Calcitonin inhibits osteoclast formation in mouse haematopoetic cells independently of transcriptional regulation by receptor activator of NF-κB and c-Fms

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Abstract

The effects of calcitonin (CT) on osteoclast formation and gene expression have been studied in cultured mouse spleen cells and mouse bone marrow macrophages (BMMs). CT inhibited the formation of multinucleated osteoclasts and resorption pits in spleen cell cultures and BMM as well as in CD115$^+$ CD3$^-$ CD45R$^-$ sorted BMM cultures, incubated in the presence of macrophage colony-stimulating factor and receptor activator of NF-κB ligand (RANKL). No effect on apoptosis by CT was observed. CT did not affect the mRNA expressions of RANK and c-Fms, or the mRNA expressions of a wide variety of transcription factors and genes important for osteoclast differentiation and activity. CT induced inhibition of tartrate-resistant acid phosphatase (TRAP), positive multinucleated osteoclast formation was not associated with any decrease of total TRAP activity, resulting in a large number of TRAP$^+$ mononucleated cells in CT-treated cultures. CT did not affect the mRNA expression of dendritic cell-specific transmembrane protein, d2 isoform of vacuolar (H$^+$) ATPase v$_o$ domain, a disintegrin and metalloproteinase domain 8 (ADAM8), ADAM12, DNAX-activating protein or Fc receptor common γ chain suggested to be involved in fusion of mononucleated osteoclast progenitor cells. The inhibitory effect by CT was mimicked not only by compounds activating cAMP and protein kinase A (PKA) but also by a cAMP analogue activating the exchange protein directly activated by cAMP (Epac) pathway. It is concluded that CT, through cAMP/PKA/Epac cascades, inhibits osteoclast formation and that this effect is not associated with decreased transcription of genes known to be important for osteoclast progenitor cell differentiation, fusion or function.

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Introduction

Calcitonin (CT) is a 32-amino acid peptide belonging to a small family of peptides also including α-CT gene-related peptide (α-CGRP), β-CGRP, amylin, adrenomedullin, intermedin and CT receptor-stimulating peptide (Wimalawansa 1997, Katafuchi *et al.* 2000, 2003, 2004, Chang *et al.* 2004, Roh *et al.* 2004). CT was discovered as an acute hypocalcaemic hormone released from the parathyroid glands (Copp & Cheney 1962), but shortly thereafter shown to be secreted by thyroid C-cells (Foster *et al.* 1964, Zaidi *et al.* 2002). The hypocalcaemic response is mainly due to inhibition of bone resorption (Friedman & Raisz 1965), caused by activation of CT receptors (CTRs) in mature osteoclasts leading to contraction, ceased motility and decreased bone-resorbing activity (Chambers *et al.* 1984).

The CTR is expressed in several cells including cells in the central nervous system, in renal epithelial cells and abundantly in mature osteoclasts (Nicholson *et al.* 1986, Findlay & Sexton 2004). Early osteoclast progenitor cells do not express CTRs; the expression of CTRs is induced in the mononucleated osteoclast progenitor cells late during differentiation (Lee *et al.* 1995, Quinn

et al. 1999). Several isoforms of the CTR have been cloned (Lin *et al.* 1991, Goldring *et al.* 1993) and found to be members of the seven-transmembrane G-protein-coupled receptor superfamily. CTR is coupled to Gα and Gα$_q$ proteins and downstream signalling has been linked to both adenylate cyclase–cAMP–protein kinase A (PKA) and Ca^{2+}/protein kinase C (Purdue *et al.* 2002). CT has also been shown to stimulate the phosphorylation of the mitogen-activated protein kinase extracellular signal-regulated protein kinase (ERK1/2) in HEK293 stably expressing the rabbit CTR C1a (Chen *et al.* 1998) as well as in rabbit and murine osteoclasts (Zhang *et al.* 2002).

The physiological role of CT as an inhibitor of bone resorption has recently been challenged by the findings that mice deficient in the gene encoding CT, and the tissue-specific splice variant α-CGRP, do not exhibit decreased bone mass because of increased bone resorption, as expected, but instead the opposite, namely increased bone mass due to enhanced bone formation (Hoff *et al.* 2002). Similarly, heterozygous CTR-deficient mice exhibit increased bone mass (Dacquin *et al.* 2004). The increased bone mass observed in CT/α-CGRP-deficient mice is due to the absence of CT as shown by the fact that mice selectively lacking α-CGRP exhibit osteopenia caused by

decreased bone formation (Schinke *et al.* 2004). In contrast, inactivation of the amylin gene leads to decreased bone mass due to increased bone resorption which indicates that amylin may be a more important regulator of physiological bone resorption than CT (Dacquin *et al.* 2004).

Although mature osteoclasts express CTR and respond to CT, much less is known about effects of CT on osteoclast differentiation. Mononucleated osteoclast progenitor cells have a haematopoietic origin and are most closely related to the monocyte/macrophage precursor cells (Takahashi *et al.* 2002). Activation of the c-Fms receptor on these cells by macrophage colony-stimulating factor (M-CSF), released by stromal cells, or osteoblasts, upon stimulation by osteoclast-forming hormones or cytokines, leads to expansion of the precursor pool of osteoclast progenitor cells and increased cell survival through an anti-apoptotic signal. In addition, increased expression of receptor activator of NF-κB ligand (RANKL), by stromal cells or osteoblasts, stimulates its cognate receptor RANK on the osteoclast progenitor cells causing an unique osteoclast differentiation signal (Teitelbaum 2000, Boyle *et al.* 2003, Teitelbaum & Ross 2003, Lerner 2004, Asagiri & Takayanagi 2007).

Osteoclastogenesis also involves activation of immuno-receptor tyrosine-based activation motif (ITAM)-dependent calcium signalling in osteoclast progenitor cells (reviewed by Asagiri & Takayanagi 2007). ITAM domains are present in the Fc receptor common γ chain (FcRγ) and DNAX-activating protein (DAP12) which are essential for osteoclastogenesis. FcRγ and DAP12 associate with different ligand-recognizing immunoglobulin-like receptors such as osteoclast-associated receptor (OSCAR), paired immunoglobulin-like receptor A (PIR-A), triggering receptor expressed in myeloid cells (TREMs) and signal-regulatory protein β1 (SIRPβ1), of which several have been found to be expressed in osteoclast progenitor cells. The critical role of these co-stimulatory signals is demonstrated by the finding that $FcR\gamma^{-/-}/DAP12^{-/-}$ mice are osteopetrotic and lack osteoclasts (Koga *et al.* 2004, Mócsai *et al.* 2004). Recently, dendritic cell-specific transmembrane protein (DC-STAMP) has been found to be highly upregulated during osteoclastogenesis and suggested to be important in osteoclast progenitor cell fusion (Rho *et al.* 2002, Yagi *et al.* 2006). In addition, the d2 isoform of vacuolar (H^+) ATPase v_o domain (Atp6v0d2) has been associated with osteoclast fusion, since Atp6v0d2-deficient mice cannot form multinucleated osteoclast whereas the expression of several osteoclast differentiation genes was unaffected (Lee *et al.* 2006).

Studies on effects by CT on osteoclast differentiation are limited. CT has been found to inhibit parathyroid hormone (PTH)-stimulated multinucleated cell formation in feline marrow-derived mononuclear cell cultures (Ibbotson *et al.* 1984) and D_3-stimulated multinuclear cell formation in primate marrow mononuclear cell cultures (Roodman *et al.* 1985). More recently, Cornish *et al.* (2001) have shown that CT, concentration dependently, inhibits osteoclast formation in stromal cell containing mouse bone marrow cultures stimulated

by D_3. In the present investigation, we have studied the effects of CT on osteoclastogenesis in M-CSF- and RANKL-stimulated mouse spleen cell and mouse bone marrow macrophage (BMM) cultures and using gene expression analyses studied the effect of CT on osteoclast progenitor cell differentiation. In addition, intracellular signalling pathways mediating the effect of CT were studied. We found CT, through cAMP/PKA/exchange protein directly activated by cAMP (Epac) cascades, to be a potent inhibitor of osteoclast formation by a direct effect on osteoclast progenitor cells, but to be without effect on the mRNA expression of a wide variety of different genes known to be important for osteoclast differentiation, fusion and function.

