Bone Protective Effect of Simvastatin in Experimental Arthritis

JANET L. FUNK, JIANLIANG CHEN, KATHERINE J. DOWNEY, and R. ANDREW CLARK

ABSTRACT. ***Objective.*** Emerging evidence suggests that clinically important antiinflammatory effects of HMG-CoA reductase inhibition may extend beyond cardiovascular disease to other inflammatory disorders, such as rheumatoid arthritis (RA). Protective bone-specific anabolic and antiresorptive effects of HMG-CoA reductase inhibitors have also been evaluated in normal and osteoporotic bone. The specific effect of statins on inflammation-induced bone loss has not previously been a focus of evaluation. We investigated whether simvastatin, a potent HMG-CoA reductase inhibitor, alters bone turnover in an animal model of RA, thus preventing periarticular bone loss.

Methods. Hydrolyzed simvastatin (20 mg/kg/day) was administered subcutaneously to female Lewis rats 4 days before or 8 days after induction of arthritis by intraperitoneal injection of streptococcal cell wall or vehicle. Effects of simvastatin (vs vehicle) on periarticular bone, assessed by bone mineral density (BMD), biochemical markers of bone turnover, and joint histology, were determined. Effects on joint swelling were assessed clinically and histologically.

Results. Simvastatin prevented early and late joint inflammation in association with a decrease in articular macrophage influx. Simvastatin suppressed the periarticular bone destruction occurring late in the course of disease, preserving periarticular BMD and preventing increases in periarticular osteoclasts and serum pyridinoline levels in arthritic animals, while having no effect on these measures in normal animals. Osteocalcin levels, which were decreased in arthritic animals, were unaltered by statin treatment.

Conclusion. Our results suggest that inhibition of HMG-CoA reductase may be therapeutically useful in preserving periarticular bone in RA joints via suppression of inflammation-induced bone resorption. (First Release May 1 2008; J Rheumatol 2008;35:1083–91)

Key Indexing Terms:
BONE ARTHRITIS SIMVASTATIN HMG-COA REDUCTASE JOINT

HMG-CoA reductase inhibitors (statins) were first isolated from fungi and later chemically synthesized and designed with the pharmacologic goal of inhibiting hepatic cholesterol synthesis in order to decrease cardiovascular risk[1]. Emerging evidence of additional antiinflammatory cardioprotective effects of statins has stimulated analysis of their efficacy in treatment of other inflammatory diseases, including rheumatoid arthritis (RA)[2-9]. Identification of statins as potent inducers of bone morphogenic protein (BMP-2)[10] and inhibitors of osteoclastogenesis[11,12] has also stimulated interest in statins as possible bone-protective therapeutics for osteoporosis treatment[13-15]. In RA, a growing body of literature suggests a possible antiinflammatory effect of statins[2-7]. However, their effect on inflammation-associated bone destruction has not yet been a focus of evaluation.

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Joint inflammation in streptococcal cell wall (SCW)-induced arthritis, a well characterized animal model of RA, is mediated and maintained by the same mechanisms as RA including the articular influx of inflammatory cells (e.g., macrophages) and local cytokine production[16-21]. Similarly, increased osteoclast-mediated bone resorption, driven by a local increase in RANKL production by proliferating synoviocytes, also drives pathognomonic joint destruction in both disease processes[22-26].

To test the postulate that blockade of HMG-CoA reductase could preserve periarticular bone in the setting of arthritis due to direct anabolic effects as well as inhibitory effects on bone resorption, we investigated the effect of simvastatin on periarticular bone destruction and joint inflammation in SCW-induced arthritis.

MATERIALS AND METHODS

HMG-CoA reductase inhibitor treatment. Simvastatin (Merck and Company, Inc.), an inactive prodrug, was hydrolyzed from the lactone to the active dihydroxy-open form using the protocol of Endres, *et al*[27]. Briefly, simvastatin was hydrolyzed in NaOH/ethanol, pH 10-11, for 2 h at 50°C. Statin solutions were then neutralized with 2 N HCl [final concentrations 8 mg/ml statin in phosphate buffered saline (PBS), pH7, containing 10% EtOH] and stored at –70°C prior to use. A subcutaneous mode of delivery of the active drug, as used in animal experiments investigating

noncholesterol-lowering effects of statins[27], was used to minimize hepatic first-pass clearance, as our target tissue was not the liver. A dose of 20 mg/kg/day simvastatin, which corresponds to 180 mg/day in humans after correcting for body surface area[28,29], was tested based on previous *in vivo* rodent studies demonstrating bioactivity of simvastatin at this dose[27,30]. Animals were injected subcutaneously with 2.5 µl/g statin solution (20 mg/kg/day simvastatin) or a vehicle solution (PBS, pH 7, 10% EtOH) prepared and stored in parallel with statins[27].

