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Research Article

Duration of bone protection by a single osteoprotegerin injection in rats with adjuvant-induced arthritis

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Abstract. Daily osteoprotegerin (OPG) injection for 7 or more days prevents bone loss for 3 weeks in rats with adjuvant-induced arthritis (AdA). The present experiments defined the duration of bone protection in AdA provided by a single OPG bolus. Male Lewis rats received OPG at the onset or peak of clinical disease, after which bone mineral density (BMD), erosions, and osteoclasts were evaluated. An OPG bolus (4 mg/kg subcutaneously) at onset eliminated osteoclasts, preserved BMD for 7 days, and prevented bone erosions for 4 days. In contrast, an OPG bolus (1, 3, 10, or 30 mg/kg intravenously) given at the peak of disease eradicated osteoclasts in a dose-dependent manner but had no impact on bone integrity due to extensive pre-existing bone loss. These data indicate that one OPG injection will inhibit joint erosions for several days, and confirm that bone-sparing therapy must be initiated early in disease to protect joint integrity.

Key words. Apoptosis; adjuvant arthritis; osteoclast; osteoprotegerin; rheumatoid arthritis; rat.

Bone removal during normal skeletal remodeling is controlled by the balanced interactions between the tumor necrosis factor (TNF) family molecules osteoprotegerin (OPG) and receptor activator of NF-κB (RANK) ligand [RANKL, also called OPG ligand (OPGL)] [1]. This balance is lost in various pathologic conditions, including arthritis [2]. OPG is a soluble decoy receptor that inhibits osteoclast formation, function, and survival by preventing the binding of RANKL to RANK, a membrane-bound protein of the TNF receptor family that is found on chondrocytes, dendritic cells, osteoclast precursors, and mature osteoclasts. Mice lacking RANKL or RANK exhibit osteopetrosis [3, 4]. Animals with null mutations of OPG exhibit severe osteoporosis [5], while transgenic mice that over-express OPG develop osteopetrosis [6]. Exogenous OPG prevents bone loss in rodent models of adjuvant-induced [7, 8] and collagen-induced [9] arthritis, bone metastasis [10], and ovariectomy-associated estrogen deficiency [6, 11]. OPG also inhibits bone turnover in postmenopausal (estrogen-deficient) women at risk for developing osteoporosis [12]. These facts indicate that OPG has great potential as a therapy for conditions characterized by marked bone resorption.

One such disease is rheumatoid arthritis (RA), a major consequence of which is irreversible joint destruction leading to profound disability. Growing evidence shows that the periarticular osteoporosis and skeletal erosions characteristic of RA as well as animal models of immune-mediated arthritis arise from an imbalance in the OPG/RANKL/RANK signaling pathway that controls osteoclast activity. Osteoclast numbers are increased in RA patients [13–18] and animals with experimental arthritis [7, 19–21] in response to RANKL elaborated by activated

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T cells [7, 22], synovial fibroblasts [23–25] and, to a lesser extent, osteoblasts and bone marrow stromal cells [26–28]. RANKL directly regulates osteoclast numbers by three mechanisms: regulating osteoclastogenesis from monocytes [29], synovial macrophages [24, 30], and bone marrow hematopoietic cells [29, 31]; activating mature osteoclasts [32, 33], and supporting osteoclast survival [34–36]. Thus, OPG therapy to inhibit RANKL-mediated osteoclast activity represents a logical avenue for averting bone dissolution in RA.

Previous work by our laboratory [7, 8] and others [9] has shown that a short series of OPG injections [≥ 1 mg/kg per day subcutaneously (s.c.) for 3–7 consecutive days] inhibits bone destruction in severely arthritic rats by depleting intralesional osteoclasts. These bone-sparing effects occur even in the presence of continued inflammation and elevated RANKL expression within the affected joints. However, a single OPG bolus (≥ 0.3 mg/kg s.c.) has been shown to reduce bone turnover in postmenopausal women for several weeks [12], which raises the prospect that intermittent OPG injections would be a suitable regimen for treating RA. The principal objective of the present work was to explore the duration of protection afforded by a single OPG injection in a rat model of severe immune-mediated arthritis as a means of estimating the OPG dosing frequency in such conditions. Our data indicate that a single OPG application preserves bone in arthritic joints for 4 days and that OPG accomplishes this feat, at least in part, by inducing apoptosis of intralesional osteoclasts.

