Calcitonin and Prednisolone Display Antagonistic Actions on Bone and Have Synergistic Effects in Experimental Arthritis

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We tested here the hypothesis that calcitonin and glucocorticoids, known to modulate bone metabolism, could have opposite actions on bone cells regulating expression of cytokine receptor activator of nuclear factor- κ B ligand (RANKL) and osteoprotegerin (OPG). In the U2OS osteosarcoma cell line, calcitonin (10^{-11} to 10^{-9} mol/L) reduced RANKL and augmented OPG both at the mRNA and protein levels. Cell incubation with prednisolone (10^{-8} to 10^{-6} mol/L), the glucocorticoid chosen for this study, produced opposite results. These molecular studies prompted more functional analyses whereby osteoclast bone resorptive activity was determined. Calcitonin (10^{-10} mol/L) abrogated the stimulating effect of 10 ng/ml RANKL or 10^{-9} mol/L prednisolone; similar results were obtained with OPG. Assessment of calcitonin and prednisolone effects in an *in vivo* model of rheumatoid arthritis revealed partially surprising results. In fact, calcitonin not only preserved bone morphology (as assessed on day 18) in rats subjected to arthritis and treated with prednisolone (0.8 to 4 mg/kg daily from day 13) but also synergized with the steroid to elicit its antiarthritic effects. These results suggest that calcitonin could be used as a novel cotreatment to augment efficacy and reduce side effects associated with the prolonged use of steroids. (Am J Pathol 2007, 170:1018–1027; DOI: 10.2353/ajpath.2007.060830)

The receptor activator of nuclear factor- κ B ligand (RANKL) and its receptor RANK are key regulators of bone remodeling and are essential for osteoclast development and activation.^{1,2} In the presence of macrophage colony-stimulating factor, RANKL promotes osteoclastogenesis and activates mature osteoclasts *in vitro*.¹ Consistently, RANKL-null mice display severe osteopetrosis,

stunted growth, and a defect in tooth eruption.³ Some bone-resorbing factors including glucocorticoids (GCs), vitamin D, interleukin-1, interleukin-6, and parathormone up-regulate RANKL expression.⁴ Osteoprotegerin (OPG), a secreted protein with high homology to the tumor necrosis factor receptor family, is a soluble decoy receptor to RANKL. It therefore acts as an effective inhibitor of osteoclast maturation and activation both *in vitro* and *in vivo*.⁵ OPG-null mice display severe osteoporosis associated with a high incidence of fractures.^{6,7} All these data indicate that the balance between RANKL-RANK signaling and the levels of biologically active OPG are crucial in regulating the development and activation of osteoclasts and bone metabolism. Further, many factors that inhibit or enhance bone resorption can act, directly or indirectly, through regulation of RANKL-RANK and/or OPG.

Besides specific bone pathologies, enhanced bone resorption is also observed in rheumatoid arthritis (RA), a condition characterized by progressive synovial inflammation and joint destruction. Patients with RA have lower bone mineral density and are at risk of pathological fractures.^{8,9} Bone erosion in RA is caused by osteoclast activation triggered by the production of RANKL by synovial fibroblasts and T lymphocytes and is therefore susceptible to OPG inhibition.^{3,10} However, OPG has no major anti-inflammatory effects on synovitis or pannus.¹¹

GCs are potent immunosuppressive and anti-inflammatory agents widely used in all forms of chronic inflammation although their long-term use is associated with secondary osteoporosis *in vivo*. Throughout the years, GC action on bone cells has been associated with an increase in parathormone release leading to an increase in bone resorption as well as a decreased number of bone-forming cells.^{12,13} Furthermore, GC's effect on the

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Table 1. Experimental Conditions Used for the RT-PCR Analyses: Sequence of Primers

Gene of interest	5' to 3' primers (forward/reverse)	Expected product size (bp)
hOPG	5'-GAACCCCAGAGCGAAATACA-3' 5'-CGCTGTTTTACAGAGGTCA-3	441
hRANKL	5'-GCTTGAAGCTCAGCCTT TTGCTCAT-3' 5'-GGGGTTGGAGACCTCGATGCTGATT-3'	412
hCTR	5'- GCAATGCTTTCACTCCTGAGAAAC-3' 5'-CAGTAAACACAGCCACGACAATGAG-3'	386

OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor $\kappa\beta$ (RANK) ligand; CTR, human calcitonin receptor; h, human.

osteoblast has been linked to RANKL up-regulation and OPG down-regulation.^{14,15} More recently, a direct action of GCs on osteoclast cytoskeletal rearrangements resulting in suppression of the whole bone-remodeling process has also been reported.¹⁶

Calcitonin (CT), a peptide hormone secreted in response to hypercalcemia, has the dual effect of inhibiting osteoclast recruitment as well as their resorptive activity.¹⁷ Despite CT efficacy for the treatment of bone metabolic disorders, including osteoporosis, a limitation to its therapeutic application is attributable to CT receptor down-regulation after continuous treatment.¹⁸ It is of interest that a dual relationship between GC and CT is emerging, with the former being able to restore or retain CT receptor expression in osteoclasts¹⁸ and the latter being effective in preventing GC-induced spine fractures.^{19,20} No studies have yet addressed if and how GC and CT actions could converge on the RANKL system or the potential functional consequences on this interaction on bone cells, as well as *in vivo*, and this was the main aim of the present investigation.

