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CELLS IN REGENERATING DEER ANTLER CARTILAGE PROVIDE A MICROENVIRONMENT THAT SUPPORTS OSTEOCLAST DIFFERENTIATION

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Summary

Deer antlers are a rare example of mammalian epimorphic regeneration. Each year, the antlers re-grow by a modified endochondral ossification process that involves extensive remodelling of cartilage by osteoclasts. This study identified regenerating antler cartilage as a site of osteoclastogenesis *in vivo*. An *in vitro* model was then developed to study antler osteoclast differentiation. Cultured as a high-density micromass, cells from non-mineralised cartilage supported the differentiation of large numbers of osteoclast-like multinucleated cells (MNCs) in the absence of factors normally required for osteoclastogenesis. After 48 h of culture, tartrate-resistant acid phosphatase (TRAP)-positive mononuclear cells (osteoclast precursors) were visible, and by day 14 a large number of TRAP-positive MNCs had formed (783±200 per well, mean ± S.E.M., *N*=4). Reverse transcriptase/polymerase chain reaction (RT-PCR) showed that receptor activator of NFκB ligand (RANKL) and macrophage colony stimulating factor (M-CSF) mRNAs were expressed in micromass cultures. Antler MNCs have the phenotype of osteoclasts from mammalian bone; they expressed TRAP, vitronectin and calcitonin receptors and, when cultured on dentine, formed F-actin rings and large resorption pits. When cultured on glass, antler MNCs appeared to digest the matrix of the micromass and endocytose type I collagen. Matrix metalloproteinase-9 (MMP-9) may play a role in the resorption of this non-mineralised matrix since it is highly expressed in 100 % of MNCs. In contrast, cathepsin K, another enzyme expressed in osteoclasts from bone, is only highly expressed in resorbing MNCs cultured on dentine. This study identifies the deer antler as a valuable model that can be used to study the differentiation and function of osteoclasts in adult regenerating mineralised tissues.

Key words: osteoclast, differentiation, micromass culture, deer antler, cartilage, regeneration, red deer, *Cervus elaphus*.

Introduction

Deer are the only mammals that can repeatedly regenerate appendages (Goss, 1983). In male deer, and in females of some species, the antlers are shed each year then regrow into large branched bony structures that are used for fighting (Banks and Newbrey, 1983b; Goss, 1983). Antlers provide a unique system for addressing a fundamental biological question: Why has an ability to regenerate been lost in man and other mammals? The annual growth cycle of antlers is known to be influenced by endocrine and environmental factors (Goss, 1968); however, the molecular mechanisms that regulate antler development remain unclear.

Longitudinal antler growth involves an endochondral ossification process, the replacement of a cartilage template by bone, that is a feature of skeletal development, long bone growth and bone repair. In antlers, endochondral growth takes place at the distal tip of each branch, where progenitor cells in the perichondrium proliferate and differentiate into chondroprogenitors and chondrocytes arranged in vertical trabeculae (Banks and Newbrey, 1983a; Banks and Newbrey, 1983b). Although the matrix of antler cartilage is biochemically similar to that of other hyaline cartilages, its unique feature is an extensive vascular network (Price et al., 1996; Szuwart et al., 1998). The direction of blood flow is disto-proximal; from undifferentiated tissues in the perichondrium, through cartilage, down to bone. This abundant blood supply is required to meet the metabolic demands of rapidly regenerating tissue and is also a conduit for haemopoetically derived osteoclast progenitors (Fujikawa et al., 1996; Roodman, 1996; Karsenty, 1998; Ferguson et al., 1998). This is different from the situation in the growth plates of long bones, where blood vessels from the metaphysis invade the cartilage at the base of the zone of hypertrophic chondrocytes. Vascular invasion is essential for coupling bone resorption with bone formation; it has been shown recently that suppression of angiogenesis leads to impaired bone formation, a reduction in chondrocyte resorption and a reduction in ‘chondroclast’ numbers (Gerber et al., 1999).

During endochondral ossification, osteoclasts resorb

mineralised cartilage, which is then replaced by bone. The importance of osteoclasts in the development and growth of bone becomes evident when genes required for osteoclast differentiation and activity are defective (Felix et al., 1996; Kong et al., 1999). For example, growth plate abnormalities are observed in c-*src*, c-*fos*, macrophage colony stimulating factor (M-CSF) and receptor activator of NFκB ligand (RANKL) knockout mice which are osteopetrotic because of growth plate defects (Felix et al., 1996; Kong et al., 1999). Since antlers elongate extremely rapidly (up to 1 cm a day in some species), growth involves extensive osteoclastic resorption of mineralised cartilage matrix. It has previously been reported that large numbers of osteoclasts can be extracted from antler bone (Gray et al., 1992; Gutierrez et al., 1993). However, the sites of osteoclast formation in antler *in situ* have not been investigated in any detail, and the factors that control their formation and activation have yet to be identified. The objectives of the present study were (i) to determine the sites of osteoclast formation in the growing tip of the antler *in vivo*, (ii) to develop a model that supports the differentiation of large numbers of antler-derived osteoclast-like cells *in vitro*, and (iii) to characterise the phenotype of antler-derived osteoclasts.

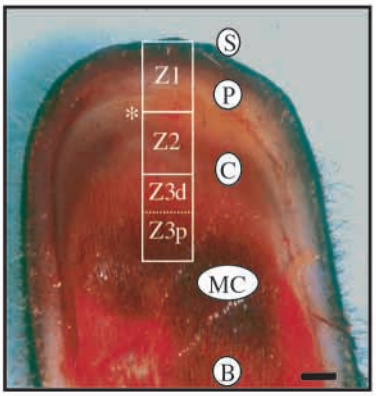

Fig. 1. The growing tip of a deer antler. Antlers were harvested 9 weeks after the previous set had been cast. Longitudinal cryostat sections were obtained from the three zones outlined; Z1, Z2 and Z3. Tissue from zones Z2 and Z3 were used for the preparation of cartilage-derived cell cultures. Zone Z1 included the velvet skin (S), the perichondrium (P) and chondroprogenitors (asterisk). Zone Z2 and the distal part of zone 3 (Z3d) were non-mineralised cartilage (C). The proximal part of zone 3 (Z3p) was mineralised cartilage. MC, mineralised cartilage; B, spongy bone. Scale bar, 0.5 cm.

Materials and methods

Tissues

Antlers were harvested at *post mortem* from red deer (*Cervus elaphus*) stags (approximately 2 years old, mass approximately 100 kg) 5 days and 4 weeks after the previous set had been cast. The distal growing tip was removed aseptically and sectioned longitudinally. Fig. 1 is a longitudinal section through an antler tip and illustrates the regions from which tissue blocks were obtained. Half of the distal tip was placed in medium [Dulbecco's medium containing 10 % foetal bovine serum (FBS), penicillin/streptomycin (PS) (100 i.u./100 mg ml^{-1}) and fungizone (F, 2.5 mg ml^{-1})] prior to tissue culture. Tissue fixed in 4 % paraformaldehyde (PFA; pH 7.4) was paraffin-embedded for routine histology. The remaining tissue was cut into 0.5 cm^3 blocks from zones 1–3 (Z1, Z2, Z3; Fig. 1) and embedded in orthochlorotoluene (OCT compound; Merck, UK) prior to snap-freezing in isopentane slurry at −70 °C. Frozen tissue sections (10–12 μm) were fixed in 4 % PFA (pH 7.4) for 20 min at 4 °C before staining.