Materials and Methods

Experimental animals

CsA mice from our own inbred colony were used in all experiments. Animal care and experiments were approved and conducted in accordance with accepted standards of humane animal care and use as deemed appropriate by the Animal Care and Use Committee of Umeå University, Umeå, Sweden.

Materials

Materials used are presented as supplementary data (see supplementary data in the online version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol195/issue 3/).

Spleen cell cultures

Cells were obtained from spleens of 5- to 9-week-old mice. The spleens were dissected free of adhering tissues, and cells were released by rubbing the spleens against the bottom of a Petri dish, in which grooves had been made by a scalpel. Erythrocytes were lysed in red blood cell lysis buffer (0·16 M NH_4Cl, 0·17 M Tris, pH 7·65) and the remaining cells were seeded, at a cell density of 10^6 cells/cm², on plastic coverslips placed in 24-well plates. The cells were cultured in 0·5 ml α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine (0·7 mM), 100 U/ml benzylpenicillin, 100 μg/ml streptomycin and 100 μg/ml gentamycin sulphate. Cells were allowed to settle overnight in complete medium and thereafter the medium was changed and the experiment started. Osteoclast precursor cells were induced to proliferate and differentiate by the addition of M-CSF (25 ng/ml) and RANKL (100 ng/ml). To study the effect of salmon CT (sCT) on osteoclastogenesis, cells were grown in medium containing sCT at different concentrations, together with M-CSF and RANKL. Cells cultured in complete medium without M-CSF and RANKL were included in all experiments as a control. Medium was changed after 3 days. After 1–6 days, the cells were washed three times with PBS (pH 7·35) and fixed with acetone in citrate buffer/3% formaldehyde solution. Cells were then stained for

tartrate-resistant acid phosphatase (TRAP) activity using the Sigma Diagnostics Acid Phosphatase Leukocyte staining kit and by following the manufacturer's instruction. Multinucleated (no. of nuclei ≥ 3), TRAP-positive cells were counted as osteoclasts (TRAP$^+$-MuOCL). Osteoclasts formed in the spleen cell cultures stimulated by M-CSF and RANKL were able to form pits when cultured on slices of devitalised bovine bone and the stimulation of osteoclastogenesis was associated with increased mRNA expression of several osteoclast genes including *ctr*, *trap* and *cathepsin K* (data not shown; present study). No osteoclasts were formed when cells were treated with either M-CSF or RANKL alone and the stimulation caused by M-CSF and RANKL was abolished by osteoprotegerin (OPG; data not shown). No osteoclasts were formed in the presence of PTH or 1,25(OH)$_2$–vitamin D3 (D$_3$; data not shown), indicating the lack of stromal cells in the spleen cell cultures.

Bone marrow macrophage cultures

Highly purified BMMs were isolated according to Takeshita *et al*. (2000). These cells did not express alkaline phosphatase, RANKL, OPG or CTR mRNA, but mRNA for RANK, c-Fms, cathepsin K and TRAP, as assessed by quantitative real-time PCR (data not shown).

For osteoclastogenesis experiments, BMMs were seeded either on 0·8 cm^2 glass chamber slides or 0·32 cm^2 96-well plates at a density of 10^4 cells/cm^2 in α-MEM/10% FBS containing either 100 ng/ml M-CSF (controls) or 100 ng/ml M-CSF $+50$ ng/ml RANKL, with and without sCT or human CT (hCT), 6-MB-cAMP, 8-pMeOPT-2$'$-*o*-cAMP, 8-bromo-5$'$-AMP or forskolin.

After 4–5 days, with a change of medium after 3 days, the cultures were harvested and the cells fixed with acetone in citrate buffer/3% formaldehyde and subsequently stained for TRAP. The TRAP-positive cells with three or more nuclei were considered osteoclasts, and the number of multi-nucleated osteoclasts was counted (TRAP$^+$-MuOCL). Osteoclasts formed in these cultures stimulated by M-CSF and RANKL were able to form pits when cultured on slices of bovine bone and osteoclast formation was associated with increased mRNA expression of CTR, TRAP and cathepsin K. Osteoclasts were not formed in the presence of PTH or D$_3$, indicating the lack of stromal cells in the cultures. Osteoclast formation caused by M-CSF and RANKL was abolished by OPG (data not shown).

Fluorescence-activated cell sorting (FACS)

Crude bone marrow and BMM cells, obtained (by culturing bone marrow cells for 6 days in the presence of M-CSF) as described above, were washed with PBS/3% FBS and stained with antibodies (0·4 μg/10^6 cells) against the macrophage markers mouse CD11b and CD115, or the lymphoid cell markers CD3e and CD45R. The cells were then analysed using a flow cytometer (FACS Calibur; Becton Dickinson, San Jose, CA, USA).

Cell sorting

BMM cells, obtained as described above, were washed with PBS/3% FBS and incubated with antibodies (0·4 μg/10^6 cells) against the macrophage marker mouse CD115 and the lymphoid cell markers CD3 and CD45R. CD115$^+$ CD3$^-$ CD45R$^-$ cells were sorted by flow cytometry in the flow cytometer cell sorter FACSVantage DiVa (BD Biosciences) and seeded in 0·32 cm^2 96-well plates at a density of 10^4/cm^2 in α-MEM/10% FBS containing either 100 ng/ml M-CSF (controls) or 100 ng/ml M-CSF $+50$ ng/ml RANKL, with and without sCT (10^{-9} M). After 4–5 days, with a change of medium after 3 days, the cultures were harvested and the cells fixed with acetone in citrate buffer/3% formaldehyde and subsequently stained for TRAP.

TRAP activity assay

Spleen cells were isolated, as described above, and seeded in 24-well plates at a cell density of 10^6 cells/cm^2. The cells were allowed to settle overnight before medium was changed, and then cultured for an additional 4 days (as described above). The cells were then washed in PBS and lysed in Triton X-100 (0·2% in H$_2$O). After centrifugation, supernatant was collected and kept at -20 °C until analyses. TRAP activity was determined using *p*-nitrophenyl phosphate as substrate at pH 4·9, in the presence of tartrate (0·17 M) according to the manufacturer's instructions (Sigma). The activity of the enzyme was assessed as the OD$_{405}$ of liberated *p*-nitrophenol, and normalised to the amount of cell protein analysed using the BCA protein assay kit (Pierce, Rockford, IL, USA). The enzyme assays were performed under conditions where the reaction was proportional to amount of enzyme and reaction time.

cAMP formation

BMMs were seeded in 24-well plates at a density of 10^4 cells/cm^2 in α-MEM/10% FBS containing either 100 ng/ml M-CSF (controls) or 100 ng/ml M-CSF $+100$ ng/ml RANKL. After 96 h, cells were washed in serum-free α-MEM, and thereafter incubated in HEPES-buffered α-MEM containing 10^{-5} M rolipram for 30 min, after which CT (10^{-9} M, final concentration) was added. After 2–15 min, cells were harvested and cAMP was extracted from the cells, using 90% *n*-propanol. Propanol was removed by evaporation, and the remaining sample was resuspended in assay buffer and analysed using a cAMP [^{125}I] RIA Kit, according to the manufacturer's instructions.

Pit formation

Bone slices with a diameter of 5 mm and a thickness of 100 μm were prepared from bovine femur, washed in ethanol and PBS and then ultrasonicated. The slices were placed at the bottom of 96-well plates, and BMMs (3200 cells/well) were added in 125 μl α-MEM/10%FBS with or without test substances.