Materials and Methods

All materials were purchased from Sigma-Aldrich Co. (Poole, UK) unless otherwise specified in the text. Preliminary time course and concentration-response experiments were performed to select the optimal conditions for the experiments listed below. Elcatonin (eCT), a stable analog of eel CT, was a gift from Asahi Chemical Industry (Institute for Life Science Research, Shizuoka, Japan), and salmon CT (sCT), used for the *in vivo* studies, was obtained from Sigma-Aldrich. In our experience, the two preparations of CT are fully interchangeable; in addition, sCT was selected for the *in vivo* analyses for its wide use in clinical settings. OPG and RANKL protein used for the bone resorption experiments were kindly provided by Dr. David Lacey, Amgen Inc., Thousand Oaks, CA.

Cell Culture

The osteosarcoma cell lines U2OS and Saos-2 and the breast cancer cell line T47D (all from the American Type Culture Collection catalog; distributed by LGC Promochem, Teddington, Middlesex, UK) were cultured in McCoy's medium with 10% fetal calf serum (Gibco, Paisley, UK), 100 U/ml penicillin, and 100 μ g/ml streptomycin in an atmosphere of 5% CO₂/95% air at 37°C and passaged two times a week.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Subconfluent cells were incubated with RPMI without phenol red and with 1% stripped bovine serum for 24 hours before stimuli addition. Plates were washed with phosphate-buffered saline (PBS) and RNA extracted with a phenol-free total RNA isolation kit (RNAqueous; Ambion Inc., Austin, TX); total RNA was then reverse-transcribed with random primers (RETROscript; Ambion Inc.) and cDNA used for PCRs (Hyaid; Thermofisher Scientific Inc., Waltham, MA). The sequences of the primers and the PCR conditions used are summarized in Tables 1 and 2. Primers for 18S RNA (QuantumRNA; Ambion Inc.) (expected size, 324 bp) were used as internal control to normalize the results obtained with densitometry analysis, as performed using Scion Image from NIH Software, Bethesda, MD.

OPG and RANKL Protein Determination

To quantify OPG and RANKL protein contents, cells (10⁵/well) were incubated for 4 or 24 hours with 10⁻⁸ to 10⁻⁶ mol/L prednisolone (prednisolone 21-hemisuccinate, sodium salt) or 10⁻¹¹ to 10⁻⁹ mol/L eCT. OPG was measured with enzyme immunoassay (EIA) development reagents from R&D Systems (Abingdon, UK) following the

Table 2. Experimental Conditions Used for the RT-PCR Analyses: PCR Conditions

Gene of interest	Denaturation	Annealing	Extension	Final extension	Cycle numbers
hOPG	95°C, 45'	60°C, 30'	72°C, 30'	72°C, 10'	27
hRANKL	95°C, 45'	55°C, 30'	72°C, 30'	72°C, 10'	35
hCTR	95°C, 45'	60°C, 30'	72°C, 1'	72°C, 10'	35

OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor $\kappa\beta$ (RANK) ligand; CTR, human calcitonin receptor; h, human.

supplier instructions, and RANKL was measured by EIA using paired antibodies from Peprotech (London, UK) as previously described.²¹ Detection limits for these assays were as follows: 25 pg/ml for OPG and 50 pg/ml for RANKL. Whereas OPG was readily detected in the cell incubation media, RANKL was not detected in the media (data not shown). Thus, cell pellets were incubated with 2 mmol/L Triton in PBS for 10 minutes, centrifuged, and the supernatant analyzed. Relevantly activated T cells produce soluble RANKL, whereas cell-associated RANKL is a feature of osteoblasts and fibroblasts and is functionally active in inducing efficient osteoclastogenesis.²²

CT Receptor

The expression of CT receptor on U2OS cell surface was monitored by flow cytometry. In brief, 5×10^5 treated cells were washed twice with PBC (PBS containing 0.2% bovine serum albumin and 1.3 mmol/L CaCl_2) and labeled with 20 $\mu\text{g}/\text{ml}$ mouse anti-human CT receptor antibody (1 hour at 4°C; Serotec, Abingdon, UK). Then, cells were washed with ice-cold PBC and incubated for 30 minutes at 4°C with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (Serotec). Finally, cells were washed and resuspended in PBC before flow cytometry analysis with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Receptor functionality was confirmed by measuring intracellular accumulation of cAMP.²³ For this purpose, cells were plated in 24-well plates ($5 \times 10^5/\text{well}$), serum-starved for 24 hours, and then stimulated for 30 minutes with 10^{-12} to 10^{-9} mol/L eCT in the presence of 10^{-3} mol/L isobutylmethylxanthine. Samples were then lysed and processed for cAMP EIA determination following the kit manufacturer procedure (Amersham Biosciences, Buckinghamshire, UK).

GC Receptor Binding Assay

Subconfluent U2OS cultures were kept for 24 hours in RPMI medium without phenol red and with 2% bovine serum (charcoal stripped to remove endogenous GC). The binding assay was performed as previously described.²⁴ Briefly, triplicate samples of 10^6 cells were incubated with 1.57 to 50 pmol [^3H]dexamethasone (Amersham Biotechnology, Abingdon, Oxon, UK) at 37°C for 1 hour, in the presence or absence of 50×10^{-6} mol/L unlabeled dexamethasone, to determine the values for nonspecific binding. Samples were then washed three times with ice-cold PBS and analyzed with a β -counter (LS6000IC; Beckman). Scatchard plot analysis was performed to measure the affinity constant K_D and the maximal binding B_{max} . To construct displacement curves, cells were prepared as above and incubated with 50 pmol of [^3H]dexamethasone in the presence or absence of 10^{-9} to 10^{-5} mol/L unlabeled prednisolone for 1 hour at 37°C. Samples were then processed as above, and count per minute (cpm) values were used to calculate the IC_{50} .