Cultures of antler cartilage-derived cells

All products for tissue culture were purchased from GibcoBRL (Paisley, Scotland) except where specified otherwise. Tissue was dissected from the non-mineralised region of the antler tip [zone Z2 and the distal part of zone Z3 (Z3d) in Fig. 1], diced into 2–4 mm^3 pieces, washed with Hanks Balanced Salt Solution containing PS and F (HBSS/PS/F) and incubated in 0.125 % trypsin-EDTA for 15 min at 37 °C. Following two washes in culture medium (BGJb medium supplemented with 10 % FBS, PS and F) and one wash in HBSS/PS/F, the tissue was incubated with hyaluronidase (1 mg ml^{-1}; Sigma, Poole, UK) for 30 min at 37 °C. The tissue was then washed twice with HBSS/PS/F and digested further with 0.25 % type I bacterial collagenase (Sigma, Poole, UK) in HBSS/PS/F at 37 °C for 2 h (samples were vortexed every 20 min). The tissue digest was filtered through a nylon mesh (pore size, 125 μm), and the cell suspension was centrifuged (200 ***g***) for 10 min. Cells were washed twice in HBSS/PS/F, and the cell pellet was then resuspended in culture medium and the cells counted.

Micromass cultures of cartilage-derived cells were established as described previously (Gay and Kosher, 1984). In brief, 2×10^5 cells in 10 μl of culture medium were spotted onto glass coverslips or dentine slices in the centre of 12-well culture dishes and incubated for 2 h at 37 °C in 5 % CO_2/95 % air before the addition of 1 ml of culture medium to each well, producing a micromass of diameter 4 mm. The medium was replaced after 48 h and thereafter did not contain the antimycotic. In some experiments, after 4 days of culture, cells were incubated with or without parathyroid-hormone-related protein (PTHrP) (1-34) (Bachem, Essex, UK) at 10^{-7} mol l^{-1} for 5 days. The medium containing test factors or vehicle control was changed every 2 days. Dentine slices were prepared as described previously (Nesbitt and Horton, 1997).

Histochemical staining in tissue sections and in vitro

Tartrate-resistant acid phosphatase (TRAP)

TRAP staining solution [4 % solution of 2.5 mol l^{-1} acetate buffer (pH 5.2), 12.5 mg ml^{-1} naphthol AS-BI phosphoric acid, 0.67 mol l^{-1} L-(+)tartrate buffer (pH 5.2), 15 mg of fast Garnet salt] was freshly prepared with reagents from a commercial leukocyte staining kit (Sigma, Poole, UK) and filtered before use. Sections and cells, fixed in PFA, were TRAP-stained for

10–20 min at 37 °C as described previously (Takahashi et al., 1988). The numbers of TRAP-positive multinucleated cells in control and treated wells were counted at days 4, 9 and 14. The results presented are representative of four experiments and are expressed as the mean ± S.E.M. for four wells. One-way analysis of variance (ANOVA) using Sheffe's test for *post-hoc* analysis was used for statistical analysis. A level of $P<0.05$ was considered statistically significant.

Von Kossa staining for mineral in tissue sections and micromass cultures

Sections and cells, fixed in PFA, were rinsed in distilled water and placed in 2 % silver nitrate solution for 30 min at room temperature (25 °C). Following three washes in distilled water, sections/cultures were immersed in 2.5 % sodium thiosulphate for 5 min. Finally, they were washed in distilled water. Sections were counterstained with von Gieson stain to detect collagen and cells counterstained with an alkaline phosphatase histochemical stain (see below).

Von Gieson stain for collagen

Sections were stained in von Gieson solution (50 % saturated picric acid solution, 0.09 % acid fuchsin) for 3 min. Sections were then blotted dry prior to dehydration through alcohol (70 %, 90 %, 100 % ethanol). Finally, sections were cleared in xylene then mounted. Extracellular collagen stained red.

Alkaline phosphatase histochemical stain

At days 2 and 9, cultures were fixed in PFA. Cells were stained in the dark for 30 min in a mixture of 0.1 mol l^{-1} Tris/HCl (pH 9), 0.1 % with Fast Red TR as a coupler and 0.02 % naphthol AS-MX phosphate (Sigma, Poole, UK) as artificial substrate.

Alcian Blue stain

Cells were fixed with PFA after 2 and 9 days of culture, washed with 0.1 mol l^{-1} phosphate-buffered saline (PBS; pH 7.4), rinsed twice in 3 % acetic acid (pH 2.5) for 30 min and then incubated with Alcian Blue staining solution [1 % Alcian Blue in 3 % acetic acid] for 30 min at room temperature. After rinsing twice in 3 % acetic acid (pH 1.0) and then distilled water, the micromasses were dehydrated in ethanol (70 % for 5 min, 95 % for 5 min, 100 % for 5 min).

Reverse transcriptase/polymerase chain reaction (RT-PCR)

The expression of aggrecan and type I and type II collagen was studied in primary cultures of cartilage-derived cells by RT-PCR. To determine whether mature osteoblasts were present in micromass cultures at 9 days, we studied the mRNA expression of osteocalcin. We used deer antler cultured bone cells as a positive control. We also studied the presence of RANKL and M-CSF mRNAs in micromass cultures at 9 days. A portion (2 μg) of extracted total RNA (Ultraspec RNA; Biogenesis, Poole, UK) was reverse-transcribed and RT-PCR analysis undertaken. Sequences of primer pairs used for amplification; the predicted sizes of the products and the annealing temperature are summarised in Table 1. RT-PCR cycling conditions were as follows: 94 °C for 4 min, 35 cycles of 94 °C for 1 min, annealing temperature (see Table 1) for 1 min and 72 °C for 1 min, then 72 °C for 10 min. The identity of the PCR product was confirmed by sequencing using a genetic analyser (Perkin-Elmer, Norwalk, CT, USA).

Localisation of F-actin, vitronectin receptors, cathepsin K, MMP-9 and type I collagen by immunofluorescence and scanning confocal microscopy

The primary antibodies used were monoclonal mouse anti-human vitronectin receptor (VNR) (23C6; Horton et al., 1985), monoclonal mouse anti-human cathepsin K (a kind gift from Ian James and Maxim Goven, SmithKline Beecham, USA; Drake et al., 1996), polyclonal rabbit anti-human matrix metalloproteinase-9 (MMP-9) (Chemicon, UK) and polyclonal rabbit anti-human type I collagen (Chemicon, UK). All antibodies were used at a concentration of 10 μg ml^{-1}. Secondary antibodies were FITC-conjugated goat anti-mouse and Cy5-conjugated goat anti-rabbit antibodies (Dako A/S, UK). In brief, cells were fixed in 3.5 % PFA, 2 % sucrose solution and permeabilised before staining with antibody (Nesbitt and Horton, 1997). Dentine slices (or glass coverslips) were saturated with washing solution (5 % new-born calf serum, 0.02 % sodium azide in PBS, pH 7.4) for 30 min at room temperature. Cells (and cryostat-fixed tissue sections for VNR

Table 1. *Primer sequences used for reverse transcriptase/polymerase chain reaction*

Species	Name	Forward primer (5′–3′)	Reverse primer (5′–3′)	T_a (°C)	PCR band size (bp)
Human	Aggrecan	ATGCCCAAGACTACCAGTGGATCGG	CGTCCTGGAAGCTCTTCTCAGTGG	60	500
Bovine	Type I collagen	CTTGGTCTCGTCACAGATCA	TGTTCAGCTTTGTGGACCTC	60	235
Bovine	Type II collagen	CATTGGTCCTTGCATTACTCCCAAC	AGTCGCTGGTGCTGCTGACGCTG	50	390
Bovine	Osteocalcin	TGGCCCTGGCCACACTCTGC	GGCTGCAAGCTCTAGACTGG	55	285
Bovine	M-CSF	AACTGCAACAACAGCTTTGC	TGGTCGCTGCTTGGCACTGC	63	252
Human	RANKL	GGCCAAGATCTCYAACATGAC	GCATCYTGATCCGGATCCAG	58	336

T_a, annealing temperature; bp, base pair; PCR, polymerase chain reaction; M-CSF, macrophage colony stimulating factor; RANKL, receptor activator of NFκB ligand.

localisation) were incubated with the primary antibody diluted in washing solution for 30 min at room temperature, then rinsed in the same solution for 30 min prior to incubation with secondary conjugated antibodies for 30 min at room temperature. The cells were then washed for 30 min, and the same procedure was repeated for the secondary antibody. F-actin microfilament structures were probed with phalloidin-conjugated Rrhodamine (5 units ml^{-1}; Molecular Probes, Leiden, The Netherlands) for 30 min at room temperature. Confocal microscopy was performed as reported previously (Nesbitt and Horton, 1997) using a Leica TCS NT confocal microscope (Milton Keynes, UK). No specific staining was observed when primary antibodies were omitted. Photomultiplier tube voltage thresholds for confocal microscopy were set to gate out background fluorescence given by immunoglobulin G (IgG)-negative controls.