Animal procedures. To induce arthritis, female Lewis rats at either 7 or 9 weeks of age (Harlan, Indianapolis, IN, USA) were administered a single intraperitoneal (IP) injection of peptidoglycan-polysaccharides (25 µg rhamnose/g body weight) isolated from the sonicated cell wall of Group A *Streptococcus pyogenes* (Lee Laboratories, Grayson, GA, USA) or vehicle (saline) alone[19,21,24-26,31]. At the indicated times, SCW-injected (n = 10/group) and vehicle-injected (n = 4/group) animals were treated either with a single daily dose of simvastatin [20 mg/kg/day subcutaneously (SC)] or with vehicle alone[27]. Joint inflammation in each distal limb was scored daily in a blinded fashion using standard criteria and an arthritis index scale of 0–4/limb[19,21,24-26,31]. Circulating white blood cell counts on Day 29 or 42 were determined using a Hemavet 880 analyzer (CDC Technologies, Oxford, CT, USA) and cell differentials were determined by manual counting[24,25]. Serum cholesterol, creatinine, and alanine aminotransferase (ALT) levels were measured at the end of the experiment (Day 29 or 42) using an Endocheck-Plus chemistry analyzer (Hemagen Diagnostics, Columbia, MD, USA) and daily weights were recorded[24,25].

The University of Arizona IACUC approved all animal procedures.

Histology. All tissue specimens were fixed in 10% formalin; joints were subsequently decalcified in 10% EDTA, pH 7.0; and tissues were embedded in paraffin. Multinucleated osteoclasts, identified by tartrate-resistant acid phosphatase (TRAP) staining, were counted in hind-limb distal tibial growth plates 29 days after injection of SCW (or vehicle) as described[24,25]. An index of articular cartilage destruction in distal tibias was determined as described using hematoxylin and eosin (H&E) stained sections of hind ankle joints obtained on Day 29 (0 = normal; 1 = minimal destruction, 2 = at least 50% destroyed, 3 = entirely destroyed)[24,25]. Use of H&E (vs toluidine-blue) stained sections for assessment of cartilage integrity has been previously verified in this model, as loss of proteoglycan matrix does not appear to occur in SCW arthritis in the absence of cartilage invasion by synovium[24]. Granuloma formation at Day 29 or 42 was assessed in H&E stained liver and spleen sections using standard criteria[21,24,25,31]. Macrophages were identified in the synovium using ED1 antibody (vs IgG-negative control) and standard immunohistochemical staining techniques as described[24,32]. All histological analyses were performed in a blinded fashion.

Biochemical markers of bone turnover. Serum levels of osteocalcin, a marker of bone formation, were determined using a commercial immunoradiometric assay specific for rat (Immutopics, San Clemente, CA, USA) in samples collected 29 days after SCW administration. Serum levels of pyridinoline, a marker of cartilage and bone destruction[24,33-35], were assayed by competitive enzyme immunoassay (Metra Biosystems, Mountain View, CA, USA).

Bone mineral density (BMD). BMD of the distal 25% of the hind femur and L2 to L6 of the anteroposterior lumbar spine was determined at weekly intervals *in vivo* by dual energy X-ray absorptiometry at the times indicated (Piximus, GE Lunar, Madison, WI, USA)[24,25].

Osteoclastogenesis assay. As described[25,26], bone marrow cells isolated from 3 tibias per treatment group 29 days after SCW (or vehicle) injection were combined and plated at 2×10^5 nucleated cells/well in 24-well plates with 800 µl of alpha-modified Eagle's medium (α-MEM)/15% fetal calf serum (FCS) containing 50 ng/ml macrophage-colony stimulating factor (M-CSF; Intergen, Purchase, NY, USA) + 300 ng/ml RANK-activating antibody (R&D Systems, Minneapolis, MN, USA). One-half of the media was replaced with fresh M-CSF and RANK-activating antibody-containing media after 2 days. On Day 5, the number of TRAP-positive (Acid Phosphatase Leukocyte TRAP Kit 387-A; Sigma, St. Louis, MO, USA) cells containing more than 3 nuclei was counted in each well (4 wells/treatment group).