After 7 days, the cells were removed with trypsin, ultrasonicated and the bone slices stained with toluidine blue to detect the resorption pits.

Gene expression in spleen cell and BMM cultures

Spleen cells were isolated, as described above, and seeded in Petri dishes with a growth surface of 20 cm^2, at a cell density of 10^6 cells/cm^2. After allowing the cells to settle overnight, medium was changed and the experiment started. Cells were cultured in medium (controls), in medium containing M-CSF (25 ng/ml) and RANKL (100 ng/ml), or in complete medium supplemented with M-CSF, RANKL and sCT (10^{-9} M). At 24, 48, 72 or 96 h, the cells were collected using a cell scraper and total RNA was extracted.

BMMs were seeded at a density of 10^4 cells/cm^2 in 10 cm^2 six-well plates and cultured in α-MEM/10% FBS added to either M-CSF (100 ng/ml) or M-CSF (100 ng/ml) + RANKL (50 ng/ml), in the absence or presence of sCT (10^{-9} M). At 48 and 96 h, total RNA was extracted.

RNA isolation and first-strand cDNA synthesis

Total RNA was extracted using either TRIzol reagent (Invitrogen) or using the RNAqueous-4PCR kit (Ambion, Cambs, UK) according to the manufacturer's instructions. Then the samples were subsequently digested with DNA-free Kit (Ambion). The quality of the RNA preparations was analysed in 1·5% agarose gel electrophoreses and visualised using ethidium bromide. Single-stranded cDNAs were synthesised from 1 µg total RNA using a First-Strand cDNA Synthesis Kit with avian myeloblastosis virus (AMV) and oligo(dT)$_{15}$ primers, according to the manufacturer's protocol (Roche). To ensure that there was no genomic DNA in the samples, reactions without AMV reverse transcriptase were included as a negative control. The mRNA expression was then analysed, using semi-quantitative reverse transcriptase-PCR (RT-PCR) or quantitative real-time PCR.

Semi-quantitative reverse transcriptase-PCR

First-strand cDNA was amplified by PCR using a PCR core kit (Roche) and PC690G Gradient Thermal Cycler (Corbett Research, Mortlake, NSW, Australia) or Mastercycler Gradient (Eppendorf, Hamburg, Germany). The PCRs for GAPDH, 36B4, TRAP, cathepsin K, chloride channel-7 (ClC-7), c-Fms, c-Jun, c-Fos, TREM-3, DC-STAMP, Atp6v0d1 and ATP6i were performed by PCR standard protocol. The conditions for PCR were: denaturing at 94 °C for 2 min, annealing at various temperatures for 35 s, followed by elongation at 72 °C for 40 s; in subsequent cycles denaturing was performed at 94 °C for 35 s. The PCRs for CTR, TREM-2, NFAT-2, integrin α$_v$, integrin β$_3$, Atp6v0d2 and matrix metalloproteinase-9 (MMP-9) were initiated with hot start, using HotStar Taq polymerase kit (Qiagen Ltd). Annealing temperatures were 55 °C (DC-STAMP), 57 °C (GAPDH, cathepsin K, c-Fms, c-Jun, c-Fos, ClC-7, integrin α$_v$, integrin β$_3$, MMP-9 and TREM-2), 58 °C (Atp6v0d1,

Atp6v0d2 and TRAP), 61 °C (36B4, NFAT-2, TREM-3 and ATP6i) and 67 °C (CTR). The PCRs for RANK and DAP12 were performed with a step-down technology, where the primer annealing temperature was 65 °C for the first ten cycles, and then decreased by 5 °C every five cycles down to 45 °C. The sequences of primers, GenBank accession numbers and the positions for the 5′- and 3′-ends of the nucleotides for the predicted PCR products are listed in supplementary table 1 (see supplementary data in the online version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol195/issue3/). The expressions of these factors were compared at the logarithmic phase of the PCR, the products separated in electrophoreses on a 1·5% agarose gel, and visualised using ethidium bromide. Primers were designed using ABI Prism Primer express (Applied Biosystems, Foster City, CA, USA) or Omiga (Genetic Computer Group Inc., Madison, WI, USA). The identity of the PCR products was confirmed using a QIAquick purification kit (Qiagen Ltd) and a Thermo Sequence-TM II DYEnamic ET terminator cycle sequencing kit (Amersham) with sequences analysed on an ABI377 XL DNA sequencer.

Quantitative real-time PCR

Quantitative real-time PCR analyses of NFAT-2, RANK, TRAP, cathepsin K and CTR were performed by the TaqMan Universal PCR Master Mix (Applied Biosystems) and a sequence detection system (ABI Prism 7900 HT Sequence Detection System and Software, Applied Biosystems) with fluorescence labelled probes (reporter fluorescent dye VIC at the 5′-end and quencher fluorescent dye TAMRA at the 3′-end) as described previously (Ahlen *et al*. 2002). The sequences of primers and probes, GenBank accession numbers and the positions for the 5′- and 3′-ends of the nucleotides for the predicted PCR products are listed in supplementary table 2 (see supplementary data in the online version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol195/issue3/). Primers and probes were designed using ABI Prism Primer express (Applied Biosystems). The expressions of c-Fms, ATP6i, integrin β3, MMP-9, OSCAR, a disintegrin and metalloproteinase domain 8 (ADAM8) and ADAM12 were analysed using TaqMan Gene Expression assays. To control variability in amplification due to differences in starting mRNA concentrations, β-actin was used as an internal standard. The relative expression of target mRNA was computed from the target C_t values and the β-actin C_t value using the standard curve method (User Bulletin no. 2, Applied Biosystems).

Apoptosis

Spleen cells were seeded in a 96-well plate at a density of 10^6 cells/cm^2. The cells were allowed to settle overnight in the complete medium after which the medium was changed to medium containing 25 ng/ml M-CSF and 100 ng/ml RANKL. At 72 h, sCT (10^{-9} M) was added to the cultures, which were then incubated for 4 or 14 h before harvest.

Cultures without CT were used as a control. Cells were washed in PBS and lysed. DNA fragmentation was then analysed using Cell Death Detection ELISA (Roche).

Statistical analyses

All statistical analyses were performed by one-way ANOVA with Levene's homogenicity test, and *post hoc* Bonferroni's test, or where appropriate, Dunnett's T3 test or using the independent sample *t*-test (SPSS for Windows, Apache Software Foundation). All experiments have been performed at least twice with comparable results, and all data are presented as means ± s.e.m.

Results

Effects of CT on osteoclast formation in spleen cell cultures

Treatment of mouse spleen cells with M-CSF (25 ng/ml) and RANKL (100 ng/ml) resulted in an increased number of TRAP$^+$-MuOCL, which was evident after 72 h and maximal after 96 h, after which time point the number declined (Fig. 1A). The addition of sCT (10^{-9} M) to spleen cells stimulated by M-CSF and RANKL resulted in 70% inhibition of the number of osteoclasts formed at 96 h

(Fig. 1A). The inhibitory effect of sCT was concentration dependent (10^{-14}–10^{-9} M) with apparent half maximal inhibition obtained at 10^{-12} M (Fig. 1B). The degree of inhibition caused by adding sCT during the last 24 h of a 96-h culture was identical to that obtained when sCT was present during the complete culture period (Fig. 1C). Treatment with M-CSF and RANKL induced a time-dependent enhanced expression of CTR mRNA, a response which was abolished by sCT (Fig. 1D).