Materials and methods

Animals

Male Lewis rats (Charles River, Wilmington, MA) weighing 180–200 g were acclimated for 1 week and then randomly assigned to treatment groups (n = 6/group). The small group size was used because interindividual variability between untreated arthritic rats is minimal in this model [37]. Animals were given tap water and fed pelleted rodent chow (No. 8640; Harlan Teklad, Madison, WI) ad libitum; calcium and phosphorus contents were 1.2% and 1.0%, respectively. At necropsy, all animals were sacrificed by carbon dioxide inhalation. These studies were conducted in accordance with federal animal care guidelines and were preapproved by the Amgen Institutional Animal Care and Use Committee (AIACUC).

Induction of adjuvant arthritis

Adjuvant arthritis (AdA) was induced on day 0 as described elsewhere [37] by a single intradermal injection at the base of the tail of heat-killed *Mycobacterium tuberculosis* H37Ra (0.5 mg; Difco, Detroit, MI) suspended in 0.05 ml paraffin oil (Crescent, Hauppauge, NY). The clinical onset of arthritis was at day 9 as indicated by hind paw swelling.

Experimental design

The effects of a single OPG dose on joint integrity and intralesional osteoclasts were investigated in two parallel experiments. The test material, a recombinant fusion protein (Amgen, Thousand Oaks, CA) combining the OPG ligand-binding domain of human OPG with the constant domain of human immunoglobulin G_1, was injected in phosphate-buffered saline (PBS). This molecule effectively binds rat RANKL as indicated by its ability to significantly reduce bone destruction in rats with AdA [7, 8]. Concurrent negative control groups received PBS or were not treated, as our past experience with this model has shown that the disease course for these two 'treatments' is similar [unpublished data].

During the first experiment, rats were given OPG (4 mg/kg once by s.c. bolus) at disease onset (day 9). This regimen was selected for investigation because s.c. injection of OPG at 4 mg/kg per day for 7 days (days 9–15) prevents structural dissolution in AdA for almost 4 weeks [8]. Animals were necropsied over time – at days 10 (onset + 1 day), 13 (onset + 4), 16 (onset + 7), or 19 (onset + 10) – to follow the progression of disease, particularly the recovery of osteoclast populations. Rats in the second experiment were given a single OPG dose [1, 3, 10, or 30 mg/kg by intravenous (i.v.) bolus] at the peak of clinical disease (day 16, or onset + 7). This exposure route was chosen based on a prior mouse study [36] to explore the impact of a single OPG injection delivered at the peak of disease; for this purpose, we considered that the rapid peak in circulating OPG levels elicited by i.v. administration would be preferable to the gradual rise provided by the s.c. route. Animals were necropsied on day 18 to examine the impact of OPG administration on osteoclast integrity during active disease.

Assessment of arthritis

Bone mineral density (BMD) in the tibiotarsal region (hock) was measured by dual-energy X-ray absorptiometry (DEXA) using a fan beam X-ray densitometer (Model QDR-4500 A; Hologic, Waltham, MA). At necropsy, hind paws were removed at the fur line (just proximal to the hock) and stored in 70% ethanol. Paws were scanned while oriented horizontally relative to the detector, after which BMD was calculated in a 29 × 25 mm rectangle centered at the calcaneus using proprietary software (Hologic). In the AdA model, hind paw BMD of male Lewis rats with untreated arthritis is comparable to that of non-arthritic animals until day 13 (onset + 4) after adjuvant inoculation, after which BMD falls precipitously ($p \leq 0.05$) by 10% (day 15, onset + 6) to 20% (day 16, onset + 7) [37]. In contrast, age-matched normal rats increase their

Table 1. Histopathology criteria for lesion scores in adjuvant-induced arthritis.