Tumor necrosis factor-α (TNF-α) release from bone marrow cells. Bone marrow cells, isolated as described above, were plated at 1×10^6 nucleated cells/well and treated with 1 µg/ml lipopolysaccharide (LPS; 055:B5; Difco, Detroit, MI, USA). In one experiment, cells were treated *in vitro* with hydrolyzed simvastatin (10 µM) for 1 h prior to LPS stimulation. Supernatants were harvested at 24 h and stored at –70°C prior to assay of TNF-α using a commercial rat-specific ELISA (R&D Systems).

Statistical analysis. Values are presented as mean ± SEM with statistical significance determined by ANOVA with post-hoc testing, Student's t-test, or by Fisher's exact test, as appropriate, using Instat software (Graphpad, San Diego, CA, USA).

RESULTS

Effect of simvastatin on joint swelling. Joint inflammation in vehicle-treated animals injected with SCW at 7 weeks of age followed the usual course (Figure 1A, 1C)[20,24,25,31]; an acute phase of joint swelling peaking 3–4 days after SCW injection, followed by a brief nadir in disease activity that precedes a chronic, persistent phase of joint inflammation associated with progressive, erosive joint destruction (Figure 2A, normal joint; Figure 2C, untreated SCW joint)[19,21]. Daily simvastatin treatment in these animals (20 mg/kg/day, begun 4 days prior to SCW injection) prevented both early and late joint inflammation (Figure 1A; Figure 2D, simvastatin-treated SCW joint at Day 29), achieving statistical significance as early as Day 7 after SCW injection. In a replicate experiment using slightly older animals (9 weeks) that experience less of a nadir in joint swelling between the early and chronic phase[21], a statistically significant antiinflammatory effect of simvastatin was documented as early as 3 days after SCW injection (Figure 1B). The antiinflammatory effect of simvastatin in both experiments persisted throughout the month-long course of disease (Figure 1A, 1B), inhibiting joint swelling on Day 29 by an average of 68% ($p < 0.02$). Simvastatin was also effective in the treatment of existing arthritis (Figure 1C). However, as commonly seen in animal RA models[25,36], the degree of inhibition occurring with delayed treatment (8 days post-SCW injection), while significant, was less than that occurring with pretreatment (34% vs 68% inhibition of joint swelling, respectively).

Effect of simvastatin on other markers of inflammation. Simvastatin had no effect on SCW-induced leukocytosis (Table 1), which is primarily due to an increase in circulating neutrophils[21]. However, simvastatin selectively inhibited the increase in circulating monocytes that also occurs in response to SCW injection (Table 1). Simvastatin pretreatment or delayed treatment also inhibited over 60% of the influx of ED-1-positive monocytes/macrophages into the arthritic synovium (Table 2). The granulomatous inflammatory response that occurs in liver at sites of hepatic SCW deposition[21,24,25] was unaffected by treatment with simvastatin (65% incidence in untreated SCW-injected rats vs 61%