Expression of c-Fms and RANK mRNA in CT-treated cells

When assessed at 96 h by semi-quantitative RT-PCR, it was observed that M-CSF and RANKL stimulation of spleen cells caused a substantial increase of both c-Fms and RANK mRNA, which was unaffected by sCT (Fig. 2A). To study whether sCT treatment had affected the c-Fms and RANK mRNA expressions during earlier stages of osteoclast progenitor cell differentiation, the c-Fms and RANK mRNA expressions were analysed using quantitative real-time PCR at 24–96 h. As shown in Fig. 2B, the c-Fms mRNA expression was enhanced by treatment of the spleen cells with M-CSF and RANKL for 72 h, but sCT did not affect c-Fms mRNA level at any time point. Similarly, the RANK mRNA expression was unaffected by sCT at all time points (Fig. 2C).
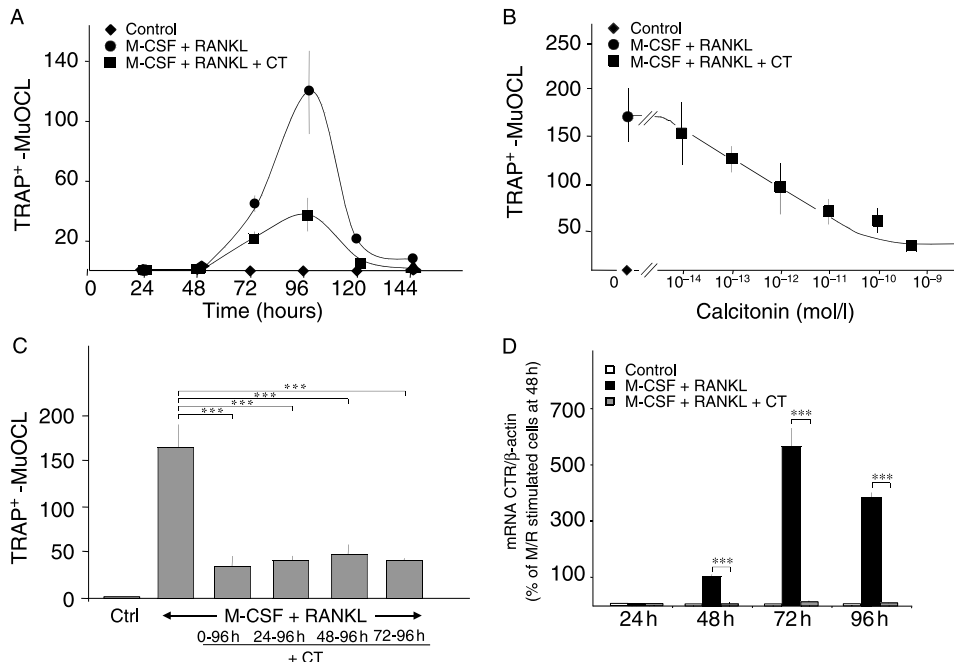

Figure 1 The inhibitory effect of sCT on osteoclast (TRAP$^+$-MuOCL) formation in spleen cell cultures stimulated by M-CSF and RANKL is dependent on time (A) and concentration (B) and can be obtained by adding calcitonin during the last 72, 48 or 24 h of a 96-h culture (C). The expression of the CTR was upregulated by RANKL but was inhibited by the addition of calcitonin, as assessed by quantitative real-time PCR (D). sCT was added to a final concentration of 10^{-9} M in (A), (C) and (D). Symbols and columns represent the mean of six wells per group and s.e.m. is given as vertical bars when larger than the height of the symbol. ***Statistical significance at $P < 0.001$.
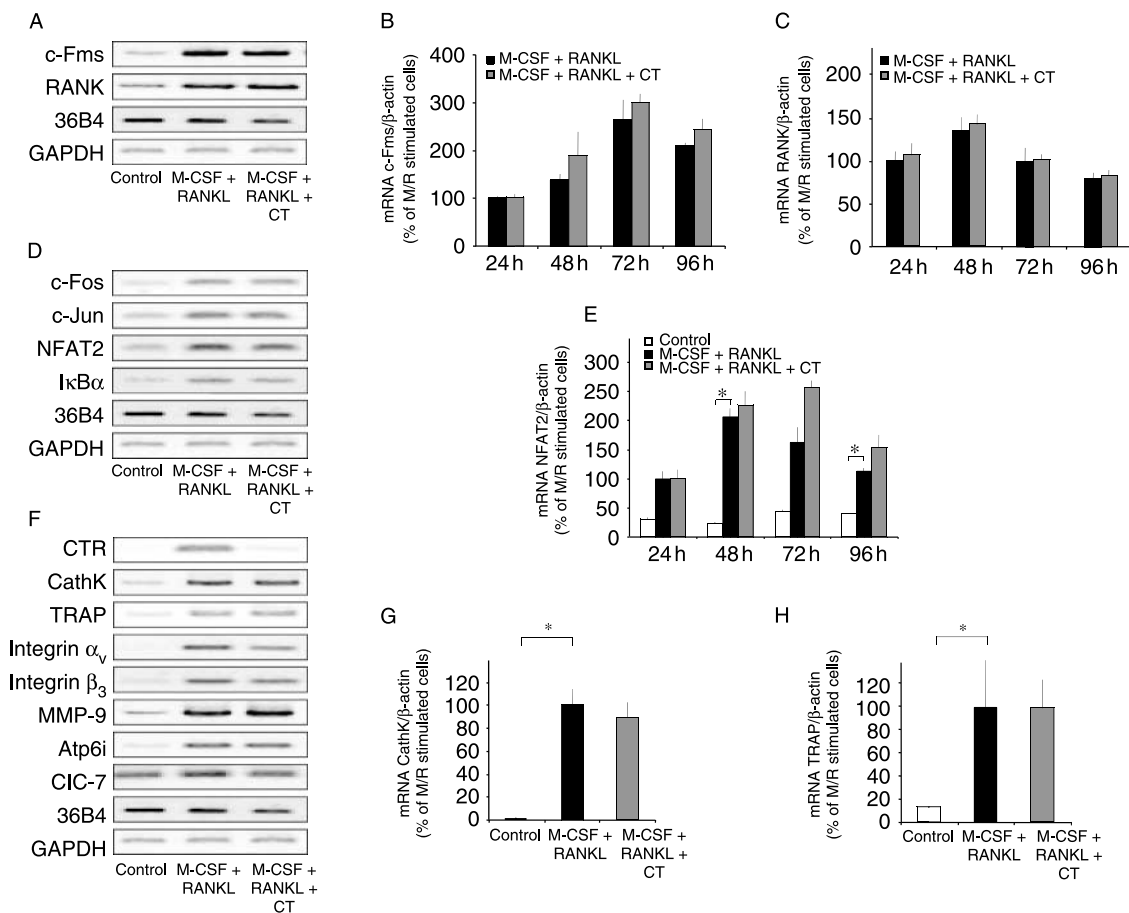
Figure 2 Analysis of mRNA expression in spleen cell cultures. PCR analyses were performed in untreated cells (control) and in cells stimulated with M-CSF and RANKL, with or without sCT (10^{-9} M). Semi-quantitative RT-PCR analyses of mRNA expression of the receptors c-Fms and RANK at 96 h are shown in (A). In (B and C), the quantitative real-time PCR analyses of c-Fms (B) and RANK (C) at 24–96 h are shown. In (D), the semi-quantitative RT-PCR analyses of the mRNA expression of the transcription factors c-Fos, c-Jun, NFAT-2 and the mRNA expression of IκBα, the inhibitory component of the transcription factor NF-κB, at 96 h are shown. In (E), the quantitative real-time PCR analysis of NFAT-2 for 24–96 h is shown. In (F), the semi-quantitative RT-PCR analyses of the mRNA expression of calcitonin receptor (CTR) and seven genes important for osteoclast function (cathepsin K, TRAP, MMP-9, the proton pump subunit ATP6i, ClC-7 and integrins α_v and β_3), cultured for 96 h are shown. In (G and H), the quantitative real-time PCR analysis of cathepsin K (G) and TRAP (H) at 96 h is shown. In semi-quantitative RT-PCR analyses, the expressions were compared with the two house-keeping genes, *36B4* and *GAPDH*. Columns represent the mean of four wells and s.e.m. is shown as vertical bars. *Statistical significance at $P < 0.05$.