Erosion score	
0	normal
1	minimal loss of cortical or trabecular bone at a few sites
2	mild loss of cortical or trabecular bone at modest numbers of sites (generally tarsals)
3	moderate loss of bone at many sites (usually the trabeculae of the tarsals, but sometimes the cortex of the distal tibia)
4	marked loss of bone at many sites (usually as extensive destruction of trabeculae in the tarsals, but sometimes with partial loss of cortical bone in the distal tibia)
5	marked loss of bone at many sites (with fragmenting of tarsal trabeculae AND full thickness penetration of cortical bone in the distal tibia)
Osteoclast score	
0	normal (essentially no osteoclasts)
1	few osteoclasts (lining less than 5% of most affected bone surfaces)
2	some osteoclasts (lining between 2–25% of most affected bone surfaces)
3	many osteoclasts (lining between 26–50% of most affected bone surfaces)
4	myriad osteoclasts (lining more than 50% of most affected bone surfaces)

hind paw BMD by approximately 13% during the same period [8].

After DEXA analysis, ethanol-fixed hind paws were decalcified by immersion in eight changes of a 1:1 mixture of 8 N formic acid and 1 N sodium formate (solution changed daily), divided longitudinally along the median axis, and processed into paraffin. A 4-μm-thick section was processed to reveal multinucleated osteoclasts using an indirect immunoperoxidase method and proprietary rabbit anti-human monoclonal antibody (Amgen) directed against the marker protein cathepsin K [23]; hematoxylin and eosin (HE) were used as counterstains. Bony components of the arthritic process in the distal tibia and tarsal bones were evaluated for each hind paw by a board-certified veterinary pathologist (B. B.) using tiered, semi-quantitative grading criteria (table 1) and a 'blinded' analytical paradigm. Lesion scores for bone erosion and osteoclasts were acquired as described previously [8]; both scores increase substantially over time in arthritic rats but do not rise above basal values in normal animals (table 2). Morphologic features of cathepsin-K-labeled multinucleated cells among different treatment groups were also compared to evaluate the capacity for OPG to induce apoptosis in this osteoclast population.

Statistical analysis

All results were expressed as the mean ± SD. The BMD data (a continuous variable) were assessed using Kruskal-Wallis analysis of variance (ANOVA) and the Mann-Whitney U test, while the histopathologic lesion scores (ordinal variables) were analyzed using the χ^2 test. A p value of 0.05 was used to delineate significant differences between groups.

Results

Experiment 1: incipient disease

The mean baseline hind paw BMD for the arthritic animals (Days 9–16) exceeded by 4% the mean value recorded for non-arthritic control rats at day 16 (fig. 1A). In fact, hind paw BMD of arthritic animals in the current study was increased by 10% relative to the range of BMD reported for non-arthritic male Lewis rats of comparable age [37] during the period encompassed by our arthritis studies. Since animals were randomly assigned to treatment groups, we have no explanation for this elevation other than 'biologic variation'.

Table 2. Progression of histopathologic lesions in hind paws of male Lewis rats with adjuvant-induced arthritis.

	Day of onset	Onset +1 day	Onset +3 days	Onset +4 days	Onset +7 days
Arthritic rats					
n	12	12	6	6	18
Erosion score	0.2 ± 0.4	0.2 ± 0.4	2.7 ± 1.0	3.2 ± 0.8	4.4 ± 0.7*
Osteoclast score	0.6 ± 0.7	0.7 ± 0.7 *	3.0 ± 1.5	3.7 ± 0.5	4.0 ± 0*
Normal rats					
n		6			18
Erosion score	ND	0	ND	ND	0
Osteoclast score	ND	0	ND	ND	0

Values represent the mean ± SD and were derived by comparison of scores from two studies conducted during the same calendar quarter. Scores for arthritic rats include those for groups that received either no treatment or vehicle, but not OPG. 'ND' denotes 'not determined'; values for these normal control groups were not acquired because criteria for lesion scores were defined so that 'normal' = 0. * denotes a significant difference from time-matched normal rats, $p \leq 0.05$, by the χ^2 test.