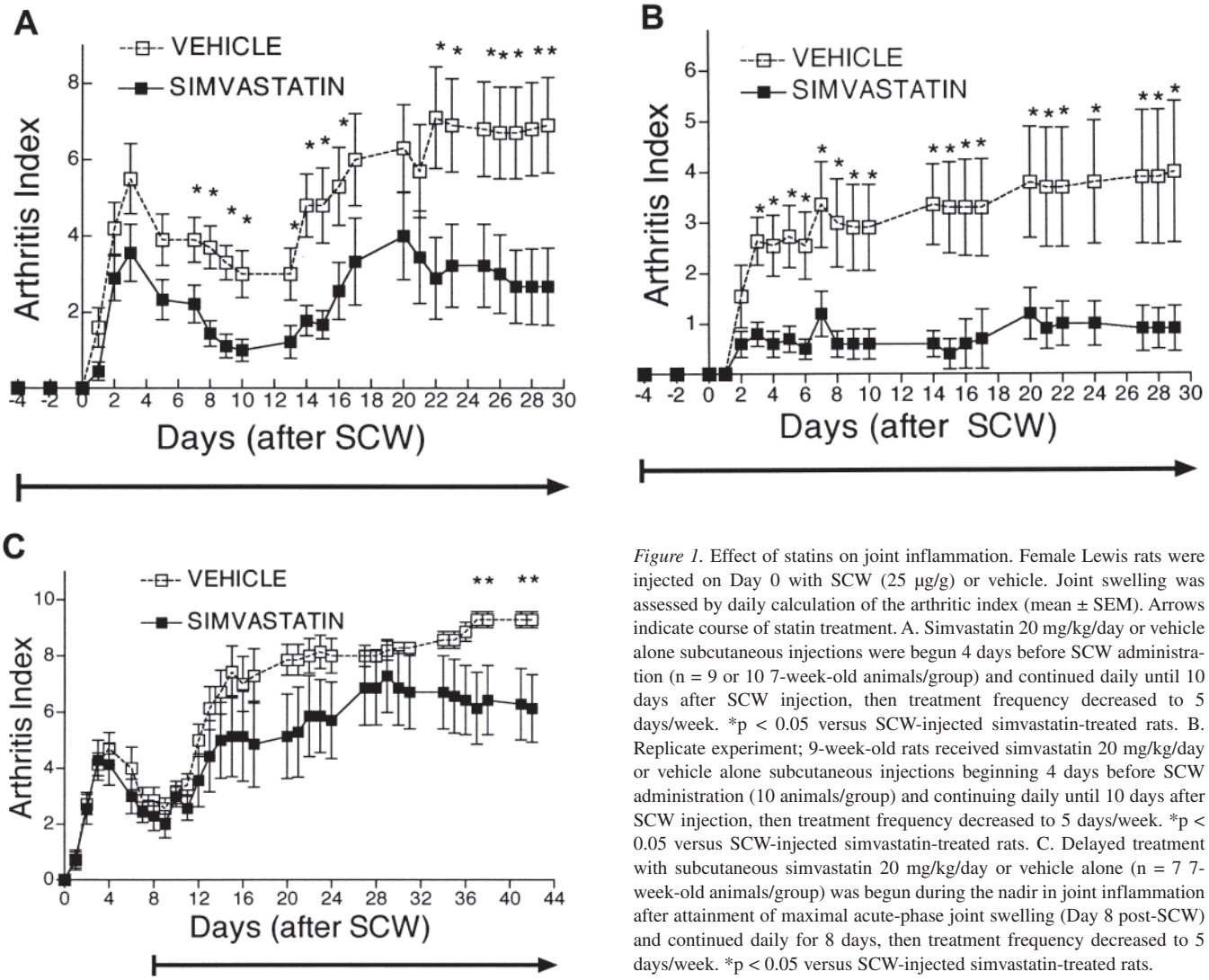

Figure 1. Effect of statins on joint inflammation. Female Lewis rats were injected on Day 0 with SCW (25 μg/g) or vehicle. Joint swelling was assessed by daily calculation of the arthritic index (mean ± SEM). Arrows indicate course of statin treatment. A. Simvastatin 20 mg/kg/day or vehicle alone subcutaneous injections were begun 4 days before SCW administration (n = 9 or 10 7-week-old animals/group) and continued daily until 10 days after SCW injection, then treatment frequency decreased to 5 days/week. *$p < 0.05$ versus SCW-injected simvastatin-treated rats. B. Replicate experiment; 9-week-old rats received simvastatin 20 mg/kg/day or vehicle alone subcutaneous injections beginning 4 days before SCW administration (10 animals/group) and continuing daily until 10 days after SCW injection, then treatment frequency decreased to 5 days/week. *$p < 0.05$ versus SCW-injected simvastatin-treated rats. C. Delayed treatment with subcutaneous simvastatin 20 mg/kg/day or vehicle alone (n = 7 7-week-old animals/group) was begun during the nadir in joint inflammation after attainment of maximal acute-phase joint swelling (Day 8 post-SCW) and continued daily for 8 days, then treatment frequency decreased to 5 days/week. *$p < 0.05$ versus SCW-injected simvastatin-treated rats.

incidence in simvastatin-treated SCW-injected rats; $p > 0.05$ for n = 18–20 animals/group).