Expression of transcription factors involved in osteoclast differentiation in the absence and presence of CT

Stimulation of spleen cells for 96 h with M-CSF and RANKL caused increased mRNA expression of c-Fos, c-Jun and NFAT-2, as assessed by semi-quantitative RT-PCR (Fig. 2D). As a consequence of NF-κB activation caused by RANKL–, M-CSF/RANKL-stimulated spleen cells expressed increased mRNA for inhibitory κBα (IκBα; Fig. 2D) M-CSF/RANKL-induced increase of mRNA for c-Fos, c-Jun, NFAT-2 and IκBα was unaffected by sCT (Fig. 2D). sCT-insensitive stimulation of NFAT-2 mRNA by M-CSF/RANKL treatment was also documented at 24–96 h by quantitative real-time PCR (Fig. 2E).

Effects of CT on the mRNA expression of genes known to be expressed in terminally differentiated osteoclasts

Treatment of spleen cells with M-CSF and RANKL for 96 h resulted in increased mRNA expression of CTR, TRAP, cathepsin K, MMP-9, ATP6i and the integrins α_v and β_3, but not of ClC-7, as assessed by semi-quantitative RT-PCR and using either GAPDH or 36B4 as house-keeping genes (Fig. 2F). The addition of sCT (10^{-9} M) abolished the M-CSF/RANKL induction of the *CTR* gene, but did not affect the mRNA expression of any of the other seven osteoclastic genes at the end of the 96-h cultures (Fig. 2F). Using quantitative real-time PCR, it was confirmed that sCT did not affect the enhanced mRNA expression of TRAP and

cathepsin K (Fig. 2G and H). The enhanced mRNA expression of cathepsin K and TRAP was observed already after 24-h treatment with M–CSF/RANKL and CT did not affect the mRNA expression at any time point (24–96 h; data not shown).

Effects of CT on the number of TRAP⁺ mononucleated cells

The decreased number of TRAP$^+$-MuOCL in M–CSF/RANKL-stimulated spleen cell cultures treated with sCT (10^{-9} M) was not associated with any decrease of total TRAP activity induced by the M–CSF/RANKL treatment (Fig. 3A). In agreement with the biochemical analysis of TRAP activity, morphological assessment of TRAP-stained spleen cell cultures showed the presence of large numbers of TRAP$^+$, mononucleated cells in the sCT-treated cultures stimulated by M–CSF/RANKL (Fig. 3B and 3C).

Effects of CT on apoptosis

Using a one-step sandwich ELISA kit, recognizing fragmented DNA bound to histones, we were unable to detect any effect of sCT (10^{-9} M) on apoptosis after 4 h of treatment (M-CSF+RANKL $100\pm13\%$, M-CSF+RANKL+sCT $98\pm13\%$; OD$_{405}$/well). Nor did we observe any effect after 14 h of sCT treatment (M-CSF+RANKL $100\pm27\%$, M-CSF+RANKL+sCT $109\pm29\%$).

Expression of FcRγ, DAP12 and associated immunoglobulin-like receptors in the absence and presence of CT

Using semi-quantitative RT-PCR, we found that stimulation of spleen cells for 96 h with M–CSF (25 ng/ml) and RANKL (100 ng/ml)) resulted in increased mRNA expressions of OSCAR at 48–96 h and of NKG2D at 72 and 96 h, whereas the mRNA expressions of DAP12, FcRγ, TREM-2, TREM-3, SIRPβ1 and PIR-A6 were unaffected at all time points (supplementary figure; see supplementary data in the online version of the Journal of Endocrinology at http://joe.endo-crinology-journals.org/content/vol195/issue3/). The addition of sCT (10^{-9} M) to the M–CSF/RANKL-stimulated spleen cells did not affect the mRNA expressions of DAP12 or FcRγ, nor the receptors to which these ITAM harbouring molecules dimerise (supplementary figure; see supplementary data in the online version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol195/issue3/).

Effect of CT on osteoclast formation, pit formation and gene expression in BMMs

It has been shown that T-cell-derived cytokines such as granulocyte–M–CSF, interferon-γ, interleukin-4 (IL-4) and IL-13 can negatively influence osteoclast progenitor cell differentiation (Takayanagi *et al.* 2000, Moreno *et al.* 2003, Shinoda *et al.* 2003), and that T-cells are required for the inhibitory effects of IL-12 and IL-18 (Horwood *et al.* 1998, 2001). We, therefore, studied whether CT had any effect on osteoclastogenesis in a population of BMM, highly enriched for osteoclast progenitor cells, obtained by culturing mouse bone marrow cells in the presence of M–CSF for 6 days on plastic dishes, to which neither T- and B-cells, nor stromal cells, can adhere. The BMM cells used were devoid of T- and B-cells as indicated by the lack of cells expressing CD45R and CD3, as assessed by FACS analysis (Fig. 4A). CD115/c-Fms was expressed on 75% of the cells and all cells expressed the macrophage marker CD11b/Mac-1. As a comparison, crude
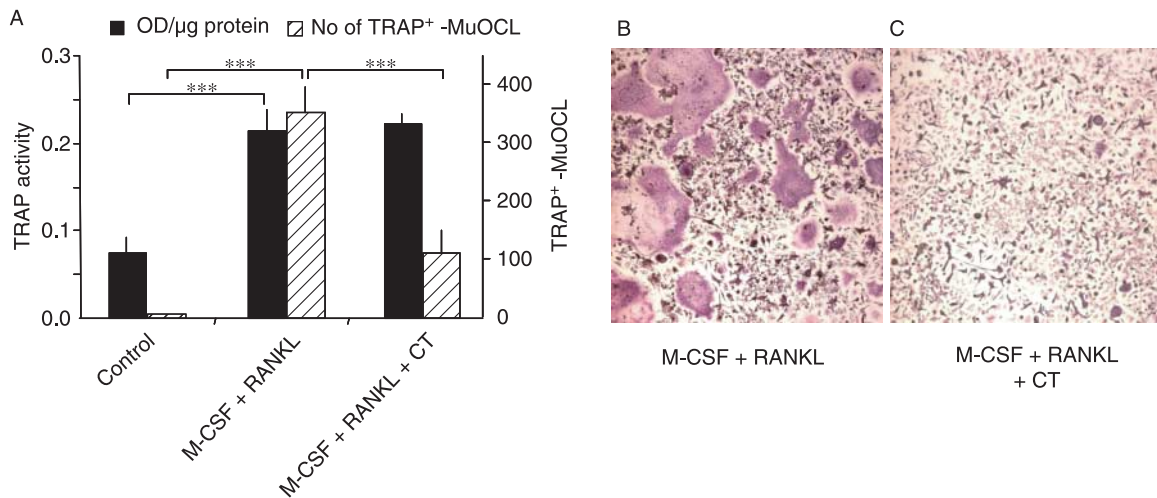

Figure 3 The number of TRAP$^+$-MuOCL was counted and in parallel cultures TRAP activity was quantified. Treatment with sCT (10^{-9} M) results in decreased amounts of TRAP$^+$ multinucleated osteoclasts associated with unchanged levels of total TRAP activity (A). TRAP staining of spleen cells cultured for 96 h in medium containing M-CSF and RANKL (B) and in medium containing M-CSF, RANKL and calcitonin (C). Decreased number of TRAP$^+$-MuOCL was associated with a large number of TRAP$^+$ mononuclear osteoclast progenitor cells (C). ***Statistical significance at $P<0.001$.

bone marrow cells contain CD3, CD45R and CD11b–positive cells, but almost completely lack CD115-positive cells (Fig. 4A). As appears in Fig. 4B, sCT inhibited M-CSF/RANKL–induced formation of TRAP$^+$-MuOCL in the BMM cultures to a degree comparable with the inhibition seen in spleen cells (Fig. 1B), with half maximal inhibition at 10^{-12} M. hCT also concentration dependently inhibited formation of TRAP$^+$-MuOCL, but was considerable less potent than sCT (Fig. 4B). Similarly, TRAP$^+$-MuOCL formation in a population of initially non–adherent BMMs (Roach *et al.* 1997) stimulated by M-CSF and RANKL was reduced by 80% by sCT (10^{-9} M; data not shown). The addition of sCT to BMM stimulated by M-CSF and RANKL resulted in a large number of TRAP$^+$ mononucleated cells (Fig. 4C). The number of resorption pits on devitalised bone slices was abolished by treatment of BMM with sCT (Fig. 4D). The number of TRAP$^+$-MuOCL seen was clearly decreased by CT when culturing CD115$^+$ CD3$^-$

CD45R$^-$ sorted BMM with M-CSF and RANKL in combination with CT (Fig. 4E). Also in these cultures, the inhibitory effect of CT on osteoclastogenesis was associated with the appearance of TRAP$^+$ mononucleated cells.