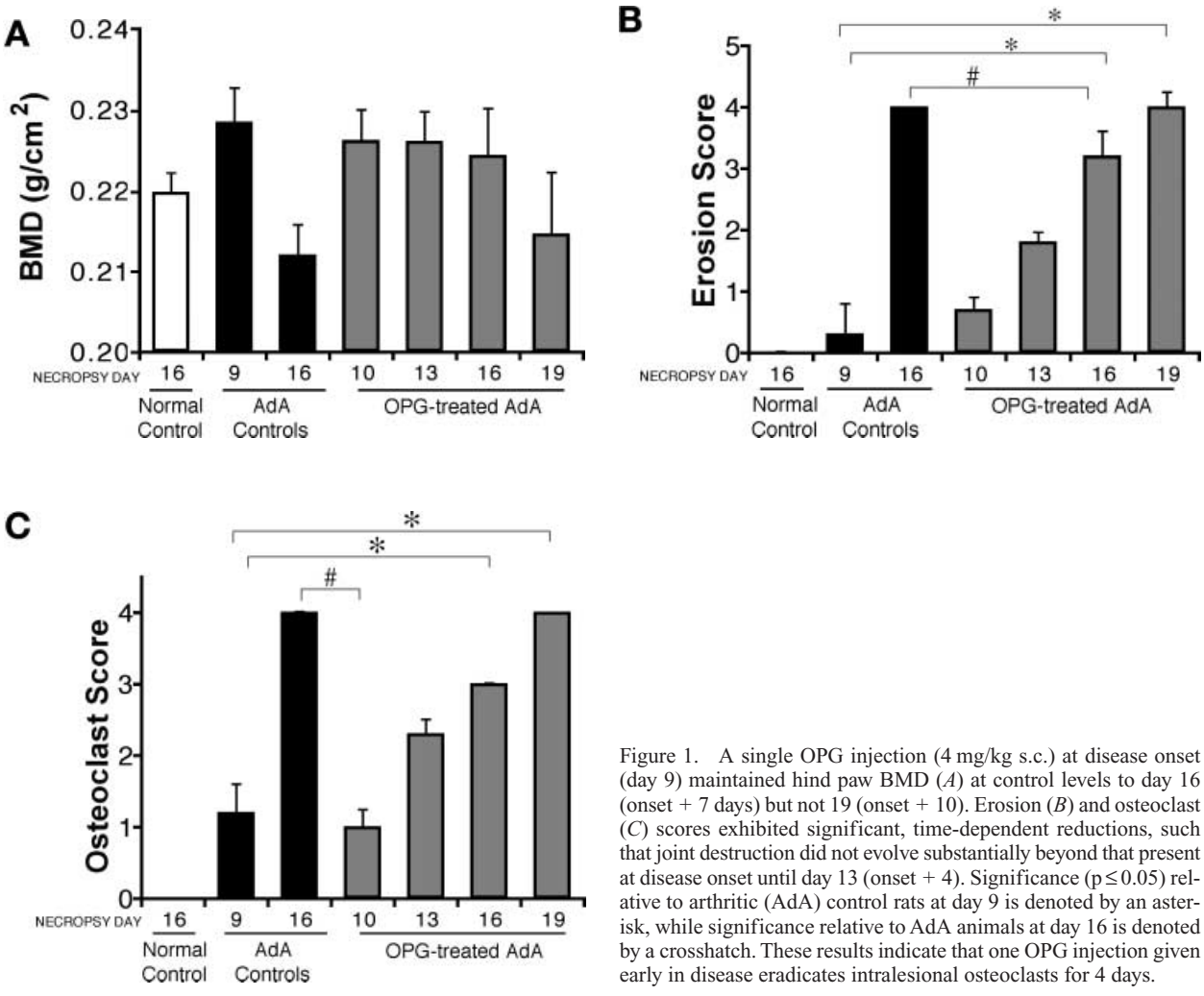

Figure 1. A single OPG injection (4 mg/kg s.c.) at disease onset (day 9) maintained hind paw BMD (*A*) at control levels to day 16 (onset + 7 days) but not 19 (onset + 10). Erosion (*B*) and osteoclast (*C*) scores exhibited significant, time-dependent reductions, such that joint destruction did not evolve substantially beyond that present at disease onset until day 13 (onset + 4). Significance ($p \leq 0.05$) relative to arthritic (AdA) control rats at day 9 is denoted by an asterisk, while significance relative to AdA animals at day 16 is denoted by a crosshatch. These results indicate that one OPG injection given early in disease eradicates intralesional osteoclasts for 4 days.

After a single OPG dose (4 mg/kg s.c.) at disease onset (day 9), hind paw BMD (fig. 1A) was maintained at the initial level until day 16 (onset + 7 days) but not day 19 (onset + 10). In these animals, erosion scores (fig. 1B) at days 10 (onset + 1 day) and 13 (onset + 4) were comparable to those of untreated AdA rats at day 9. Erosion scores at day 16 of rats given one s.c. bolus of OPG on day 9 were significantly greater than values in untreated arthritic rats at day 9 (fig. 1B). However, the moderate scores were significantly lower than the marked grades characteristic of untreated AdA rats at day 16. Erosion scores of marked severity did not occur in OPG-treated animals until day 19 (fig. 1B).