Toxicity monitoring. Daily administration (5–7 days/wk) of 20 mg/kg simvastatin (Table 1) for up to 34 days had no effect on liver function as assessed by measurement of serum ALT concentrations in normal or SCW-treated animals, including SCW-treated animals that developed hepatic granulomas. Body weight and serum creatinine levels were also unaffected by simvastatin treatment (Table 1). Consistent with the known lack of effect of statins on cholesterol levels in rats[1,37], serum cholesterol was also not altered by simvastatin treatment in normal or SCW-treated animals (Table 1).

Effect of simvastatin on BMD. BMD of the distal femur of untreated SCW-injected animals versus control animals was decreased as early as 3 days after SCW injection (Figure 3A), and ultimately was 30% to 40% lower than in normal controls at Day 27 or Day 41, respectively, during the chronic destructive phase of arthritis (Figure 3A, 3B). Simvastatin (20 mg/kg/day) pretreatment or delayed treatment prevented 30% of the decreased BMD that occurred at Weeks 4–6 during the chronic phase of joint destruction (Figure 3A), but had no effect on earlier BMD decreases in SCW-injected animals. In control animals, simvastatin had no effect on BMD of the distal femur (Figure 3A, 3B) or spine (L2–L6; data not shown) at any timepoint. Lumbar spine BMD in untreated arthritic animals, which was not different from that in controls, was also unaltered by simvastatin treatment (data not shown).

Effect of simvastatin on bone turnover and cartilage destruction. At Day 29 in untreated arthritic SCW-injected animals, serum pyridinoline levels increased and serum osteocalcin levels were decreased compared to control animals (Table 3), suggesting that uncoupled bone turnover with increased bone resorption and suppressed bone formation both contributed to periarticular bone loss and destruction at this late timepoint, when bone erosion by the invading synovium is prominent (Figure 2C). Simvastatin pre-