Semi-quantitative RT-PCR revealed that RANKL induced an increase in mRNA expression of OSCAR, DC-STAMP but not of DAP12 and FcRγ in BMM cells at 96 h (Fig. 5A). Similar to the observation in spleen cells, sCT did not affect the mRNA expressions of DAP12, FcRγ or OSCAR in BMM. Neither was DC-STAMP expression affected by sCT (Fig. 5A). Quantitative real-time PCR showed that CTR, cathepsin K, TRAP, ATP6i, integrin β_3, MMP-9 and OSCAR mRNA were increased by RANKL (Fig. 5B–H). The inhibition of osteoclastogenesis caused by sCT in the adherent BMM was associated with decreased mRNA expression of CTR (Fig. 5B), with no effect on the mRNA expressions of the other genes assessed (Fig. 5C–H).
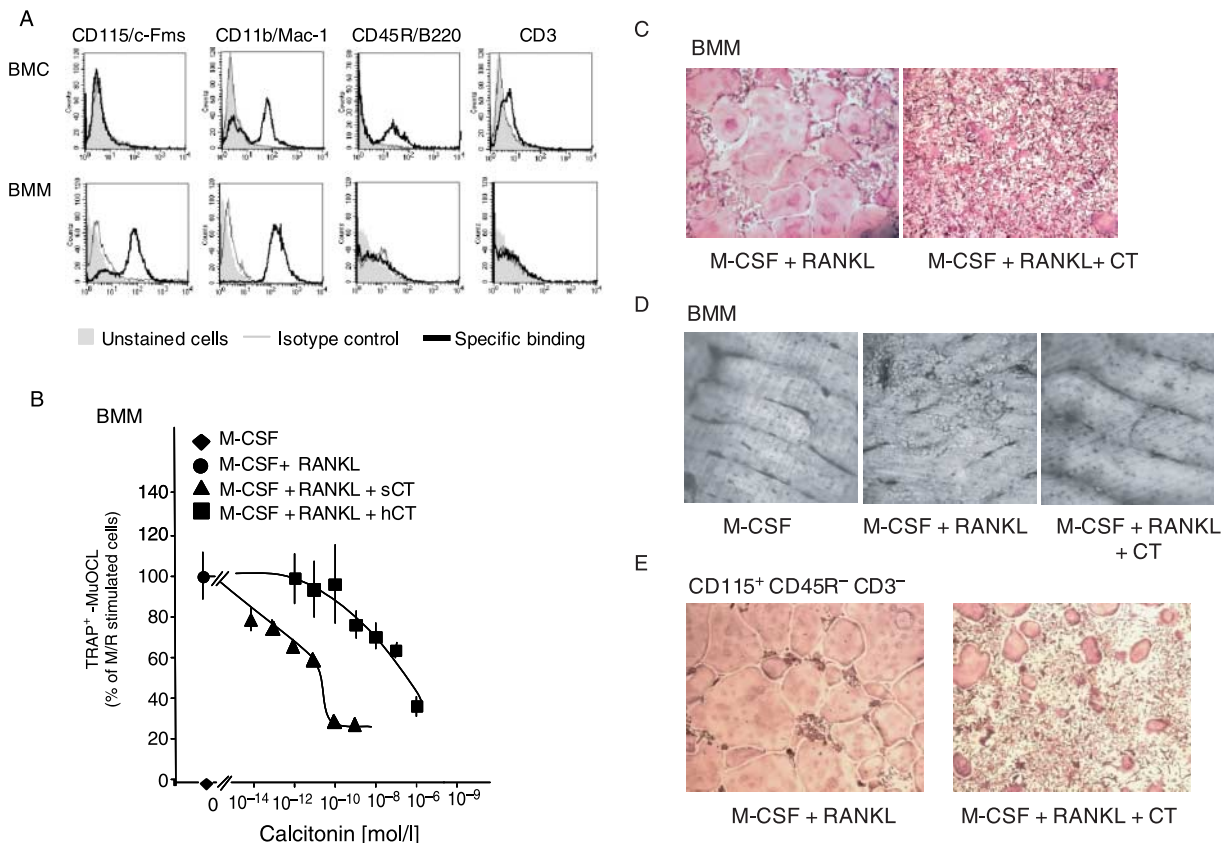

Figure 4 Flow cytometry analysis of bone marrow cells (BMCs) reveals that there is a population of cells which express CD11b and CD45R and that some cells express CD3, whereas no cells that express CD115 can be detected (A). In bone marrow macrophage (BMM) cultures, all cells are positive for CD11b and ∼75% express CD115 on their cell surface. No cells that express CD45R or CD3 can be detected in these BMM cultures (A). Salmon and human calcitonin inhibit the formation of osteoclasts in bone marrow macrophage cultures, stimulated by M-CSF and RANKL for 96 h, in a concentration-dependent manner (B). TRAP staining shows multinucleated TRAP$^+$ cells in BMM cultures stimulated by M-CSF and RANKL (C, left) and mononucleated TRAP$^+$ cells in cultures treated with calcitonin (C, right). Calcitonin inhibits bone resorption by BMM cells stimulated with M-CSF and RANKL (D, right) compared with cultures without calcitonin (D, centre). TRAP staining shows that M-CSF and RANKL induce formation of multinucleated TRAP$^+$ cells in CD115$^+$ CD3$^-$ CD45R$^-$ sorted BMM cultures (E, left) and that calcitonin inhibits the multinucleation process in these cultures (E, right).
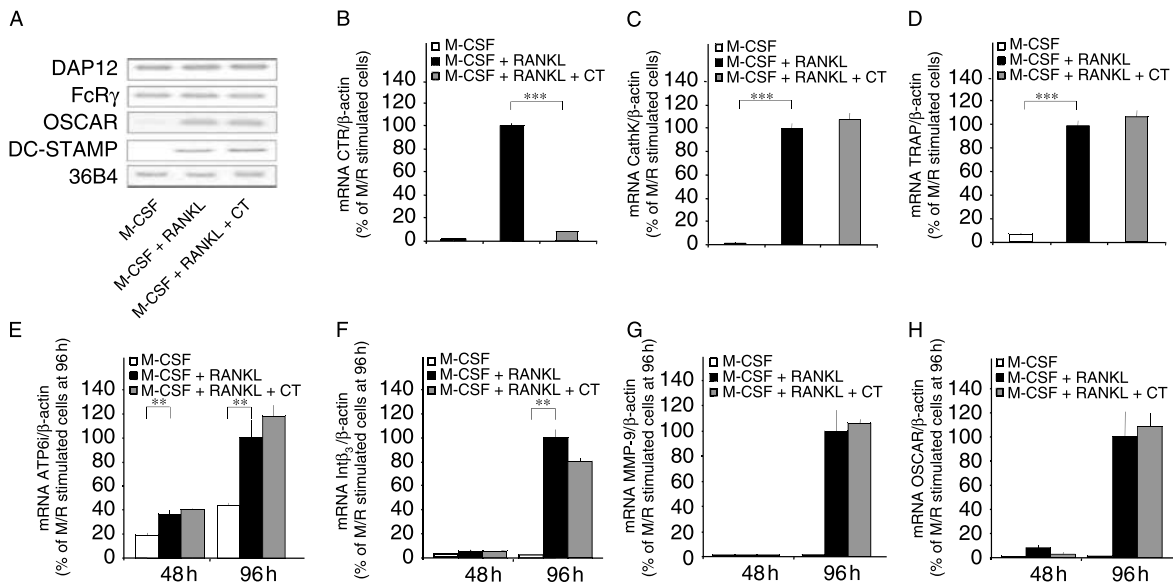
Figure 5 sCT (10^{-9} M) does not affect the mRNA expression of DAP12, FcRγ, OSCAR and DC-STAMP in M-CSF/RANKL-stimulated BMM cells at 96 h, as assessed by semi-quantitative RT-PCR (A). In M-CSF/RANKL-stimulated BMM cells, sCT (10^{-9} M) inhibits the mRNA expression of CTR, without affecting the increased mRNA expressions of cathepsin K, TRAP, ATP6i, integrin β$_3$, MMP-9 and OSCAR, as assessed by quantitative real-time PCR (B–H). The expressions of cathepsin K and TRAP were analysed at 96 h and the expressions of ATP6i, integrin β3, MMP-9 and OSCAR were analysed at 48 and 96 h. Columns represent the mean of four wells and S.E.M. is shown as vertical bars. **Statistical significance at $P<0.01$, ***statistical significance at $P<0.001$.