Relative to arthritic control rats on day 9, a single OPG dose (4 mg/kg s.c.) at day 9 significantly reduced the osteoclast score on day 10 (fig. 1C). Osteoclasts subsequently increased in a time-dependent manner. The osteoclast population at day 13 was still comparable to those of untreated arthritic rats on day 9; levels similar to those of untreated rats with marked AdA did not evolve until day 16 (fig. 1C). At days 10 and 13, cathepsin-K-labeled osteoclast remnants (stellate to crescentic, anuclear bodies) consistent with cell fragmentation were scattered in paw sections of OPG-treated rats but were absent in paws of either untreated or normal rats. Small osteoclasts (oval cells with one or two nuclei) were also common in paws of animals given OPG, while multinucleated osteoclasts were depleted.

Experiment 2: peak disease

In chronic arthritis, a single OPG injection also depleted intralesional osteoclasts but yielded a different pattern of bone damage. Mean osteoclast scores for rats given higher OPG doses (1, 3, 10, or 30 mg/kg i.v.) at the peak of clinical disease (day 16, onset + 7) were significantly lower than those of non-treated and vehicle-treated arthritic animals (fig. 2A). However, the substantial decrease in intralesional osteoclasts had no impact on erosions due to the extensive pre-existing bone loss found in advanced disease (fig. 2B).