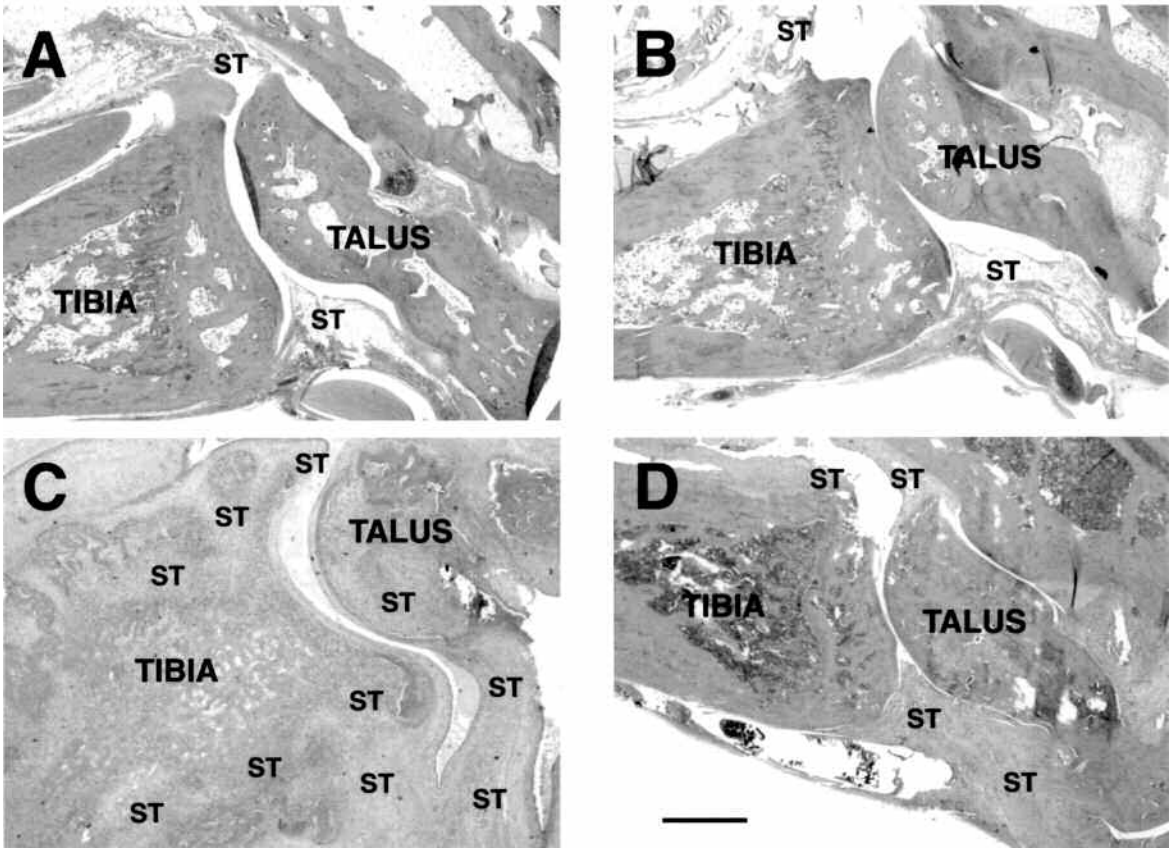

Figure 2. Histologic features of articular pannus formation. A. Normal H&E-stained talo-tibial joint from vehicle-injected animal (Day 29). B. Talo-tibial joint from nonarthritic simvastatin-treated animal (Day 29) is also unremarkable. C. Talo-tibial joint from untreated SCW-injected arthritic animal (Day 29 post-SCW) shows characteristic tumor-like growth of synovial tissue with invasive destruction of articular cartilage and periarticular bone. D. Talo-tibial joint with average degree of clinical joint swelling from simvastatin-treated SCW-injected animal (Day 29 post-SCW) provides representative example of simvastatin inhibition of synovial pannus formation and associated destruction of cartilage and bone. ST: talo-tibial synovial tissue. Bar = 1 mm.

vented the increase in pyridinoline in SCW-injected animals at Day 29, while having no effect on osteocalcin (Table 3). In control animals, neither biochemical marker was significantly altered by simvastatin treatment (Table 3). Because destruction of both bone and cartilage can contribute to increased serum pyridinoline in arthritic animals, effects of simvastatin on increased periarticular bone-resorbing osteoclasts and on articular cartilage destruction in SCW-injected animals were each assessed histologically (Table 3). Simvastatin treatment significantly inhibited (–30%) the increase in periarticular osteoclasts in arthritic joints, while osteoclast numbers in normal animals were unchanged (Table 3). Cartilage destruction in arthritic animals was also inhibited (–47%) by simvastatin treatment (Table 3).

Table 1. Effect of simvastatin on complete blood count, serum chemistries, and body weight. Female Lewis rats were injected on Day 0 with streptococcal cell wall (SCW, 25 g/g body weight) to induce arthritis or with vehicle alone. Subcutaneous injections of simvastatin 20 mg/kg/day or vehicle alone were begun 4 days before SCW injection and continued daily until 10 days after SCW injection, then treatment frequency decreased to 5 days/week. Blood samples were obtained 29 days after SCW injection (7–11 normal animals/group, 14–20 SCW-injected animals/group for assay of complete blood counts and serum chemistries. All values are mean ± SEM; statistical significance determined by ANOVA with post-hoc analysis.

	WBC, 10^6/μl	Monocytes, 10^6/μl	Cholesterol, mg/dl	ALT, U/l	Creatinine, mg/dl	Weight, g
Vehicle control	5.1 ± 0.7	0.07 ± 0.03	75.3 ± 3.5	22.5 ± 2.5	0.24 ± 0.03	190.3 ± 5.6
Simvastatin	5.5 ± 0.4	0.12 ± 0.08	84.9 ± 2.4	28.4 ± 3.5	0.23 ± 0.02	189.9 ± 4.5
SCW	13.4 ± 1.6*	0.94 ± 0.25**	80.8 ± 2.6	22.8 ± 1.5	0.26 ± 0.02	185.9 ± 4.1
SCW/simvastatin	12.7 ± 1.1*	0.33 ± 0.08†	86.1 ± 3.0	23.4 ± 1.9	0.25 ± 0.02	190.5 ± 3.3

* $p < 0.01$ versus vehicle-only rats. ** $p < 0.05$ versus vehicle-only rats. † $p < 0.01$ versus untreated SCW-injected rats. WBC: white blood cells.

Table 2. Effect of simvastatin on influx of ED-1-positive monocytes/macrophages into the arthritic synovium. Female Lewis rats were injected on Day 0 with streptococcal cell wall (SCW, 25 g/g body weight) to induce arthritis or with saline vehicle. Subcutaneous injections of simvastatin 20 mg/kg/day or vehicle alone were begun either 4 days before (pretreatment) or 8 days after (delayed treatment) SCW injection and continued daily until 10 days (pretreatment) or 16 days (delayed treatment) after SCW injection, then treatment frequency decreased to 5 days/week. Hind ankle joints (4 normal rats/group, 8–14 SCW-injected rats/group) were obtained for immunohistochemical analysis of ED-1-positive macrophages in synovial tissue 29 days (pretreatment) or 42 days (delayed treatment) after SCW injection. All values are mean ± SEM; statistical significance determined by ANOVA with post-hoc analysis.