Rat Osteoclast Isolation and Bone Resorption Assay

Newborn Sprague-Dawley rats were killed by cervical dislocation and legs removed for isolating osteoclast-rich cell population as previously described.²⁵ The ability of osteoclasts to resorb bone was measured using 24-well plates coated with a film of calcium phosphate apatite (OAAS; OCT Inc., Chunan, Korea). The osteoclast suspension was applied to the 24-well plates and allowed to sediment, and the unattached cells were washed away with minimal essential medium. After 18 hours of incubation with the compounds to be tested, the 24-well plates were treated with a sodium hypochlorite solution [10% (v/v)], washed with distilled water, and dried. The 24-well plates were then analyzed with an inverted microscope interfaced with an Argus-10 image-processing system (Hamamatsu Photonics, Enfield, UK). The number of excavation pits was counted for each well, and the pit area was recorded. The resorbed area on the bone surface was calculated as the sum of the areas of individual excavations and expressed as a percentage of control values.

Collagen II-Induced Arthritis (CIA)

Female Lewis rats (150 ± 20 g body weight; Harlan UK Ltd., Bicester, UK) were fed on a standard chow pellet diet and had free access to water and maintained on a 12-hour light/dark cycle. Animal work was performed under license from the Home Office in accordance with the Animals (Scientific Procedures) Act, 1986. Bovine nasal collagen II (4 mg/ml) was dissolved in acetic acid (0.01 mol/L) and then emulsified with the same volume of ice-cold Freund's incomplete adjuvant. On day 0, rats were anesthetized with halothane and injected intradermally at the base of the tail with collagen II/adjuvant suspension (400 μg of collagen II per rat). The first signs of arthritis were evident between days 11 and 13, with maximal inflammation observed at days 16 to 18.²⁶

Salmon CT was dissolved in PBS with 0.1% bovine serum albumin and given daily intraperitoneally at the dose of 2 $\mu\text{g}/\text{kg}$ per rat. Prednisolone was dissolved in PBS with 0.1% bovine serum albumin and given daily intraperitoneally at doses of 0.6 and 3 mg/kg (corresponding to 1.2 and 6.2 $\mu\text{mol}/\text{kg}$), alone or with sCT. Control treatments consisted of PBS with 0.1% bovine serum albumin. CIA-induced inflammation was confined to ankle joints and footpads of the hind legs (with digit involvement in severe cases). Hind ankles were scored clinically on an arbitrary scale, ranging from 0 (no inflammation) to 3 (severe inflammation, involving ankles, footpads, and digits). In addition, between days 0 and 18, hind paw volumes were measured using a plethysmometer (Ugo Basile, Milan, Italy), and values were averaged to give a measurement of inflammation for each animal. Body weight was also monitored during the study.

Tissue slices for histology were prepared along classical protocols. In brief, on day 18 animals were killed by cervical dislocation and the hind limbs removed, fixed in

buffered formol-saline solution, and then placed in formic acid-formal saline solution for ~1 week for decalcification. X-ray analysis by Faxitron imaging confirmed decalcification. Tissue was then sampled, using cross sections of the digits, and tissue blocks were processed to paraffin wax using a Shandon hypercenter with standard protocol (Thermo Electron Corporation, Waltham, MA). Slides were prepared from paraffin blocks at 5- μ m thickness; these were dewaxed, rehydrated through graded alcohols, and stained using hematoxylin and eosin. Sections were mounted in Canada balsam and viewed by standard transmission light microscopy. Slides were prepared and examined blind to the treatment group by a histopathologist.

Statistical Analysis

Experiments were repeated at least three times, each in triplicate. *In vivo* experiments were performed with 10 rats per group. In all cases, data are reported as means $\pm$ SEM and were analyzed by analysis of variance followed by Dunnett post hoc analysis. A *P* value less than 0.05 was taken as significant.

Results

CT and GC Receptor Expression

Initially, we validated the presence of specific receptors for CT and GC in the osteosarcoma cell line U2OS. To detect the GC receptor, we used [3 H]dexamethasone as tracer for experiments of binding. Scatchard plot analysis revealed that U2OS cells possess $32,532 \pm 2993$ binding sites per cell, with an affinity for the receptor of 10.4 ± 1.3 nmol/L (K_D) and a B_{max} of 53.6 ± 3.8 pmol/L ($n = 3$ experiments) (Figure 1A). Because prednisolone was the GC selected for the majority of the functional experiments, we verified its ability to compete for [3 H]dexamethasone binding to GC receptor. Increasing concentrations of unlabeled prednisolone produced a significant displacement with a calculated IC_{50} of 87.8 ± 3.4 nmol/L ($n = 3$ experiments) (Figure 1B). Complete displacement of [3 H]dexamethasone binding was observed at 10^{-5} mol/L prednisolone.

The specific mRNA for CT receptor was found in U2OS cells but not in another osteosarcoma cell line, Saos-2 (Figure 2A). The assay was validated by using the human breast cancer cell line T47D as positive control. Thus, U2OS cells were selected for further experiments. Flow cytometry analysis shows specific and marked presence of the CT receptor in U2OS cells (Figure 2B). The functionality of this receptor was assessed by measuring cAMP accumulation, which was augmented after cell incubation with 10^{-9} mol/L eCT (Figure 2C).