Bone resorption in vitro

Dentine has been well characterised as a suitable model for the analysis of bone resorption by osteoclasts (Chambers et al., 1984; Boyde et al., 1984). The bone resorption assay has been described in detail elsewhere (Selander et al., 1994). In brief, cells were removed from the surface of dentine slices by wiping with a soft brush. Dentine slices were then incubated with peroxidase-conjugated wheatgerm agglutinin lectin (Sigma, Poole, UK) diluted 1:40 in PBS for 40 min at room temperature. After washing with PBS, DAB solution [3,3′-diaminobenzidine, 0.52 mg ml^{-1} in PBS containing 0.1 % H_2O_2] was added for 1–5 min, and the slices were then washed in PBS. The slices were mounted using glycerol, and resorption pits were visualised as brown-coloured areas.

Localisation of calcitonin receptors by autoradiography

Calcitonin (CT) receptors were localised as described previously (Takahashi et al., 1988) in micromasses of antler cartilage cells cultured for 2 and 9 days on dentine slices. At both time points, cells were incubated in α-MEM containing ^{125}I-labelled salmon CT (1.7 kBq per well; Amersham, UK) for 1 h at room temperature. Non-specific binding was assessed with a 1500-fold excess of unlabelled salmon CT (Sigma, UK). After incubation, cells were fixed for 10 min in PFA and stained for TRAP activity. Dentine slices were then dipped in LM-1 emulsion (Amersham Co., UK) and exposed for 2 weeks at 4 °C in the dark. Slices were developed and fixed (5 min each) at room temperature (Ilford imaging, UK), and the presence of silver grains was observed by light microscopy.

Results

TRAP activity and vitronectin receptor expression in antler tissue sections

Tissue sections were examined for TRAP activity and VNR expression to establish the location of osteoclasts in three regions of the antler; zones Z1–Z3, which extended from the velvet skin to the cartilage (Fig. 1). Zone Z1 included the velvet skin (epidermis and dermis), the perichondrium (a fibrous component and subjacent mesenchymal cells) and chondroprogenitors. No staining for TRAP was detected in zone Z1 (data not shown). In zone Z2, haematoxylin and eosin staining of paraffin sections showed chondrocytes arranged in vertical trabeculae between which were vascular spaces and a region of perivascular tissue. There was no mineralisation of the matrix in this region and no mature osteoclasts were observed (Fig. 2A). TRAP-positive cells were first identified in perivascular tissues in lower regions of this zone (Fig. 3a) and throughout zone Z3 (Fig. 3b). Mineralisation was first seen in the centre of the cartilaginous trabeculae in the lower regions of Z3 (Z3p, Fig. 3c). Haematoxylin and eosin staining of paraffin sections revealed the presence of multinucleated osteoclast-like cells in the lower mineralised cartilage region (Fig. 2B). TRAP expression was also studied in antlers harvested 5 days after the previous set has been shed. This was a very early stage of development, when the antler was composed of a blastema of densely packed mesenchymal cells. As expected, no staining for TRAP was present in epidermal and dermal tissues, although groups of TRAP-positive cells were identified in mesenchymal tissue (Fig. 3d).

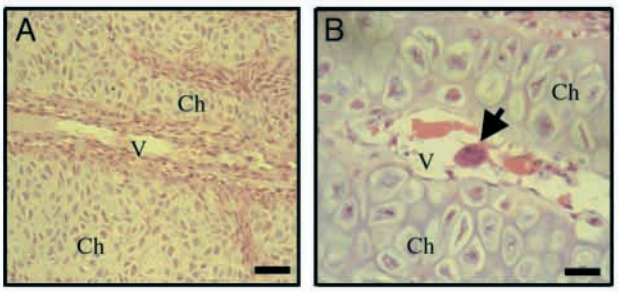

Fig. 2. Histology of antler cartilage. Paraffin-embedded tissue sections were stained with haematoxylin and eosin. (A) In zone Z2, the region from which cells in the micromass cultures were derived, vascular spaces (V) were separated by chondrocytes (Ch) arranged in vertical trabeculae. The matrix was not mineralised, and no mature osteoclasts were observed in this region. (B) In the mineralised cartilage region (zone Z3p), multinucleated osteoclast-like cells were observed (arrow) in the vascular spaces (V). Scale bars: A, 30 μm; B, 15 μm.

VNRs were localised in cells associated with vascular spaces in antler cartilage (Fig. 4). Although VNR-expressing cells were first identified in the lower part of zone Z2 (data not shown), the number of cells in which positive staining was detected increased in the perivascular tissues of zone Z3 (Fig. 4).

Micromass cultures of cartilage-derived antler cells

Having identified non-mineralised cartilage as a tissue that contained osteoclast progenitors, we established micromass cultures from this region to determine whether the cells could support osteoclast differentiation *in vitro*. Primary cultures of antler cartilage-derived cells expressed types I and II collagen and aggrecan mRNAs (Fig. 5A). After 48 h in culture, cells from non-mineralised antler cartilage (Z3d) formed discrete micromasses. Cells with the morphology of chondrocytes were