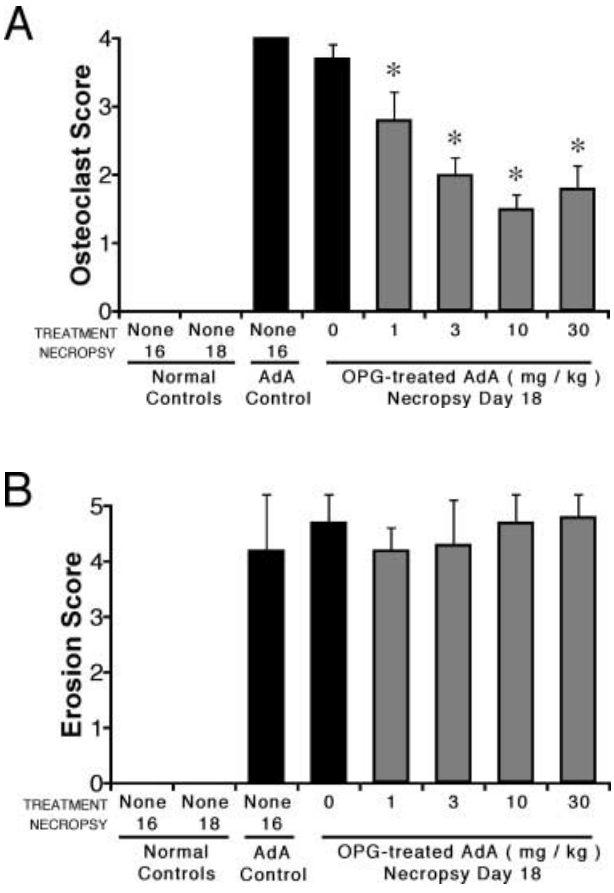

Figure 2. A single OPG injection (1, 3, 10, or 30 mg/kg) at the peak of clinical disease (day 16, or onset + 7 days) significantly ($p \leq 0.05$) reduced osteoclast scores relative to arthritic (AdA) control rats at day 16 by 48 h (asterisks in *A*). The large decrease in intralesional osteoclasts had no impact on erosions (*B*) due to extensive pre-existing bone loss.

Interestingly, osteoclasts in bones of the hind paw exhibited one of three patterns, depending on individual disease status (fig. 3). In normal rats, scattered osteoclasts were found only in the distal tibia, just proximal to the growth plate, while essentially no osteoclasts were observed in tarsal bones. Most were small to medium-sized cells and crescent shaped, a morphology typical of 'resting' cells. In animals with AdA, however, osteoclasts were plentiful in marrow cavities of bones comprising the intertarsal and tibiotarsal joints. In cathepsin-K-labeled sections of untreated AdA rats, intralesional osteoclasts at these sites assumed one of two conformations. Active lesions contained myriad, oval to round, medium-sized to very large osteoclasts (features typical of 'activated' cells). In contrast, lesions in which the inflammatory process was receding had modest numbers of crescentic to oval, small to large osteoclasts, as well as many fragments and occasional apoptotic large osteoclasts (consistent with the occurrence of 'physiologic' apoptosis in a spontaneously regressing lesion). A single i.v. bolus of OPG (10 or 30 mg/kg) on day 16 eliminated all but a few osteoclasts by day 18, and those remaining were fragmented. Treatment with a lower OPG dose (1 or 3 mg/kg i.v.) provided a modest reduction in the intralesional osteoclast population. The morphology of the remaining cathepsin-K-labeled cells at these two low doses ranged from crescentic to round and from small to very large, a range of features similar to that found in naturally regressing lesions.

Discussion

Irreversible erosion of bone and cartilage in arthritic joints is a hallmark of immune-mediated arthritis. OPG is the first agent that essentially halts osteoclast-induced joint erosions in active immune-mediated arthritis [7]. OPG performs this feat by inhibiting RANKL, one of two principal molecules (along with colony stimulating factor-1) that are necessary and sufficient for induction of osteoclastogenesis [38]. The joint erosions in RA likely reflect an imbalance in the RANKL signaling pathway such that endogenous OPG levels are inadequate to counteract the excess RANKL elaborated in this disease [2, 25, 30].

Data from the present experiments extend our prior findings [7, 8] regarding the bone-protective efficacy of OPG in the Lewis rat model of AdA. Our current findings showed that a single s.c. bolus of OPG (4 mg/kg) given at disease onset yields significant reductions in clinical and histopathologic indices of bone damage for 4 days (fig. 1), even in the presence of extensive inflammation. Furthermore, our present results have confirmed that OPG will not preserve joint integrity if therapy is initiated in advanced disease (after articular bone has already been eroded) even though osteoclasts were eradicated (compare figs 1 and 2). These data were well correlated with the outcome of our previous studies, in which bone destruction and osteoclast production were significantly reduced in a schedule-dependent manner by repeated s.c. injection of OPG [8]. Taken together, our entire collection of data with OPG therapy in Lewis rats with AdA permit two deductions with respect to the use of OPG in RA. First, our results show that intralesional osteoclast populations will recover (fig. 1) in joints in which inflammation cannot be quelled, indicating that periodic readministration of OPG throughout the course of RA will be necessary to preserve joint integrity. More importantly, our findings demonstrated that a short series of OPG injections substantially extends the interval between treatments relative to that provided by a single OPG bolus. The erosion-free interval in AdA provided by a 7-day treatment course is 19 days [8], while that achieved by a single OPG dose in the present study was 4 days (fig. 1B). A plausible explanation for this phenomenon is that multiple OPG doses target not only mature ('resting') osteoclasts available for immediate activation but also osteoclast precursors in which