Effects of CT and Prednisolone on RANKL and OPG mRNA and Protein

RT-PCR analysis showed that after 2 hours of incubation of U2OS cells with 10^{-10} mol/L eCT, there was a de-

Figure 1. GC receptor binding assay in U2OS cells. **A:** Scatchard plot analysis with different concentrations of the tracer [3 H]dexamethasone indicates the existence of a single specific binding site in U2OS cells. The parameters calculated from this analysis are the following: affinity constant (K_D) of 10.4 ± 1.3 nmol/L, with a maximal number of binding sites (B_{max}) of 53.6 ± 3.8 pmol/L, corresponding to $32,532 \pm 2993$ sites per cell. The results are expressed as the means $\pm$ SEM of three experiments performed in triplicate. **B:** Dexamethasone binding was displaced with unlabeled prednisolone in a concentration-related manner with a calculated IC_{50} of 87.8 ± 3.4 nmol/L, demonstrating that U2OS cells are also capable of binding prednisolone. The results are expressed as the means $\pm$ SEM of three experiments. All experiments were performed in triplicate. ***P* < 0.01.

crease in RANKL mRNA coupled with an increase in OPG mRNA (Figure 3A). This was paralleled by changes at the protein level: eCT produced a decrease in the cellular content of RANKL as seen after 24 hours of incubation with U2OS cells, although significantly different only at the concentration of 10^{-11} mol/L (Figure 3B). The release of OPG in the medium was also modulated by eCT such that significant increases were detected at the 4-hour, but not 24-hour, time point (Figure 3C). U2OS cell incubation with prednisolone produced opposite effects on the RANKL/OPG system. In fact, early (2 hours) incubation with this GC yielded an increase in RANKL mRNA level, and a decrease in OPG mRNA (Figure 4A). In terms of protein expression, cell-associated RANKL content was not modified by any of the concentrations used (Figure 4B), whereas a significant reduction in OPG cellular output was produced both at 4 and 24 hours of incubation with prednisolone (Figure 4C).

Figure 2. Expression of a functional calcitonin receptor in U2OS cells. **A:** Expression of the specific mRNA for the calcitonin receptor as detected by RT-PCR. U2OS cells show a positive band at the expected size (386 bp) (lane 1), whereas another human osteosarcoma cell line, Saos-2, did not express CTR (lane 2). The breast cancer cell line T47D was used as positive control for the receptor expression (lane 3). **B:** FACS histogram showing immunofluorescence due to the anti-CT receptor mAb compared with control antibody. **C:** Increase in intracellular cAMP accumulation in U2OS cell following 30 minutes of stimulation with eCT. Values for **C** are mean $\pm$ SEM of three experiments performed in triplicate.

Combined Action of CT and Prednisolone on Bone Resorption

The functional relevance of the cellular changes described above was tested by means of bone resorption assay using an osteoclast-enriched cell population. Data in Figure 5 illustrate this set of experiments. Both 10⁻⁹

Figure 3. CT modulates RANKL and OPG expression in U2OS cells. **A:** Cell incubation with CT (10⁻¹⁰ mol/L, 2 hours) down-regulates RANKL mRNA expression and up-regulates OPG mRNA expression, as detected by RT-PCR. The representative blot shows the RANKL product (441 bp), the OPG product (412 bp), and the 18S (324 bp), used as internal control. Graph on the right presents these data in a semiquantitative manner, with normalization for each specific product versus 18S RNA band. The values obtained in U2OS cells in the absence of treatment are taken as 100%, with a calculated decrease in RANKL mRNA of 79% and increase in OPG mRNA of 59%. Data are means $\pm$ SEM of three experiments. **B:** RANKL protein levels as measured in U2OS cells by EIA. Cells were incubated with eCT for 4 or 24 hours. **C:** OPG protein released by U2OS cells as measured by EIA. Cell incubation as in **B**. For **B** and **C**, results are expressed as the means $\pm$ SEM of three experiments performed in triplicate. **P* < 0.05 versus control (concentration 0).

mol/L prednisolone and 10 ng/ml RANKL (equivalent to $\sim 4 \times 10^{-10}$ mol/L) increased bone resorption to a similar extent. Equally, OPG and 10⁻¹⁰ mol/LM eCT inhibited almost completely osteoclast resorptive activity. OPG was active at 100 ng/ml (equivalent to 1.4×10^{-9} mol/L) but not at 30 ng/ml (Figure 5A). However, either OPG concentration was effective in significantly attenuating the stimulating effect of 10⁻⁹ mol/L prednisolone on resorption (Figure 5B). Likewise, 10⁻¹⁰ mol/L eCT was able to challenge both RANKL-induced and prednisolone-induced bone resorption producing a significant reduction of osteoclast activity (Figure 5C). None of these actions was consequent on widespread toxicity because no changes in trypan blue uptake could be measured when the cells were incubated with or without 100 ng/ml OPG or 10⁻¹⁰ mol/L eCT (data not shown).