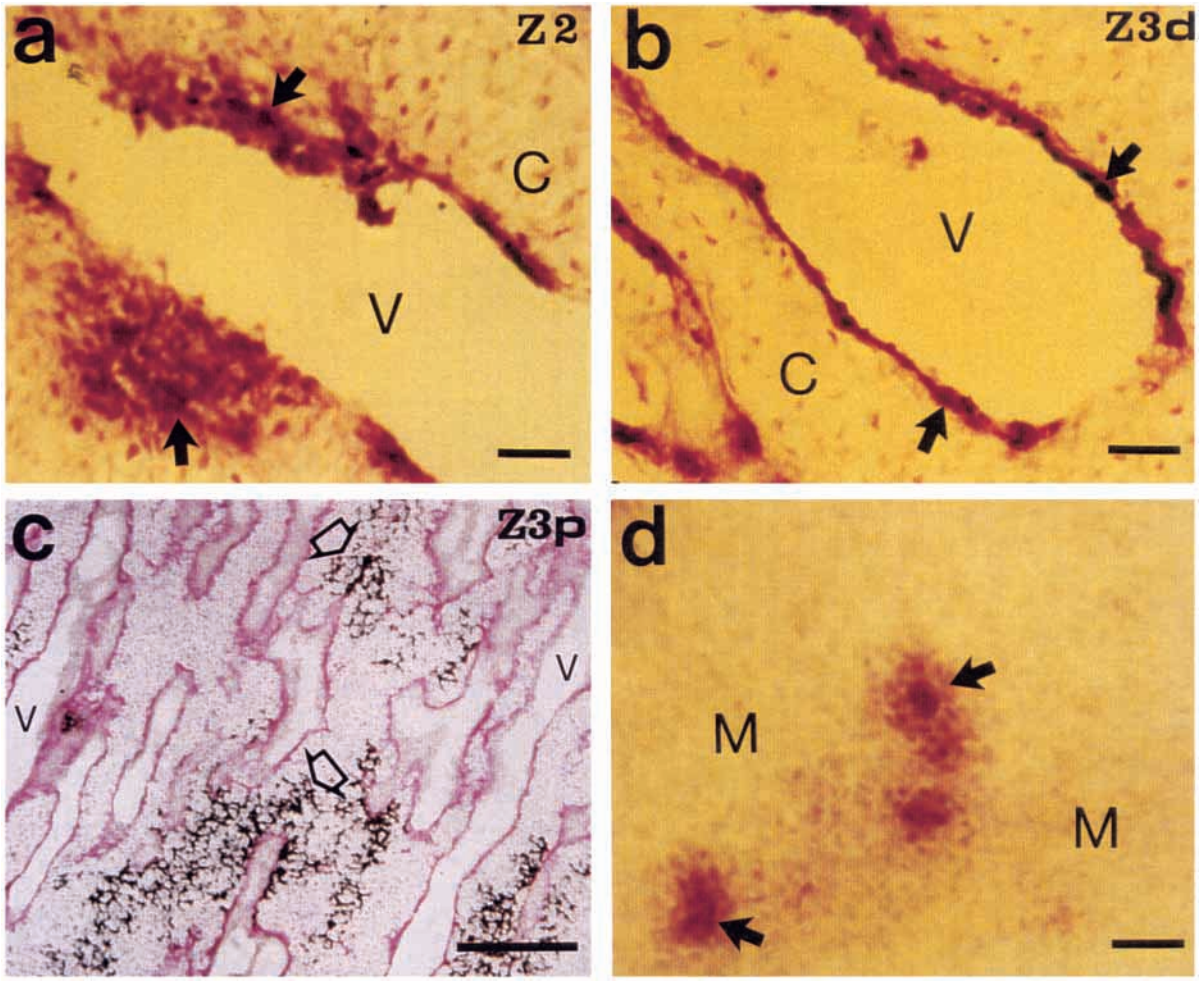

Fig. 3. Staining for tartrate-resistant acid phosphatase (TRAP) and mineral localisation in antler tissue sections. In zones Z2 and Z3, the chondrocytes are arranged in vertical trabeculae (C) separated by vascular spaces (V). TRAP-positive cells (red stain) were first observed in perivascular tissues in the proximal region of zone Z2 (arrows) (a) and throughout zone Z3d (arrows) (b). Mineralisation of cartilage was detected by von Kossa staining in the proximal region of zone 3 (Z3p) (open arrows in c). In the early antler blastema (5–7 days of growth), clusters of TRAP-positive cells were identified in undifferentiated mesenchymal tissue (M) (arrows in d). Scale bars: a,b,d, 50 μm; c, 200 μm.

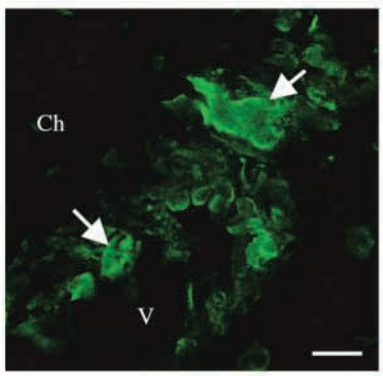

Fig. 4. Localisation of vitronectin receptor (VNR)-positive cells in antler cartilage. Immunofluorescence and scanning laser confocal microscopy showed that cells from zone Z3d express the osteoclast cell membrane marker VNR (green stain, arrows) and were adjacent to the vascular spaces (V). Ch, chondrocytes arranged in vertical trabeculae. Scale bar, 20 μm.

present in the central region (Fig. 6a). Over time in culture, the cells at the periphery of the micromasses proliferated with polygonal/fibroblastic morphology, and by day 9 the well was covered by a dense monolayer of this stroma (Fig. 6d). We detected no osteocalcin mRNA expression at this time, showing that no mature osteoblasts were present in micromass cultures after 9 days (Fig. 5B). Cultured antler bone cells expressed osteocalcin mRNA and were used as a positive control (Fig. 5B). However, RT-PCR showed that cartilage-derived cells cultured in micromasses for 9 days expressed RANKL and M-CSF mRNAs (Fig. 5C). Cells cultured from the mineralised cartilage failed to form discrete micromasses (data not shown). Although Von Kossa staining detected very small areas of mineral deposition on the surface of micromasses, an organised mineralised matrix did not form during the culture period (Fig. 6g). After 2 days in culture, cells in the micromass synthesised proteoglycans (PGs), detected by Alcian Blue staining, and expressed high levels of

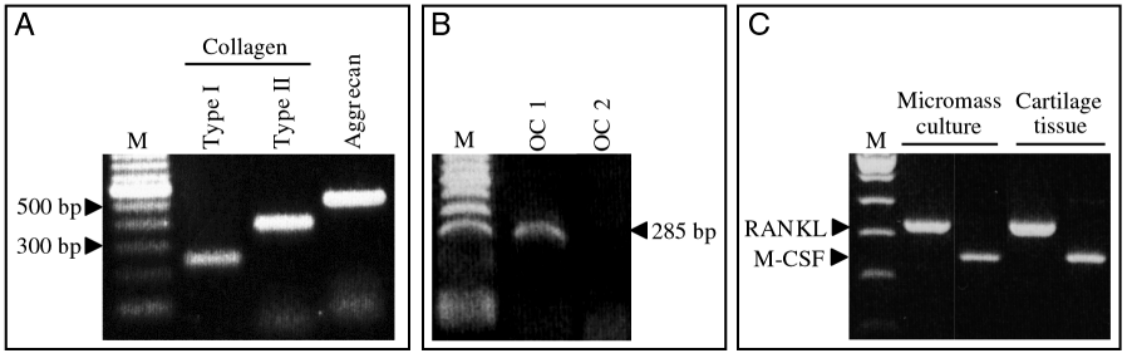

Fig. 5. mRNA expression in cartilage-derived cells studied by reverse transcriptase/polymerase chain reaction (RT-PCR). (A) Types I and II collagen and aggrecan mRNAs were expressed in primary cultures of cartilage-derived cells. (B) In micromass cultures at day 9, osteocalcin mRNA was not detected (lane OC 2), although it was present in deer antler bone cells used as a positive control using the same set of bovine osteocalcin primers (lane OC 1). (C) Receptor activator of NFκB ligand (RANKL) and macrophage colony stimulating factor (M-CSF) mRNA expression were observed in micromass cultures at day 9 and in cartilage tissues of deer antler. M, 100 base pair DNA ladder.