	ED-1-Positive Cells (cells/mm^2)			
	Vehicle	Simvastatin	SCW	Simvastatin/SCW
Simvastatin, pretreatment	98 ± 28	62 ± 1	780 ± 62**	353 ± 59*†
Simvastatin, delayed	64 ± 33	182 ± 25	1077 ± 71**	459 ± 56*†

* $p < 0.05$ versus vehicle-only rats. ** $p < 0.001$ versus vehicle-only rats. † $p < 0.001$ versus untreated SCW-injected rats.

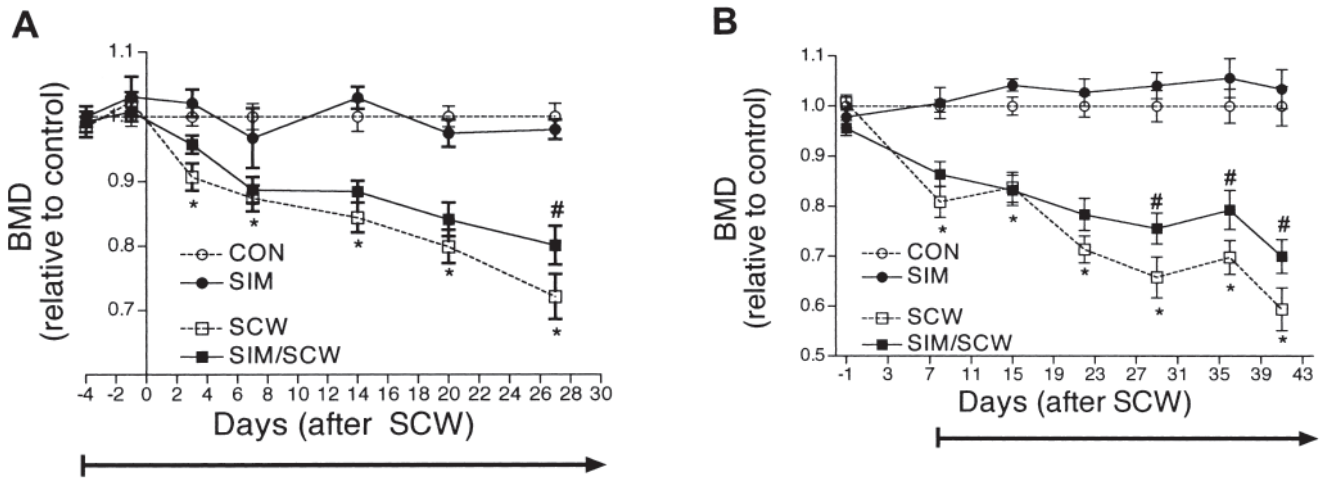

Figure 3. Effect of simvastatin on bone mineral density (BMD). Female Lewis rats were injected on Day 0 with SCW (25 μg/g) or vehicle. Effects of simvastatin or vehicle on BMD of the distal femur in SCW or vehicle-injected controls was assessed at the indicated times (mean ± SEM; n = 8/group controls, n = 14-20/group SCW-injected animals). Arrows indicate course of statin treatment. A. Simvastatin 20 mg/kg/day or vehicle subcutaneous treatments were begun 4 days before injection of SCW or vehicle alone and continued daily until 10 days after SCW injection, then treatment frequency decreased to 5 days/week. Results are expressed as fold-change relative to controls. BMD of controls was 0.201 ± 0.004 g/cm^2 on Day 27. *$p < 0.05$ vs untreated controls. #$p < 0.05$ vs untreated SCW-injected animals. B. Delayed treatment with subcutaneous simvastatin 20 mg/kg/day or vehicle alone was begun during the nadir in joint inflammation after attainment of maximal acute-phase joint swelling (Day 8 post-SCW) and continued daily for 8 days, then treatment frequency decreased to 5 days/week. Results are expressed as fold-change relative to controls. BMD of controls was 0.207 ± 0.008 g/cm^2 on Day 41. *$p < 0.05$ vs untreated controls. #$p < 0.05$ vs untreated SCW-injected animals.