CIA

Rat immunization with collagen provoked a delayed arthritic response localized at the foot and digit level, with

Figure 4. Prednisolone modulates RANKL and OPG expression in U2OS cells. **A:** Cells were incubated with prednisolone (10^{-8} mol/L, 2 hours) and analysis of mRNA expression conducted as detailed in Figure 3. Blot is representative of three experiments whose densitometric analysis after normalization with 18S RNA is shown on the right. **B:** RANKL protein levels as measured in U2OS cells by EIA. Cells were incubated with prednisolone for 4 or 24 hours. **C:** OPG protein released by U2OS cells as measured by EIA. Cell incubation as in **B**. For **B** and **C**, results are expressed as the means $\pm$ SEM of three experiments performed in triplicate. * $P < 0.05$ versus control (concentration 0).

kinetics similar to our previous studies (eg, Paul-Clark et al²⁶). Likewise, administration of a full dose of prednisolone (4 mg/kg/day) markedly inhibited disease incidence and its aggressive malformations, monitored by measuring paw volume and arthritis score: these data are illustrated in Figure 6. Interestingly, daily administration of CT or a subtherapeutic dose of prednisolone (0.8 mg/kg) failed to impact on disease incidence and arthritic indices, whereas a marked synergistic response was observed in the group of rats co-treated with CT plus 0.8 mg/kg prednisolone. Figure 6A shows the marked attenuation of disease incidence in the CT plus 0.8 mg/kg prednisolone group, and this was mirrored by reductions in paw volume (Figure 6B) and arthritic score (Figure 6C). The synergistic effect of CT could not be observed when the higher dose of prednisolone was used because it is fully therapeutic on its own (Figure 6). All these effects were not secondary to alteration in the health status of the animals: vehicle-treated rats gained ~4 g throughout the 26-day period, and this was reflected in all groups with changes ranging from 4 to 8 g of body weight gain (not significantly different for any group).

Histological analyses of the affected digits and feet confirmed these macroscopic findings, with a marked degree of synovitis and bone erosion in CIA rats compared with the naïve group (Figure 7, a and b). In the rats

Figure 5. CT and prednisolone modulate the bone resorptive activity of rat primary osteoclasts. **A:** Bone resorption after overnight cell incubation with single concentration (each concentration given in the legend). Prednisolone (10^{-9} mol/L) and RANKL (10 ng/ml) potentiated bone resorption, whereas OPG (100 ng/ml) and eCT (10^{-10} mol/L) diminished it. Data are means $\pm$ SEM of three experiments. * $P < 0.05$ and ** $P < 0.01$ versus control (unstimulated cells). **B:** Cell incubation with prednisolone (10^{-9} mol/L) and the reported concentrations of OPG reduced bone resorption. Data are means $\pm$ SEM of three experiments. ** $P < 0.01$ versus control (unstimulated cells); * $P < 0.05$ and ** $P < 0.01$ versus prednisolone alone. **C:** eCT (10^{-10} mol/L) addition to cells abolishes the effect of both RANKL (10 ng/ml) and prednisolone (10^{-9} mol/L). Data are means $\pm$ SEM of three experiments. ** $P < 0.01$ versus control (unstimulated cells) and ** $P < 0.01$ versus RANKL or prednisolone alone.

treated with the subtherapeutic dose of prednisolone, no major protective effects were observed with respect to both the inflammatory reaction and bone alterations (Figure 7, c and d). However, analysis of the joints of the rats treated with CT plus this subtherapeutic dose of prednisolone showed a marked attenuated degree of synovitis (Figure 7e), in line with the macroscopic analysis (Figure 6), and a nearly intact bone profile (Figure 7f).

Figure 6. CT and prednisolone synergize in the CIA model. Collagen II injection to rats provoked arthritis from day 11 onwards when treatment with CT (2 μ g/kg s.c.) alone or with prednisolone (0.8 or 4 mg/kg s.c.) started and continued on a daily basis. **A:** Profiles of disease incidence. **B:** Changes in right hind paw volume. **C:** Arthritic scores. Data are means $\pm$ SEM of 10 rats per group. * P < 0.05 versus vehicle; # P < 0.05 versus prednisolone (0.8 mg/kg/day) alone.

Thus, histological morphology reinforced the synergistic protective role of CT when accompanied by the low-dose prednisolone.

Discussion

In this study, we have investigated the interrelationship between prednisolone and CT, using an integrated approach in which *in vitro* biological effects on the RANKL/OPG system have been complemented by more functional data on arthritis. We propose that a CT-mediated increase in OPG and a decrease in RANKL could also contribute to the known antiresorptive activities of this hormone. In addition, we unveil a novel macroscopic joint-protective effects of CT evident after GC treatment, an effect not observed with OPG treatment.¹¹

CT is a 32-amino acid peptide hormone with potent inhibitory effects on osteoclast activity: on one hand, it down-regulates osteoclast recruitment and, on the other hand, exerts potent inhibition on the resorptive activity of mature osteoclasts.¹⁷ Whereas these actions of CT are well described, not many studies have investigated the possibility that these effects could be, at least partially, indirect. For instance, CT can produce an anabolic effect on osteoblasts *in vitro*,^{27,28} and moreover, CT receptors have been found in cells of osteoblast lineage as well as in an osteosarcoma cells line.²⁹ Here, we demonstrated that the osteoblast-like cell line U2OS expressed both message and protein for the CT receptor. In addition, the receptor was functional because marked increases in cAMP²³ could be measured after addition of low eCT concentrations. U2OS cells also expressed the GC receptor. Analysis of the experiments of binding indicated an affinity constant similar to one previously found in human osteosarcoma cell lines³⁰ as well as in our previous studies with other cellular systems.²⁴ The coexpression of these two receptors in this osteoblastic cell line prompted the second part of the study, also in view of the preclinical and clinical opposing effects exerted by GC and CT on bone resorption.^{12,13,17} In the *in vitro* experimentation, we focused on the RANKL/RANK system shown to have crucial primary effects on bone metabolism.³