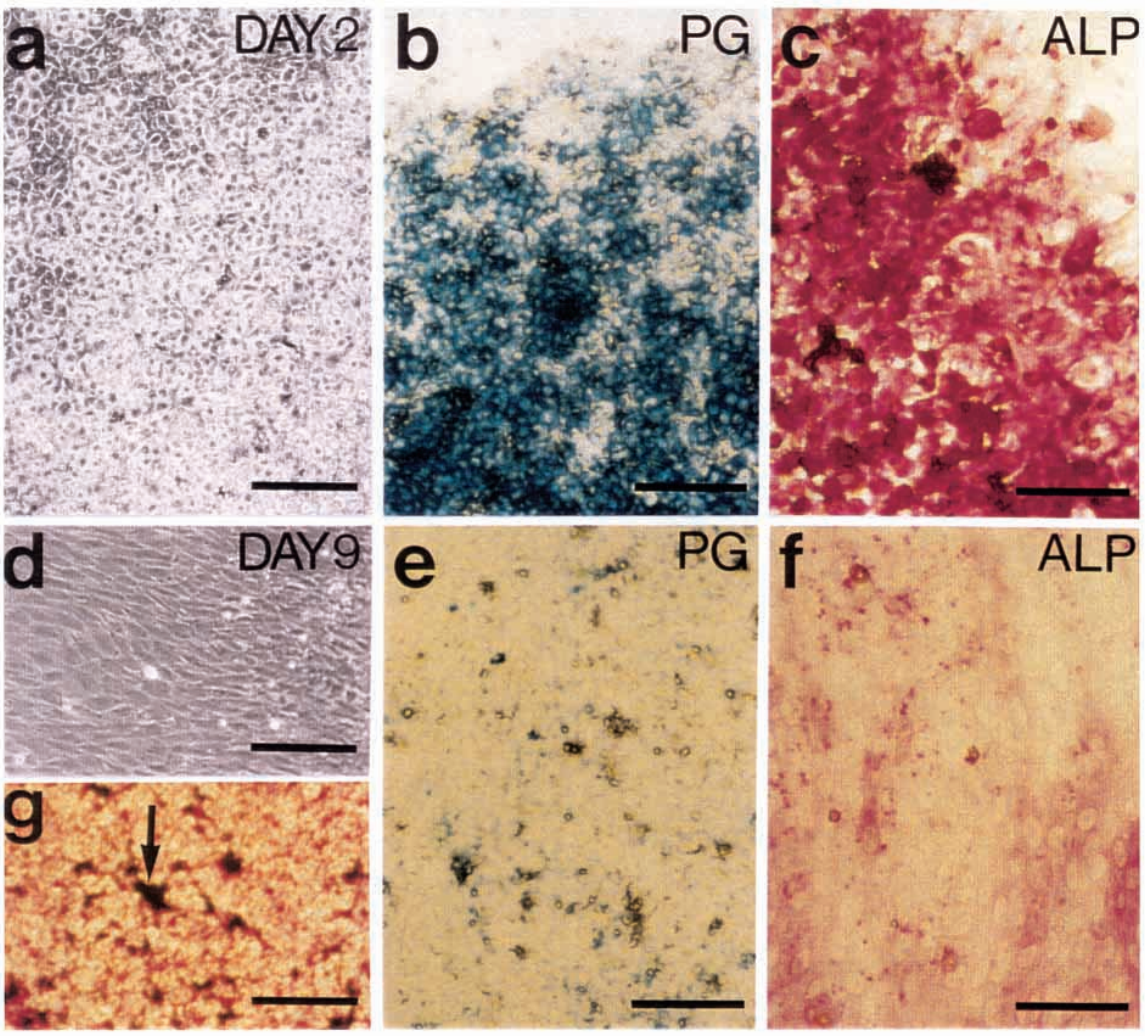

Fig. 6. Micromass cultures of cartilage-derived antler cells. Cells were extracted from zones Z2 and Z3d (non-mineralised cartilage) and cultured as a micromass. At 48 h after explant, the micromass was composed of cells with a chondrocyte morphology (a) that synthesised proteoglycans (PG), detected by Alcian Blue staining (b), and expressed high levels of alkaline phosphatase (ALP) (c). By day 9 after explant, cells with a more fibroblastic morphology at the periphery of the micromass had proliferated to form a dense monolayer (d), and small mineral precipitates were detected by von Kossa staining on the surface of the micromass (arrow in g). However, no discrete mineralised nodules formed in these cultures. At day 9, PG synthesis (e) and ALP activity (f) were much lower than at 48 h (b,c), indicating that the expression of chondrocyte phenotypic markers decreased with time in culture. Scale bars: a,b,d,e, 60 μm; c,f, 30 μm; g, 120 μm.

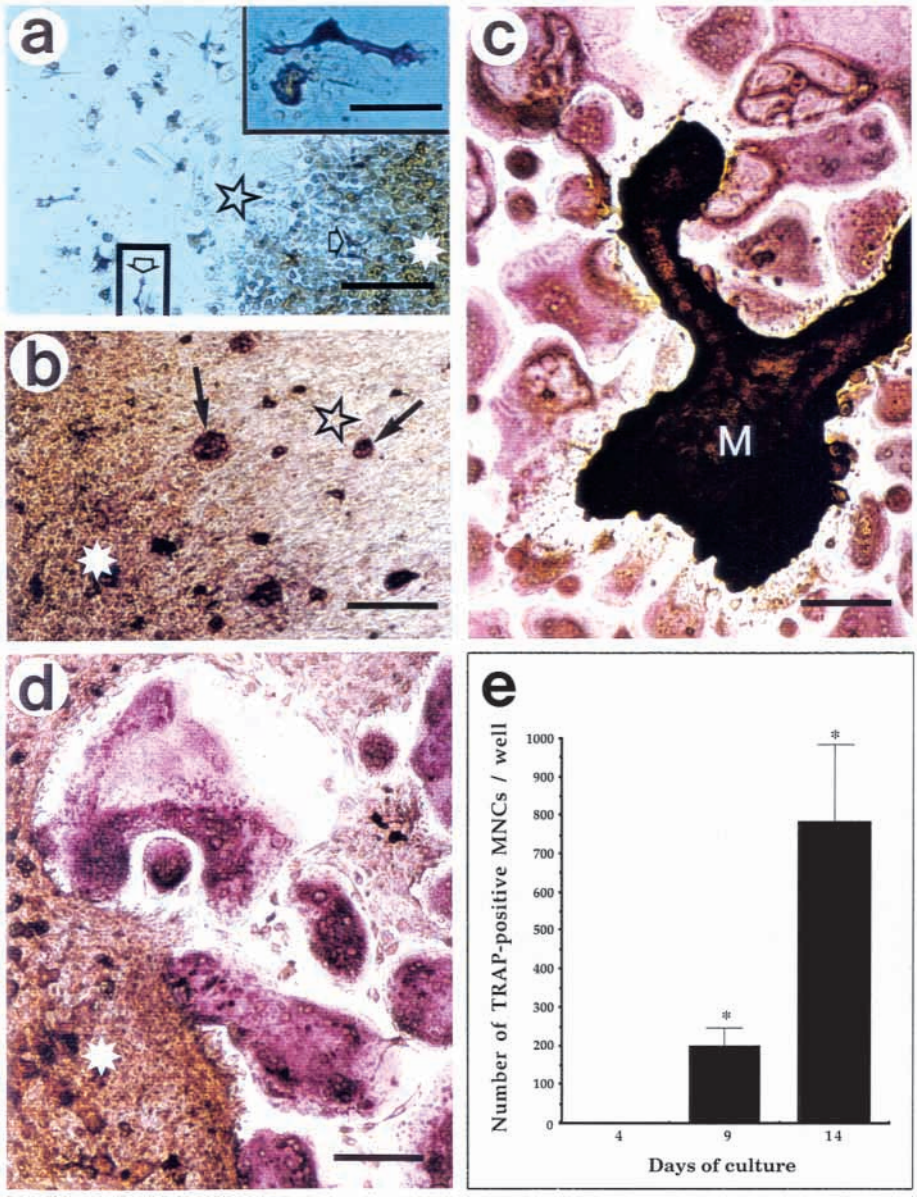

Fig. 7. Formation of osteoclast-like cells in micromass cultures. After 2 days in culture, tartrate-resistant acid phosphatase (TRAP)-positive mononuclear cells (open arrows in a) were observed in the centre (white asterisk) and on the periphery (open star) of the micromass. A higher-power image of a TRAP-positive cell is shown in the inset. In control cultures at day 9, a small number of TRAP-positive multinucleated cells (MNCs) were observed (b, arrows, and e), whereas in parathyroid-hormone-related protein (PTHrP)-treated cultures significantly more TRAP-positive MNCs (at least three nuclei) were present at this stage (c; the residual matrix of the micromass is indicated M). However, by day 14, the number of TRAP-positive MNCs (with more than three nuclei) in control cultures had increased significantly (d,e). Numerical analysis of representative experiments (e) shows the number of TRAP-positive MNCs in four wells (means + S.E.M.). (e) A time course experiment showing a significant increase in MNC formation in control cultures at days 9 and 14 (*P<0.001) compared with day 4. Scale bars, 60 μm.