CT did not affect Atp6v0d2 or Atp6v0d1 mRNA expression in M-CSF/RANKL-stimulated BMM at 48 or 96 h (data not shown). It was suggested by Lee *et al*. (2006) that ADAM8 and ADAM12 were partially involved in the mechanism downstream Atp6v0d2. However, quantitative real-time PCR analysis showed that CT did not affect ADAM8 or ADAM12 mRNA expression at 48 and 96 h in M-CSF/RANKL-stimulated BMM (data not shown).

Effects of cAMP analogues on osteoclast formation

The addition of CT to BMM cultures stimulated by M-CSF/RANKL caused a time-dependent accumulation of cAMP (Fig. 6A), which was not seen in BMM cultures stimulated with only M-CSF (Fig. 6B), in agreement with the mRNA data (Fig. 5B). Forskolin is a well-known activator of adenylate cyclase, causing enhanced intracellular accumulation of cAMP. Adding forskolin to BMM cultures stimulated with M-CSF/RANKL, mimicked the effect of CT, resulting in decreased number of TRAP$^+$-MuOCL with very many TRAP$^+$ mononucleated cells being observed (Fig. 6C and D). Since cAMP functions as a second messenger activating at least two pathways, the PKA signalling and the Epac signalling (Kawasaki *et al*. 1998, de Rooij *et al*. 1998), we examined the effect of two different cAMP analogues; 6-MB-cAMP, which activates PKA but not Epac, and 8-pMeOPT-2'-*o*-cAMP which activates Epac but does not affect PKA. Both analogues caused an inhibition of the number of TRAP$^+$-MuOCL with very many TRAP$^+$

mononucleated cells, similar to the findings in cultures treated with CT (Fig. 6C and D). On the contrary, 8-bromo-5'-AMP, a cAMP analogue which cannot activate either PKA or Epac signalling, showed no effect on the number of TRAP$^+$-MuOCL formed (Fig. 6C and E).

Discussion

In the present study, we show that sCT and hCT inhibit osteoclast formation in mouse spleen and mouse BMM cell cultures, with half maximal inhibition at 10^{-12} M for sCT and hCT being considerably less potent, in agreement with its lower affinity to the mouse CTR. This observation is in agreement with a study by Cornish *et al*. (2001) in which rat CT was used, which to the best of our knowledge, is the only previous report demonstrating a concentration-dependent inhibitory effect by CT on osteoclastogenesis. In the studies by Cornish *et al*. (2001), mouse bone marrow cultures stimulated by D$_3$ were used to study osteoclast formation and the possibility therefore may exist that CT could have exerted its effect not directly on osteoclast progenitor cells, but indirectly via contaminating cells present in the crude bone marrow cultures. This possibility could also hold true in our spleen cell cultures. Even though it could be argued that such a possibility is unlikely, given the fact that CTR expression is a highly specific expression of osteoclasts, it should not be ignored that CTR has also been shown to be present in other cell types including lymphocytes (Body *et al*. 1994).
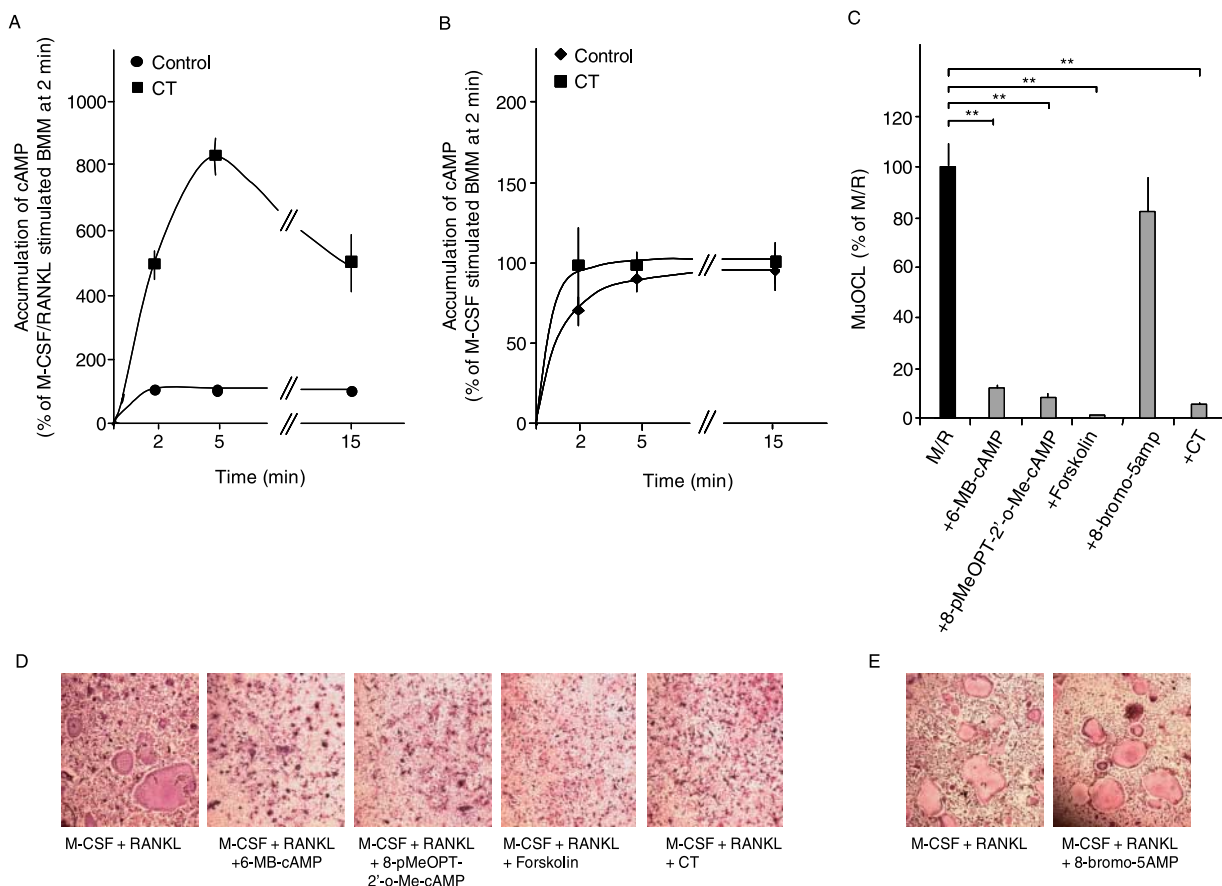
Figure 6 Addition of sCT (10^{-9} M) to BMM cultures stimulated by M-CSF/RANKL caused an accumulation of cAMP (A). In BMM cultures stimulated by M-CSF, sCT did not affect the cAMP levels (B). Forskolin (10 µM), 6-MB-cAMP (50 µM) and 8-pMeOPT-2'-o-Me-cAMP (50 µM), inhibit the formation of TRAP$^+$-MuOCL in M-CSF/RANKL-stimulated BMM cultures, similar to the effect caused by sCT (10^{-9} M; C–D). 8-bromo-5'-AMP (100 µM) did not affect the formation of osteoclasts (C and E). Symbols and columns represent the mean of four wells and s.e.m. is shown as vertical bars. **Statistical significance at $P < 0.01$. Data in (A) are based on two separate experiments, where the numbers of osteoclasts in M-CSF/RANKL-stimulated cultures were 157 ± 13/well and 219 ± 22/well respectively.