GCs increase RANKL and suppress OPG production in primary osteoblasts and osteosarcoma cells.^{14,15} In our hands, U2OS cell mRNA levels of RANKL were significantly augmented after 2 hours of incubation with prednisolone. Interestingly, and congruently with the proresorptive action of this GC, OPG mRNA levels were significantly decreased in the same culture conditions. This set of results is in line with what reported by other studies in which micromolar concentrations of hydrocortisone or dexamethasone affected OPG and RANKL mRNA.¹⁴ These actions of prednisolone on transcription were only partially reflected on translation. U2OS cell incubation with prednisolone significantly decreased OPG release as measured at both 4 and 24 hours after GC addition, but RANKL protein expression was only slightly, and not significantly, increased. The reason for this discrepancy is unclear and may warrant more detailed analysis. In any case, it was important to compare the effects of prednisolone with those of CT on these cells because we found that CT was also able to modulate these two cytokines. Short (2 hours) U2OS cell incubation with eCT markedly decreases RANKL mRNA expression counteracted by an increase in OPG mRNA expression to a similar degree (–79 versus +50%). Interestingly, these two effects related to changes in protein expression with a distinct time profile: whereas OPG protein expression was augmented at 4 hours after eCT addition, a reduction in RANKL content was significant only at the 24-hour time point. These data demonstrate that the U2OS osteoblast cell line is a sensitive target for the actions of CT. Whereas this conclusion apparently contrasts with the classic views of mechanism of action of CT,¹⁷ it is noteworthy that more recent observations provide support to our findings. For instance, CT displays antiapoptotic ef-

Figure 7. Histological analyses of arthritic joints. Selected digit joints were subjected to histological preparation as described in Materials and Methods and analyzed for monitoring synovitis and bone. **a:** Representative picture taken from a naïve rat, showing normal bone structure and adjacent tissue with no apparent signs of inflammation. **b:** Arthritic rat joint with clear focal bone resorption (**arrowheads**) and severe active inflammation (**asterisks**) in surrounding tissue. After treatment with after 0.8 mg/kg s.c. prednisolone as detailed in Figure 6, intense inflammation in periosteal tissues is evident (**c**, **asterisks**) accompanied by marked bone resorption (**d**, **arrowheads**). **e** and **f:** Animals treated with prednisolone together with CT (2 μ g/kg s.c.) displayed minimal inflammation and no significant bony abnormalities. All pictures are representative of more than five sections prepared from two to three different rats. Original magnifications, $\times 200$.

fects on osteocytic cells as well as mature osteoblasts.²⁷ This hormone stimulates proliferation, calcium uptake, and appearance of alkaline phosphatase activity (a distinct marker of osteoblast differentiation) in osteoblast-like cells.²⁸ In addition, the procalcitonin amino-terminal

cleavage peptide stimulates U2OS cell proliferation.³¹ However, the relationship of CT on osteoblast function is complex and may be bidirectional because CT receptor heterozygous knockout mice show an increase in bone formation.³²

Because the relative local expression of RANKL and OPG is instrumental in determining the degree of osteoclast-mediated resorption,²² a balance of these cytokines in favor of OPG will yield a protective effect on the skeleton. Therefore, we next tested the effects that eCT and prednisolone would exert on OPG and RANKL-regulated bone resorption. In a widely used *in vitro* model of bone resorption, eCT prevented RANKL-induced bone resorption, producing an almost complete abolition of osteoclast activity. These effects were not attributable to toxicity and were evident at the low concentration, congruent with the effects on *de novo* OPG synthesis and release. Then, as we had reported its ability to potentially stimulate bone resorption *in vitro*,²⁶ we added prednisolone to the system. At nanomolar concentrations, the GC displayed a stimulating effect on osteoclast activity, abolished by CT.

Finally, we wished to translate these findings to a more complex and pathologically relevant condition, and the choice fell to a model of RA. This pathology is characterized by increased bone erosion in addition to progressive synovial inflammation and joint destruction. Such bone degradation is caused by osteoclast activation as triggered by the production of RANKL by synovial fibroblasts and T lymphocytes.^{3,33,34} In addition, a highly effective therapy for RA is GC treatment;³⁵ however, its long-term use is associated with secondary osteoporosis.^{19,36} Besides their catabolic action on bone-forming cells, GCs also increase bone resorption via augmented parathormone release and more recently have been shown to up-regulate RANKL and decrease OPG levels,^{12–14} as well as directly affect the osteoclast activation process by preventing optimal microfilament formation.¹⁶ Therefore, the beneficial anti-inflammatory action of GCs can be impaired by an exacerbating effect on bone erosion.

In the present study, we evaluated the effect of administration of CT in combination with prednisolone in a model of RA. Whereas CT alone did not modify the incidence or severity of disease in terms of inflammation signs, there was a marked improvement in bone erosion as evident from the histological analysis. Interestingly, co-administration of CT and a subtherapeutic dose of prednisolone had a dramatic synergistic effect and reduced inflammation symptoms to the same levels of those attained by the high-dose prednisolone; importantly, CT beneficial effects on bone erosion were maintained. It is unfortunate that, in our hands, the immunoassays for OPG and RANKL did not determine the rat homologs (L.M., data not shown), and therefore we were unable to measure any changes in their plasma levels in response to prednisolone and/or CT in the rat.