ALP activity (Fig. 6b,c). However, by day 9, both PG synthesis and ALP activity had decreased (Fig. 6e,f).

Multinucleated cell formation in vitro

After 48 h of culture in medium containing 10 % FBS only, TRAP-positive mononuclear cells were visible throughout the central region and the periphery of the micromass (Fig. 7a). By day 9, TRAP-positive multinucleated cells (MNCs; more than three nuclei) had formed (200±50 mature osteoclasts per well, N=4) (Fig. 7b,e). The number of multinucleated TRAP-positive cells in each well had increased by day 14 (783±200 mature osteoclasts per well, mean ± S.E.M., N=4) (Fig. 7d,e). After 14 days, the matrix of the micromass had almost completely disappeared to reveal numerous osteoclasts on the surface of the well (Fig. 7d). We observed that treatment with PTHrP from day 4 significantly increased the number of multinucleated TRAP-positive cells (up to 36 nuclei per cell) in each well at day 9 (Fig. 7c; 581±107 mature osteoclasts per well compared with Fig. 7b, mean ± S.E.M., N=4, P<0.001).

The number of osteoclasts that could be generated *in vitro* from one antler tip was between 0.8×10^6 and 1.5×10^6. Cartilage-derived cells that had been cryopreserved immediately after digestion from the antler also supported osteoclast formation.

The osteoclast phenotype of antler MNCs

PTHrP was used to accelerate MNC formation in studies designed to characterise the phenotype of antler MNCs (Akatsu et al., 1989) within the micromass cultures seeded onto dentine slices. This was investigated using a range of markers expressed in mammalian osteoclasts.

F-actin ring and VNR localisation

Mononuclear cells expressing VNRs were identified after 2

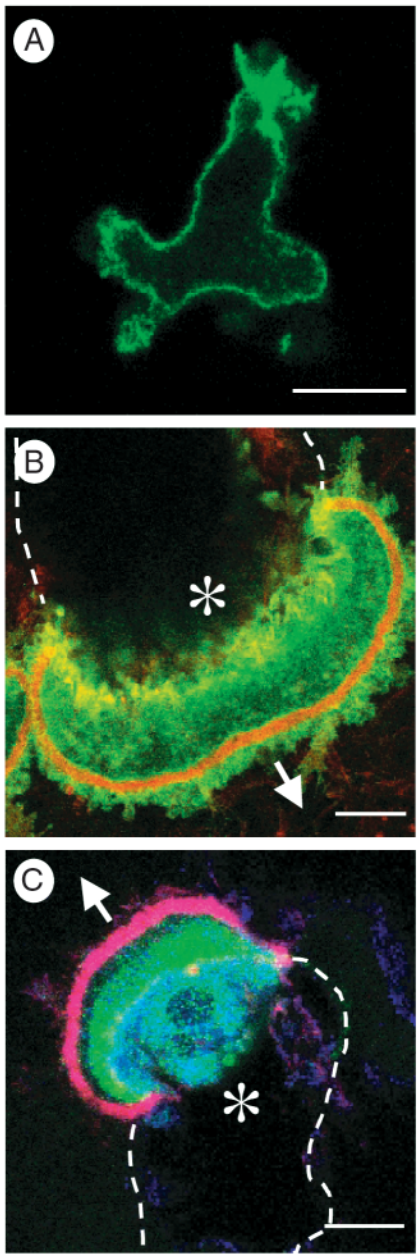

Fig. 8. Immunolocalisation of vitronectin receptor (VNR)-positive cells, F-actin, matrix metalloproteinase-9 (MMP-9) and cathepsin K in antler multinucleated cells (MNCs) cultured on dentine slices by immunofluorescent staining and scanning laser confocal microscopy. Green, VNR, cathepsin K; red, F-actin; blue, MMP-9. At day 2, mononuclear cells expressing VNRs on the plasma membrane were present (A), although no F-actin rings were identified. In parathyroid-hormone-related protein (PTHrP)-treated cultures at day 9, MNCs expressed VNRs, were polarised and formed distinct F-actin rings (B). These cells also expressed MMP-9 and cathepsin K (C). The track and direction of osteoclastic resorption are shown (broken lines and white arrows, respectively). The resorption pit is indicated behind the cell (asterisk). Scale bars, 30 μm.

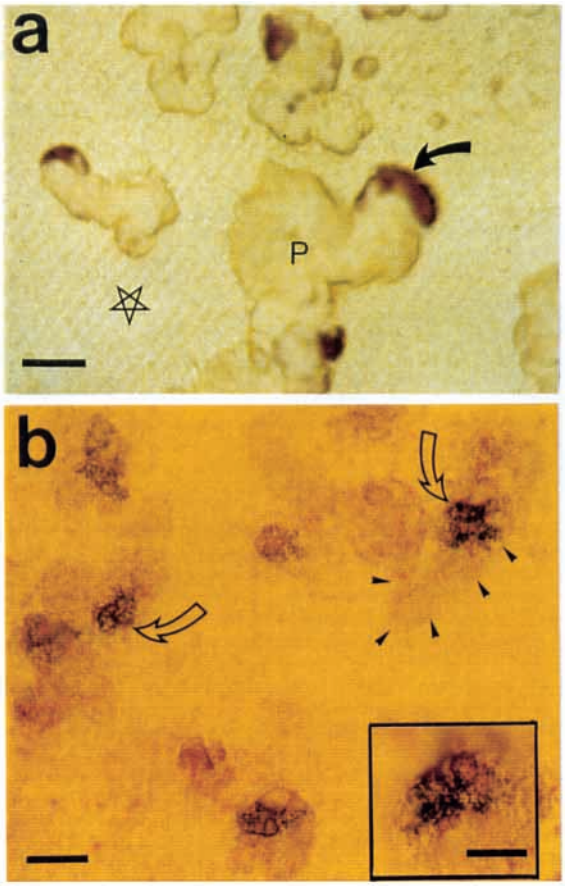

Fig. 9. Dentine resorption by antler multinucleated cells (MNCs) and the expression of calcitonin (CT) receptors. In parathyroid-hormone-related protein (PTHrP)-treated cultures, large numbers of resorption pits (P) were present at day 9 (a). Tartrate-resistant acid phosphatase (TRAP)-positive MNCs (arrow) remained attached to the dentine slices when present within deep resorption pits. The unresorbed dentine surface is indicated by an open star. Autoradiography showed that these resorbing cells expressed CT receptors visible as numerous silver grains bound to the osteoclast-like cells (b, open arrows). The edge of a resorption pit is marked with arrowheads in b. Focus variation is due to the depth of the resorption pits. Scale bars: a, 80 μm; b, 40 μm; inset, 30 μm.

days of culture, and these cells were presumptive osteoclast precursors (Fig. 8A). However, no F-actin rings or resorption pits were detected at this stage. At day 9, the proportion of VNR-positive MNCs was higher (92 % of cells) and the co-localisation of F-actin rings indicated that active bone resorption was taking place (Fig. 8B). Large numbers of resorption pits containing TRAP-positive osteoclasts were present in these cultures (346±52 per slice, mean ± S.E.M., N=8) (Fig. 9a).

Calcitonin receptors

After 2 days of culture, no CT receptors were detected by autoradiography with labelled salmon calcitonin in TRAP-positive mononuclear cells (precursors). However, by day 9 after MNC formation, numerous silver grains accumulated over approximately 80 % of TRAP-positive MNCs located in resorption pits, indicating the presence of CT receptors

(Fig. 9b). No binding occurred when excess unlabelled CT was added to the incubation mixture (data not shown).