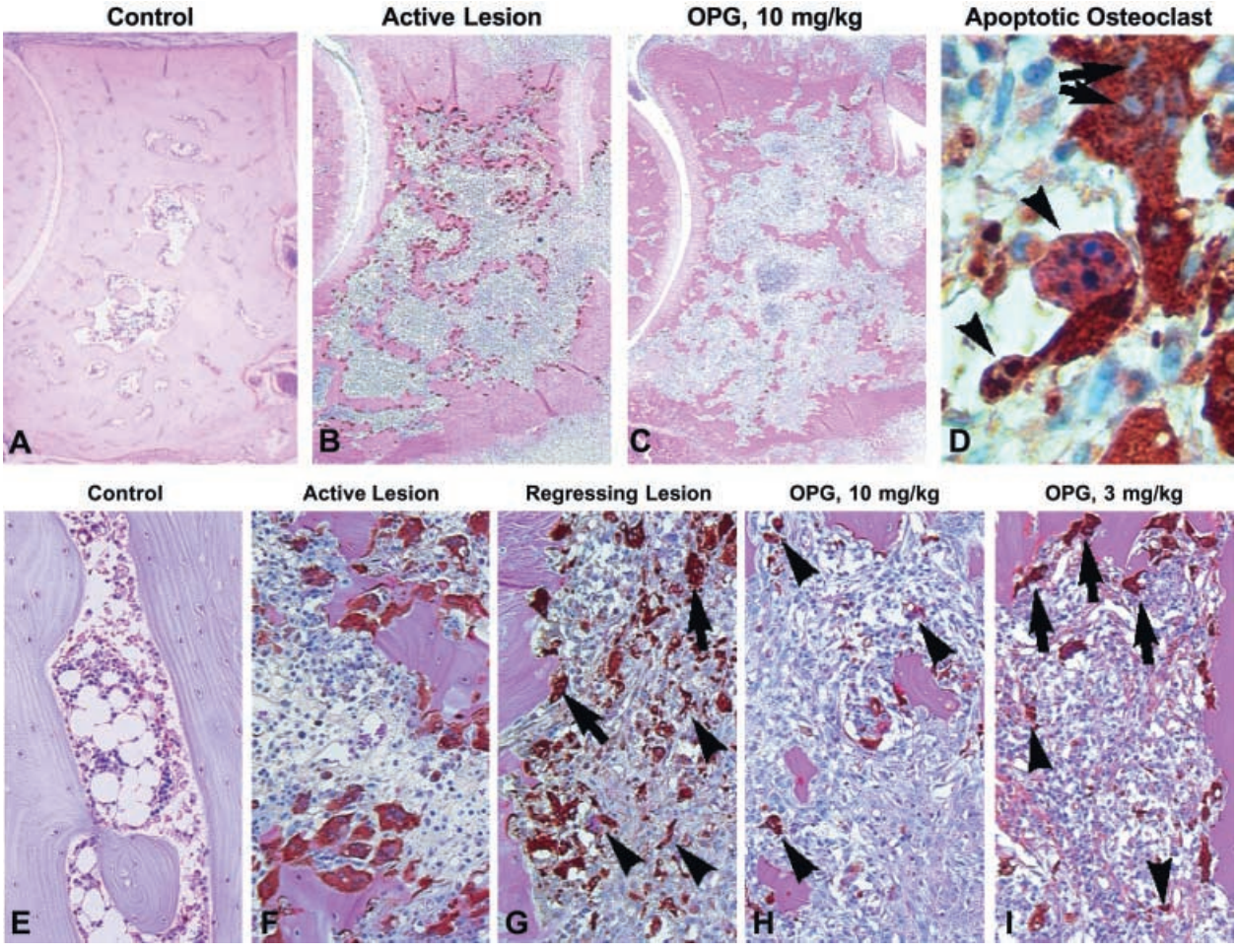

Figure 3. A single OPG injection at the peak of clinical arthritis induced dose-dependent apoptosis of intralesional osteoclasts in AdA joints. Tarsal bones of normal rats had smooth endosteal surfaces and contained essentially no osteoclasts except for a few small, crescent-shaped 'resting' cells (*A*, *E*). In untreated animals with active AdA, myriad intralesional osteoclasts lining the eroded endosteal surfaces of tarsals assumed one of two conformations depending on disease status. Osteoclasts in active lesions (characterized by extensive inflammation) were oval, medium-sized to very large 'activated' cells (*B*, *F*), while those in regressing lesions were crescentic to oval, small to large cells interspersed with many fragments ['physiologic' apoptosis (arrowheads, *G*)]. A single high-dose i.v. injection of OPG (10 or 30 mg/kg) at the peak of active disease (day 16) eliminated almost all osteoclasts (*C*, *H*); the few surviving cells resembled those found in regressing lesions [OPG-induced apoptosis (arrowheads, *H*, *I*)]. A single low-dose i.v. injection of OPG (1 or 3 mg/kg) reduced osteoclast populations modestly while leaving both 'activated' and fragmenting cells (*D*, *I*). As expected, complete elimination of osteoclasts at the peak of disease did nothing to reverse the extensive bone destruction that occurred prior to OPG treatment (compare *C* to *A*). Arrowheads (*D*, *G*, *H*) denote apoptotic osteoclasts (featuring condensed and fragmented nuclei), while arrows (*D*, *G*, *I*) define round, hematoxylin-stained nuclei of intact, multinucleated osteoclasts. Site: navicular tarsal bone. Stain: indirect immunoperoxidase stain for the osteoclast marker protein cathepsin K, with HE counterstains. Magnifications: *A*–*C*, ×30; *D*, ×500; *E*–*I*, ×250.

RANKL has initiated the differentiation program [36]. Further work will be required to identify the optimal interval between single OPG injections that will provide the best skeletal protection in immune-mediated arthritis.