A potential reason for the synergic action associated with the combined treatment of CT and prednisolone could be proposed. It is known that after repeated stimulation with CT there is an escape phenomenon due to receptor down-regulation, consequent to reduced mRNA stability and receptor binding activity. For instance, treatment of human osteoblast-like cells with GC increases transcription of CT receptor gene expression, thereby preventing loss of CT receptor function even in the presence of the agonist.¹⁸ However, other molecular mecha-

nisms behind these synergistic anti-inflammatory properties could also be postulated and deserve systematic investigation, although some of the interrelationship discussed here, converging on the OPG/RANKL system, might represent a logical starting point. In addition, early indications that CT might augment glycosaminoglycan, possibly aggrecan, synthesis from chondrocytes³⁷ could also have implications for our findings, adding this cell type to the list of potential CT targets. It is therefore possible that multiple actions of CT are responsible for its remarkable *in vivo* effects in experimental arthritis.

In conclusion, starting from analyses of CT and prednisolone effects on bone-regulating cytokines, we have revealed the novel notion that CT could be an effective anti-arthritic treatment complementary to GC, such that the latter drug would be effective in controlling inflammatory diseases without producing the side effects on the bone compartment. Moreover, our data in the CIA model suggest that CT could potentiate the anti-inflammatory activity of prednisolone itself.

References

1. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ: Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 1998, 93:165–176
2. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T: Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci USA* 1998, 95:3597–3602
3. Kong YY, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, Capparelli C, Li J, Elliott R, McCabe S, Wong T, Campagnuolo G, Moran E, Bogoch ER, Van G, Nguyen LT, Ohashi PS, Lacey DL, Fish E, Boyle WJ, Penninger JM: Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* 1999, 402:304–309
4. Wada T, Nakashima T, Hiroshi N, Penninger JM: RANKL-RANK signaling in osteoclastogenesis and bone disease. *Trends Mol Med* 2006, 12:17–25
5. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Sander S, Van G, Tarpley J, Derby P, Lee R, Boyle WJ: Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 1997, 89:309–319
6. Bucay N, Sarosi I, Dunstan CR, Morony S, Tarpley J, Capparelli C, Scully S, Tan HL, Xu W, Lacey DL, Boyle WJ, Simonet WS: Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev* 1998, 12:1260–1268
7. Mizuno A, Murakami A, Nakagawa N, Yasuda H, Tsuda E, Morinaga T, Higashio K: Structure of the mouse osteoclastogenesis inhibitory factor (OCIF) gene and its expression in embryogenesis. *Gene* 1998, 215:339–343
8. Lane NE, Goldring SR: Bone loss in rheumatoid arthritis: what role does inflammation play? *J Rheumatol* 1998, 25:1251–1253
9. Laan RF, Buijs WC, Verbeek AL, Draad MP, Corstens FH, van de Putte LB, van Riel PL: Bone mineral density in patients with recent onset rheumatoid arthritis: influence of disease activity and functional capacity. *Ann Rheum Dis* 1993, 52:21–26
10. Gravallesse EM, Harada Y, Wang JT, Gorn AH, Thornhill TS, Goldring SR: Identification of cell types responsible for bone resorption in