The expression of cathepsin K and MMP-9 in resorbing and non-resorbing osteoclasts

The phenotype of antler-derived MNCs was further characterised by determining whether these cells were able to synthesise cathepsin K and MMP-9, enzymes involved in matrix degradation that are known to be expressed in osteoclasts. Both enzymes were localised in resorbing osteoclasts cultured on dentine (100 % of cells) (Fig. 8C). Cathepsin K was predominantly localised in the ruffled border and MMP-9 was generally distributed throughout the cell (Fig. 8C). Cathepsin K was found only in osteoclasts, whereas MMP-9 was localised in other cell types in the micromass, although the level of expression was lower than in MNCs (data not shown). Neither cathepsin K nor MMP-9 was identified bound to the exposed matrix of resorption pits.

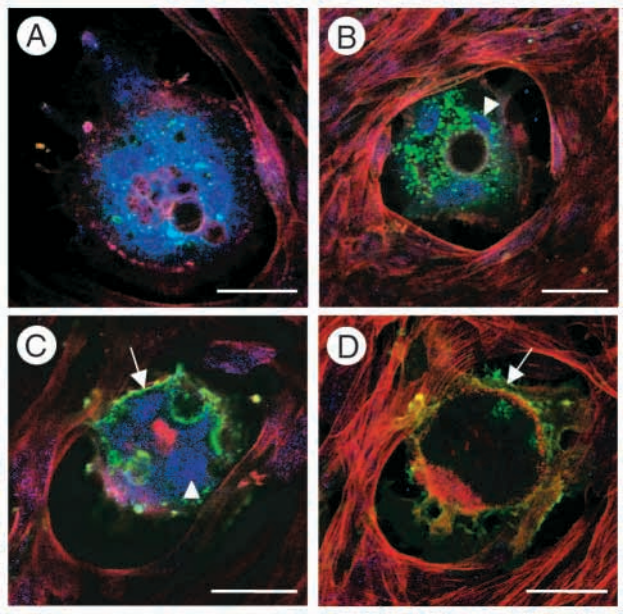

Fig. 10. Immunolocalisation of matrix metalloproteinase-9 (MMP-9), cathepsin K, vitronectin receptor (VNR)-positive cells and type I collagen in antler multinucleated cells (MNCs) cultured on glass by immunofluorescent staining and scanning laser confocal microscopy. Green, cathepsin K and VNR-positive cells; red, F-actin; blue, MMP-9 and type I collagen. Cells were treated with parathyroid-hormone-related protein (PTHrP) for 5 days. The lack of distinct F-actin rings in cells cultured on glass indicated that they were not highly polarised (A–D). However, cells expressed high levels of MMP-9 (blue stain) (A). In contrast, cathepsin K localisation showed a vesicular staining pattern throughout the cytoplasm (green stain) (A,B). Soluble type I collagen (arrowheads, blue stain) was localised in the apical region of antler cathepsin-K- and VNR-positive MNCs (B,D). Serial *x*,*y* sections were taken at 0.35 μm (C,D) (C is the apical and D is the basal surface of the VNR-positive cell adjacent to the glass substratum). The membrane of MNCs cultured on glass substrata stained positive for VNRs (arrows, green stain). Scale bars, 30 μm.

The expression pattern of these enzymes was compared when micromasses were cultured on glass coverslips for 9 days. TRAP- and VNR-positive cells cultured on glass did not form distinct F-actin rings and were surrounded by a non-mineralised collagen matrix (Fig. 10A). We observed a similar pattern of MMP-9 expression in MNCs cultured on glass and in cells cultured on dentine slices that were actively resorbing bone (100 % of MNCs, Figs 10A and 8C respectively). In contrast, cathepsin K immunoreactivity was reduced to small vesicle-like ‘spots’ throughout the cytoplasm in cells cultured on glass (Fig. 10A).

Trafficking of type I collagen fragments in osteoclasts cultured on glass

It had previously been reported that the trafficking through osteoclasts of degraded collagen and bone matrix proteins was initiated only during the bone-resorption process (Nesbitt and Horton, 1997; Salo et al., 1997). We determined whether osteoclasts in matrices other than bone, such as the dense non-mineralised matrix of micromass cultures (Figs 6, 7), played a role in the removal of degraded collagen matrix fragments. Type I collagen fragments were localised in the cytoplasm of cathepsin-K- and VNR-positive MNCs cultured on glass (Fig. 10B–D). Serial *x*,*y* sections from the apical (Fig. 10C) to the basal (Fig. 10D) surfaces of individual VNR-positive cells showed that these type I collagen fragments were localised in the apical region of the osteoclasts, away from the glass substratum.

Discussion

Antlers grow rapidly, and this requires extensive resorption of cartilage at sites of endochondral ossification in the growing tip of each branch. This study identifies sites of osteoclast differentiation in vascularised cartilage of the growing antler tip, distal to the site of bone formation. From the surface of the tip, cells expressing TRAP and VNR were first identified in the non-mineralised cartilage between the large vascular channels and the columns of chondrocytes. The number of cells expressing TRAP increased proximally in mineralised cartilage, and in this region numerous multinucleated osteoclasts were observed in paraffin sections. In this respect, the distal antler cartilage may be similar to the mesenchymal anlaga of a developing long bone, where osteoclast precursors are present at least 6 days before the appearance of mature osteoclasts (Thesingh and Burger, 1983). The cells we have identified may be equivalent to the ‘septoclasts’ identified by Lee et al. (Lee et al., 1995) on the uncalcified septa of growth plate cartilage. These authors suggested that septoclasts were phenotypically different from osteoclasts. However, the cells that we identified in tissue sections of deer antler were phenotypically identical to osteoclasts from bone (Roodman, 1996; Hayashi et al., 1998).

The results of this study are consistent with evidence presented several years ago indicating that, in foetal bone, cartilage cells may regulate osteoclast formation (Burger et al.,

1982; Burger et al., 1984; Van De Wijngaert et al., 1989). Two studies have also shown that chondrocytes may regulate osteoclast differentiation *in vitro* (Taylor et al., 1993; Suzuki, 1996). However, the nature of the molecular signals between chondrocytes and cells of the osteoclast lineage has not been established. A candidate molecule is RANKL (Takahashi et al., 1999); in recent years, its important role in regulating osteoclast differentiation in developing bone has been revealed (Hofbauer et al., 2000). RANKL is synthesised by several cell types in the bone microenvironment, including stromal cells and chondrocytes, and binds to RANK, a cell surface receptor expressed by osteoclasts (Kartsogiannis et al., 1999; Myers et al., 1999). Our observation that RANKL is expressed in antler cartilage indicates that it may also play a role in the regulation of osteoclast formation in this model. It remains to be determined whether similar processes control osteoclastic bone resorption at the pedicle bone/antler interface, which leads to casting of the previous set of antlers. This remarkable example of osteoclast action is known to be hormonally controlled (castration leads to casting); however, the local molecular mechanisms have yet to be investigated.

Having identified sites of osteoclast differentiation in the distal cartilage region of the antler tip, we established an *in vitro* model of osteoclast differentiation using micromass cultures of cells from this region. In this system, there is proliferation of osteoclast precursors since there are few mononuclear TRAP-positive cells in the cultures after 24 h; by day 4, the number of TRAP-positive mononuclear cells had increased, and by day 9 TRAP-positive multinucleated cells (more than three nuclei) had formed. The number of these multinucleated TRAP-positive cells had increased by day 14 in the absence of any factors other than 10 % FBS. In contrast, the numerous bone marrow and co-culture model systems that have been described for generating osteoclasts *in vitro* require the addition of osteotropic factors such as $1,25(OH)_2D_3$, parathyroid hormone (PTH) and M-CSF (Roodman et al., 1985; Suda et al., 1995; Sarma and Flanagan, 1996). Although RANKL induces the formation of osteoclast-like cells from spleen cells or peripheral blood mononuclear cells in the absence of osteoblasts/stromal cells (Yasuda et al., 1998; Matsuzaki et al., 1998), the addition of M-CSF to these cultures is required. We have previously shown that PTHrP is synthesised and secreted into medium conditioned by cartilage cells (Faucheux and Price, 1999) and accelerates osteoclast formation in our model. The present study shows that both RANKL and M-CSF mRNAs are also constitutively expressed in micromass cultures and may regulate osteoclast differentiation. Preliminary studies involving the addition of osteoprotegerin (the soluble decoy receptor for RANKL) to control micromass cultures failed to show inhibition of MNC formation. However, this result may reflect a problem of species cross-reactivity with the available reagents. Future studies will be directed at identifying the mechanisms that regulate RANKL, PTHrP and M-CSF expression *in vivo* and *in vitro*.