Interestingly, data from our current experiments also support the conjecture that OPG regulates osteoclast numbers in arthritis by creating a RANKL 'deficiency' that culminates in apoptosis of activated cells [34–36]. One day after a single s.c. bolus of OPG (4 mg/kg at disease onset), cathepsin-K-labeled small osteoclast precursors (characteristic of early AdA [unpublished results]) were accompanied by scattered cell fragments. These osteoclast remnants exhibited few morphologic features of apoptotic cells (e.g., chromatin margination, nuclear condensation and fragmentation, cytoplasmic eosinophilia [for a review, see ref. 39]), but this lack appeared to reflect the rapid removal of cellular debris that is typical of apoptosis. In like manner, a single i.v. bolus of OPG (10 or 30 mg/kg) at the peak of active disease (day 16) depleted osteoclasts by 48 h (fig. 3). In this instance, morphologic features of osteoclasts, including extensive fragmentation, were similar to those found in spontaneously regressing arthritic lesions even though they were found in the presence of severe inflammation (active disease), indicating that the

single OPG bolus had ignited the apoptotic process. Interestingly, although an i.v. bolus of OPG at 1 or 3 mg/kg provided a modest reduction in the intralesional osteoclast population, the variable size and appearance of the remaining cathepsin-K-labeled cells suggested that some activated osteoclasts had survived these lower doses (fig. 3). The implication of these data is that the OPG dose for use in RA will have to be set at a level high enough to eliminate all activated intralesional osteoclasts. This requirement provides further support for the use of a short OPG course rather than a single OPG injection as the more effective means of combating bone destruction in RA.

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