We, therefore, used BMM highly enriched in osteoclast precursor cells, as well as BMM cells positively sorted for CD115 and negatively for CD45R and CD3, and could show that CT inhibits osteoclast formation stimulated by M-CSF/RANKL also in these cells. These findings strongly suggest that the inhibitory effect of CT on osteoclastogenesis is due to an effect directly on osteoclast progenitor cells. The inhibitory effect of CT was obtained by adding CT during the last 24 of the 96-h cultures.

We assessed the possibility that CT-induced inhibition of osteoclastogenesis was due to increased apoptosis of osteoclast progenitor cells by an ELISA kit, recognizing fragmented DNA bound to histones. However, CT did not increase the intracellular levels of fragmented DNA.

Having established that CT inhibits osteoclastogenesis, our next aim was to determine the mechanism by which activation of CTR interferes with the downstream signalling events involved in RANK activation of osteoclast formation. First of all, we could exclude that CT affected the mRNA

expression of the receptors for RANKL and M-CSF. The knowledge about intracellular signalling mechanisms involved in osteoclast progenitor cell differentiation is still emerging. However, CT did not affect the increased mRNA expression of IκBα, c-Fos, c-Jun or NFAT-2 caused by RANK. In addition, we assessed the mRNA expression of a wide variety of genes known to be important for osteoclast function (Teitelbaum & Ross 2003). However, CT did not affect RANK-induced increased mRNA expression of TRAP, cathepsin K, MMP-9, the proton pump subunit ATP6i, ClC-7 or the integrins α_v and β_3. These observations indicate that CT inhibits osteoclast formation at a very late stage, a view compatible with the observation that addition of CT during the last 24 h was sufficient to cause the inhibitory effect. The view is also in line with the observation that a large number of TRAP$^+$ mononucleated cells was present in CT-treated M-CSF/RANKL-stimulated spleen cells and BMM, as well as with the fact that CT did not affect the increase of total TRAP activity caused by M-CSF/RANKL treatment.

DAP12, FcRγ and the immunoglobulin-like receptors to which these transmembrane signalling molecules are bound, have been suggested to be important at late stages of osteoclastogenesis, possibly at the fusion process (Paloneva *et al.* 2003). Taken together, our findings indicate that CT may play a role in osteoclast formation during the multi-nucleation process. We, therefore, analysed whether CT affected the mRNA expressions of DAP12 and FcRγ, and the receptors associated with them. We found that stimulation of osteoclast formation in spleen cell cultures with M-CSF/RANKL was associated with increased mRNA expression of OSCAR, both at early and late stages of osteoclast differentiation. We also found that the mRNA expression of NKG2D was enhanced during later stages of osteoclastogenesis. However, the mRNA expressions of TREM-2, TREM-3 and SIRPβ1, as well as the mRNA expression of PIR-A6 were unregulated both at early and late stages of osteoclastogenesis. Although the importance of FcRγ and DAP12 in osteoclastogenesis has been elegantly demonstrated, there are only very few studies reporting on the regulation of the expression of FcRγ, DAP12 and the associated receptors. In agreement with the present study, it has been shown that the expressions of OSCAR (Kim *et al.* 2002, So *et al.* 2003) and NKG2D (Humphrey *et al.* 2004) are enhanced by RANKL. However, in contrast to observations in RANKL-stimulated BMM (Humphrey *et al.* 2004), we were unable to observe any regulation of TREM-2 and TREM-3 in RANKL-stimulated spleen cells. Similar to the observations in the present study, DAP12 mRNA was unregulated by RANKL in BMM (Humphrey *et al.* 2004). Interestingly, CT did not affect the mRNA expressions of FcRγ, DAP12 or the associated receptors OSCAR, PIR-A6, TREM-2, TREM-3, NKG2D or SIRPβ1, neither at early stages of osteoclast formation nor at later stages in M-CSF/RANKL-stimulated spleen cell cultures.

DC-STAMP is a seven-transmembrane-region receptor, upregulated during osteoclast differentiation, which has been shown to be required for fusion of osteoclast progenitor cells (Yagi *et al.* 2006). Our results confirm the upregulation of DC-STAMP mRNA during osteoclastogenesis, but the enhanced DC-STAMP expression was not affected by CT. Most recently, ATP6v0d2 has been suggested to be crucial for osteoclast multinucleation (Lee *et al.* 2006). However, the mRNA expression of ATP6v0d2, or the downstream signalling effectors ADAM8 and ADAM12, was not affected by CT.

It is well documented that activation of CTR leads to stimulation of adenylate cyclase and formation of cAMP. The importance of the adenylate cyclase-activating pathway for the effect of CT in osteoclast formation was indicated by the finding that forskolin clearly mimicked the effect of CT. The downstream events of cAMP formation involve activation of the canonical PKA pathway but for some mechanisms also a non-canonical pathway mediated by Epac, a cAMP-regulated guanine nucleotide exchange factor. The latter pathway promotes exchange of GDP for GTP at the guanyl nucleotide-binding site on the small G-protein Rap1 (Kawasaki *et al.* 1998, de Rooij

et al. 1998). Active GTP-binding Rap1 interacts with Raf which can activate a MEK-ERK kinase cascade. The fact that a cAMP analogue which preferentially activates PKA mimicked the effect of CT indicates that the cAMP-PKA pathway is important. Most interestingly, however, a cAMP analogue preferentially activating Epac mimicked the effect of CT, suggesting that the non-canonical pathway is also important. It has been shown that CT rapidly and transiently can stimulate the phosphorylation of ERK1/2 in CTR-expressing cells (Chen *et al.* 1998, Zhang *et al.* 2002). This effect was paralleled by activation of protein kinase C and a rise of intracellular calcium, but the elevation of intracellular calcium was not sufficient to fully explain the phosphorylation of ERK1/2, which indicates that other mechanisms contribute. In kidney cells (Laroche-Joubert *et al.* 2002), it has recently been shown that Epac is important for ERK1/2 activation and therefore, our findings point, for the first time, that not only the PKA but also the Epac-ERK1/2 pathway is involved in the action of osteoclasts.

Our data show that CT is a potent inhibitor of osteoclast formation and that this effect is not associated with apoptosis, nor decreased transcription of a variety of genes known to be important for osteoclast progenitor cell differentiation, fusion or function. These data show that inhibition of bone resorption *in vivo* by CT may be explained not only by an acute effect on multinucleated osteoclast formation but also by an effect on osteoclast formation.

Elucidating the molecular mechanism by which CT inhibits osteoclastogenesis could be helpful in finding a novel target for the development of new inhibitors of bone resorption.

Acknowledgements

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