- rheumatoid arthritis and juvenile rheumatoid arthritis. *Am J Pathol* 1998, 152:943–951
11. Romas E, Sims NA, Hards DK, Lindsay M, Quinn JW, Ryan PF, Dunstan CR, Martin TJ, Gillespie MT: Osteoprotegerin reduces osteoclast numbers and prevents bone erosion in collagen-induced arthritis. *Am J Pathol* 2002, 161:1419–1427
12. Silvestrini G, Ballanti P, Patacchioli FR, Mocetti P, Di Grezia R, We-dard BM, Angelucci L, Bonucci E: Evaluation of apoptosis and the glucocorticoid receptor in the cartilage growth plate and metaphyseal bone cells of rats after high-dose treatment with corticosterone. *Bone* 2000, 26:33–42
13. Weinstein RS, Jilka RL, Parfitt AM, Manolagas SC: Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone. *J Clin Invest* 1998, 102:274–282
14. Hofbauer LC, Gori F, Riggs BL, Lacey DL, Dunstan CR, Spelsberg TC, Khosla S: Stimulation of osteoprotegerin ligand and inhibition of osteoprotegerin production by glucocorticoids in human osteoblastic lineage cells: potential paracrine mechanisms of glucocorticoid-induced osteoporosis. *Endocrinology* 1999, 140:4382–4389
15. Swanson C, Lorentzon M, Conaway HH, Lerner UH: Glucocorticoid regulation of osteoclast differentiation and expression of receptor activator of nuclear factor-kappaB (NF-kappaB) ligand, osteoprotegerin, and receptor activator of NF-kappaB in mouse calvarial bones. *Endocrinology* 2006, 147:3613–3622
16. Kim HJ, Zhao H, Kitaura H, Bhattacharyya S, Brewer JA, Muglia LJ, Ross FP, Teitelbaum SL: Glucocorticoids suppress bone formation via the osteoclast. *J Clin Invest* 2006, 116:2152–2160
17. Zaidi M, Chambers TJ, Bevis PJ, Beacham JL, Gaines Das RE, MacIntyre I: Effects of peptides from the calcitonin genes on bone and bone cells. *Q J Exp Physiol* 1988, 73:471–485
18. Wada S, Yasuda S, Nagai T, Maeda T, Kitahama S, Suda S, Findlay DM, Iitaka M, Katayama S: Regulation of calcitonin receptor by glucocorticoid in human osteoclast-like cells prepared in vitro using receptor activator of nuclear factor-kappaB ligand and macrophage colony-stimulating factor. *Endocrinology* 2001, 142:1471–1478
19. Adachi JD: Corticosteroid-induced osteoporosis. *Am J Med Sci* 1997, 313:41–49
20. Popp AW, Isenegger J, Buergi EM, Buergi U, Lippuner K: Glucocorticosteroid-induced spinal osteoporosis: scientific update on pathophysiology and treatment. *Eur Spine J* 2006, 15:1035–1049
21. Kinpara K, Mogi M, Kuzushima M, Togari A: Osteoclast differentiation factor in human osteosarcoma cell line. *J Immunoassay* 2000, 21:327–340
22. Nakashima T, Kobayashi Y, Yamasaki S, Kawakami A, Eguchi K, Sasaki H, Sakai H: Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-kappaB ligand: modulation of the expression by osteotropic factors and cytokines. *Biochem Biophys Res Commun* 2000, 275:768–775
23. Force T, Bonventre JV, Flannery MR, Gorn AH, Yamin M, Goldring SR: A cloned porcine renal calcitonin receptor couples to adenylyl cyclase and phospholipase C. *Am J Physiol* 1992, 262:F1110–F1115
24. Paul-Clark MJ, Roviezzo F, Flower RJ, Cirino G, Soldato PD, Adcock IM, Perretti M: Glucocorticoid receptor nitration leads to enhanced anti-inflammatory effects of novel steroid ligands. *J Immunol* 2003, 171:3245–3252
25. Mancini L, Moradi-Bidhendi N, Brandi ML, MacIntyre I: Nitric oxide superoxide and peroxynitrite modulate osteoclast activity. *Biochem Biophys Res Commun* 1998, 243:785–790
26. Paul-Clark MJ, Mancini L, Del Soldato P, Flower RJ, Perretti M: Potent antiarthritic properties of a glucocorticoid derivative, NCX-1015, in an experimental model of arthritis. *Proc Natl Acad Sci USA* 2002, 99:1677–1682
27. Plotkin LI, Weinstein RS, Parfitt AM, Roberson PK, Manolagas SC, Bellido T: Prevention of osteocyte and osteoblast apoptosis by bisphosphonates and calcitonin. *J Clin Invest* 1999, 104:1363–1374
28. Farley J, Dimai HP, Stilt-Coffing B, Farley P, Pham T, Mohan S: Calcitonin increases the concentration of insulin-like growth factors in serum-free cultures of human osteoblast-line cells. *Calcif Tissue Int* 2000, 67:247–254
29. Forrest SM, Ng KW, Findlay DM, Michelangeli VP, Livesey SA, Partridge NC, Zajac JD, Martin TJ: Characterization of an osteoblast-like clonal cell line which responds to both parathyroid hormone and calcitonin. *Calcif Tissue Int* 1985, 37:51–56
30. Bland R, Worker CA, Noble BS, Eyre LJ, Bujalska IJ, Sheppard MC, Stewart PM, Hewison M: Characterization of 11beta-hydroxysteroid dehydrogenase activity and corticosteroid receptor expression in human osteosarcoma cell lines. *J Endocrinol* 1999, 161:455–464
31. Burns DM, Forstrom JM, Friday KE, Howard GA, Roos BA: Procalcitonin's amino-terminal cleavage peptide is a bone-cell mitogen. *Proc Natl Acad Sci USA* 1989, 86:9519–9523
32. Dacquin R, Davey RA, Laplace C, Levasseur R, Morris HA, Goldring SR, Gebre-Medhin S, Galson DL, Zajac JD, Karsenty G: Amylin inhibits bone resorption while the calcitonin receptor controls bone formation in vivo. *J Cell Biol* 2004, 164:509–514
33. Kotake S, Udagawa N, Hakoda M, Mogi M, Yano K, Tsuda E, Takahashi K, Furuya T, Ishiyama S, Kim KJ, Saito S, Nishikawa T, Takahashi N, Togari A, Tomatsu T, Suda T, Kamatani N: Activated human T cells directly induce osteoclastogenesis from human monocytes: possible role of T cells in bone destruction in rheumatoid arthritis patients. *Arthritis Rheum* 2001, 44:1003–1012
34. Takayanagi H, Iizuka H, Juji T, Nakagawa T, Yamamoto A, Miyazaki T, Koshihara Y, Oda H, Nakamura K, Tanaka S: Involvement of receptor activator of nuclear factor kappaB ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis. *Arthritis Rheum* 2000, 43:259–269
35. Kirwan JR: The effect of glucocorticoids on joint destruction in rheumatoid arthritis. *N Engl J Med* 1995, 333:142–146
36. Manolagas SC, Weinstein RS: New developments in the pathogenesis and treatment of steroid-induced osteoporosis. *J Bone Miner Res* 1999, 14:1061–1066
37. Martin TJ, Harris GS, Melick RA, Fraser JR: Effect of calcitonin on glycosaminoglycan synthesis by embryo calf bone cells in vitro. *Experientia* 1969, 25:375–376