Micromass cultures do not represent a pure population of chondrocytes since antler cartilage also contains a population of perivascular cells that have yet to be fully characterised. Osteocalcin, a bone-specific protein expressed by differentiated osteoblasts, is not expressed in the region from which micromass cultures are derived, and the sites of bone formation are more proximal in the antler. The observation that primary cultures of cartilage-derived cells express both type I and type II collagen mRNAs confirms that the micromass cultures represent a mixed cell population. Furthermore, antler chondrocytes, like cells from growth plate, de-differentiate with time in culture (Aulthouse et al., 1989); this is reflected in a decrease in proteoglycan synthesis and alkaline phosphatase activity at day 9. We also observe that the proportion of cells that express type I collagen increases and the number of cells synthesising type II collagen decreases over time (C. Faucheux and J. S. Price; unpublished observations). Taylor et al. (Taylor et al., 1993) showed that inhibition of differentiation in a clonal chondrogenic cell line supported osteoclast formation in marrow co-cultures, and this was *via* a soluble factor. We suggest that, in our model, loss of the differentiated state in chondrocytes may lead to increased synthesis of the factor(s) that stimulate(s) osteoclast differentiation.

Having shown that large numbers of multinucleated cells could be generated in the micromass cultures, these cells were then characterised using a range of osteoclast markers (to distinguish them from macrophage polykaryons, which are also large multinucleated cells). Furthermore, we wished to confirm that cells that resorb mineralised cartilage (traditionally called chondroclasts) are phenotypically and functionally identical to osteoclasts. Our results support this view since antler cartilage-derived osteoclasts have the phenotypic characteristics of mammalian bone-derived osteoclasts. First, these cells stained positively for TRAP, a marker enzyme for osteoclasts (Ibbotson et al., 1984; Roodman et al., 1985). Numerous TRAP-positive MNCs were present after 9 days, showing that MNC formation is rapid in this model. A similar time scale is observed in murine calvarial-marrow cultures (Tsurukai et al., 1998), whereas osteoclast-like cells derived from human marrow cultures can take 14–21 days to form (Sarma and Flanagan, 1996). Second, mononuclear precursors and MNCs express VNRs, a member of the integrin family of adhesion molecules ($\alpha_v\beta_3$ integrin) that is required for attachment of osteoclasts to the bone surface and is expressed in committed osteoclast precursors and mature osteoclasts (Horton et al., 1985; Horton, 1997; Rodan and Rodan, 1997). Antler MNCs also express calcitonin receptors, the most specific marker of the osteoclast lineage cells (Takahashi et al., 1995). TRAP- and VNR-positive mononuclear cells in 48 h cultures do not express calcitonin receptors, indicating that these cells are precursors not yet committed to the osteoclast lineage (Hakeda et al., 1989; Kanatani et al., 1998). A previous study has shown that multinucleated cells derived from antler respond to calcitonin (Gray et al., 1992).

When cultured on dentine, antler MNCs form distinct F-

actin rings; this reorganisation of the cytoskeleton is seen only in osteoclasts that are resorbing mineralised substrata and is thought to correspond to the osteoclast clear zone that is observed *in vivo* (Lakkakorpi et al., 1989; Lakkakorpi et al., 1993; Lakkakorpi and Väänänen, 1991). Crucially, antler MNCs also form large resorption pits, and their size and shape resemble those formed by human osteoclasts isolated from giant cell tumours of bone (Nesbitt and Horton, 1997). An unexpected result is the localisation of type I collagen fragments in osteoclasts cultured on glass since, on this surface, cells do not form the distinct F-actin rings that characterise polarised resorbing cells. It is unlikely that this observation reflects collagen synthesis by osteoclasts since, in cartilage sections, type I collagen mRNA is expressed only in small flattened cells adjacent to chondrocytes (Price et al., 1996), whereas TRAP and VNR are expressed in larger cells adjacent to the lumen of vascular channels. This is the first description of endocytosis in osteoclasts that are not resorbing a mineralised matrix; however, the micromasses do contain a large amount of proteoglycan- and collagen-enriched matrix, although not significant amounts of mineral. These osteoclasts may be acting as 'professional phagocytes' for fragments of matrix degraded by proteolytic enzymes synthesised by chondrocytes (Alexander and Werb, 1991). Alternatively, the osteoclasts themselves may be actively resorbing this 'provisional' matrix. This finding challenges the traditional view that osteoclasts resorb only mineralised tissues (Väänänen, 1996). *In vivo*, resorption of non-mineralised antler cartilage by these cells may facilitate vascular invasion.

MMP-9 (or gelatinase B) may be one of the enzymes involved in this matrix degradation since the enzyme is highly expressed in antler osteoclasts cultured both on glass and on dentine. The enzyme is also present in other cells within the micromass, although the level of expression is lower than in osteoclasts. MMP-9 is highly expressed in osteoclasts from bone (Reponen et al., 1994), and Okada et al. (Okada et al., 1995) have shown degradation of collagen fragments when demineralised bone particles were incubated with MMP-9. *In vivo*, MMP-9 may play a role in vascularisation of antler cartilage since it has recently been shown that MMP-9 is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes (Vu et al., 1998).

We have also shown that osteoclasts derived from antler cartilage express cathepsin K, a proteinase that is predominantly expressed in osteoclasts and is believed to play an important role in the proteolysis of bone matrix (Drake et al., 1996; Inaoka et al., 1995). The recent observation that bone (re)modelling is impaired in cathepsin K knockout mice has confirmed that this enzyme is required for normal osteoclast activity *in vivo* (Hofbauer and Heufelder, 1999). Cathepsin K has potent endoprotease activity in acidic conditions (the pH below the ruffled border of the osteoclast is acidic) and can degrade a number of extracellular matrix proteins including collagen, elastin and osteonectin (Bossard et al., 1996; Kafienah et al., 1998). Our data indicate that MMP-9 and cathepsin K may have different functions in antler since their pattern of expression in osteoclasts is different. MMP-9 is highly expressed in cells cultured on both dentine or glass substrata, indicating that it may be involved in the degradation of both mineralised and non-mineralised matrices. In contrast, cathepsin K is highly expressed only in cells resorbing bone and may not, therefore, play a dominant role in the remodelling of cartilage. This supports the observation that the cartilage of cathepsin K knockout mice is normal (Gowen et al., 1999). Further studies are now needed to establish the detailed subcellular distribution of these enzymes in resorbing and non-resorbing cells *in vitro* and *in vivo*.

In summary, this study has identified the deer antler as a valuable *in vivo* model that can be used to study the differentiation and function of osteoclasts in rapidly growing bone. The results show that undifferentiated progenitor cells and/or cells of the chondrocyte lineage, not mature osteoblasts, support osteoclast formation at sites of endochondral ossification. The micromass cartilage cell culture system that has been developed reproducibly yields very large numbers of osteoclast-like cells in the absence of exogenously added factors and can now be used to identify the molecular mechanisms that control osteoclast differentiation in antlers. The characterisation of antler cartilage-derived multinucleated cells has provided further evidence that there is no phenotypic distinction between 'chondroclasts' and osteoclasts.

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