified by changes in rugosity alone. As far as differentiation in this system, whether it is
B.D. Boyan: We have shown that rat growth plate chondrocyte cultures produce vitamin D metabolites (Schwartz et al., 1992) as well as α2-HS-glycoprotein (Yang et al., 1991), both of which have been shown either by recruiting monocytes or enhancing their fusion. Recent reports also indicate that calcifying chondrocytes secrete factors which recruit endothelial cells. What do you postulate the C5.18 cells are doing to enhance osteoclast formation?

Authors: We did not investigate the factors produced by any of these cell populations, and as regards the possible cytokines, growth and colony stimulating factors, that may be playing a role in enhancing both osteoblast and osteoclast differentiation in this system, are extremely numerous; speculation as to their relative importance would be guess work at best. However, the C5.18 cells do behave somewhat like growth plate chondrocytes so your observations about α2-HS-glycoprotein are noteworthy.

B.D. Boyan: What is known about mouse vs rat marrow cells that could account for the species differences noted in your study? Could some of the differences not be species-specific, but reflect other aspects of the physiology of the model (e.g., age, sex, amount of bone chips in the marrow aspirate, etc.)?

Authors: Please see the Discussion for elaboration on mouse and rat species-specific differences. In regards to non-species-specific factors, the animals were all of adolescent age, and there were no bone chips in the marrow suspensions, but only aspirated marrow cells obtained by washing the endosteal surfaces of the long bones. There were sex differences, as outlined in Materials and Methods.

B.D. Boyan: What is the reproducibility of your observations?

Authors: Experiments were repeated three or four times for each species, and although the numbers were not identical from one experiment to the next, the trends in responses were the same relative to the respective marrow controls, so reproducibility is good considering the inherent variability of marrow populations.