Effects of simvastatin on ex vivo osteoclastogenesis. Consistent with reported *in vitro* effects of other statins[11,38], addition of simvastatin (10 μM) 48 h after start of *ex vivo* bone marrow cell culture completely inhibited osteoclastogenesis induced by M-CSF and a RANK-stimulating antibody in cells isolated from control animals and cultured *ex vivo* for 5 days (untreated cells, 310 ± 11 cells/well vs simvastatin-treated cells, 0 ± 0 cells/well). In contrast, *in vivo* simvastatin treatment had no effect on *ex vivo* M-CSF and RANK-stimulated osteoclastogenesis in bone marrow isolated from SCW-injected or normal animals (Table 3).

Effects of simvastatin on TNF release from bone marrow cells. LPS-stimulated TNF-α release was significantly increased from bone marrow cells isolated from arthritic SCW-injected (vs normal) animals (Table 3). However, *in vivo* simvastatin treatment had no effect on LPS-stimulated TNF release from cells isolated from normal or arthritic animals (Table 3). In contrast, *in vitro* simvastatin treatment of bone marrow cells actually enhanced LPS-stimulated TNF-α release (1.8-fold increase; $p < 0.001$, or 2.3-fold increase; $p < 0.001$, from cells isolated from untreated normal or SCW-injected animals, respectively), while having no effect on constitutive TNF-α production (data not shown).

DISCUSSION

Conflicting results in preclinical and observational human

Table 3. Effect of simvastatin on bone turnover and cartilage. Female Lewis rats were injected on Day 0 with streptococcal cell wall (SCW, 25 g/g body weight) to induce arthritis or with saline vehicle alone. Subcutaneous injections of simvastatin 20 mg/kg/day or vehicle alone were begun 4 days before SCW injection and continued daily until 10 days after SCW injection, then treatment frequency decreased to 5 days/week. Serum samples were obtained 29 days after SCW injection (8 normal animals/group or 18–20 SCW-injected animals/group) for assay of osteocalcin and pyridinoline. Hind ankle joints were obtained 29 days after SCW injection for histochemical analysis of tartrate-resistant acid phosphatase-positive osteoclasts (6–7 tibias/group) and assessment of articular cartilage destruction in distal tibia (17–18 tibias/group). Bone marrow on Day 29 was isolated and combined from 3 tibias/group for *ex vivo* assessment of macrophage colony-stimulating factor- and RANK-activating antibody-stimulated osteoclast formation or lipopolysaccharide-stimulated release of TNF-α. All values are mean ± SEM; statistical significance determined by ANOVA with post-hoc analysis.

	Osteocalcin, ng/ml	Pyridinoline, nM	Periarticular Osteoclasts, cells/mm^2	Articular Cartilage Destruction, 0–3 scale	Ex vivo Osteoclast Formation, cells/well	Ex vivo Bone Marrow TNF-α Release, pg/ml
Vehicle	161.1 ± 10.2	5.5 ± 0.7	10.6 ± 0.2	ND	478 ± 31	463 ± 53
Simvastatin	136.4 ± 10.2	5.1 ± 0.3	10.4 ± 1.3	ND	600 ± 17	721 ± 53
SCW	115.9 ± 9.2*	12.2 ± 1.4**	43.3 ± 1.9†	1.5 ± 0.2	913 ± 70†	2391 ± 597†
SCW/simvastatin	104.6 ± 8.3**	8.4 ± 1.0††	33.6 ± 2.4$^{\dagger\#}$	0.8 ± 0.2††	1017 ± 31†	2666 ± 600†

* $p < 0.05$ versus vehicle only rats. ** $p < 0.01$ versus vehicle only rats. † $p < 0.001$ versus vehicle only rats. †† $p < 0.05$ versus untreated SCW-injected rats. $^{\#}$ $p < 0.001$ versus untreated SCW-injected rats.

studies examining the effects of statins on bone turnover in osteoporosis preclude a consensus regarding their potential use as bone-protective agents[13,15,39]. As inappropriate overstimulation of the RANK pathway is an important cause of bone loss in RA and osteoporosis[39,40], the previously unstudied postulate that statins favorably alter bone turnover in the setting of inflammatory bone loss is of particular interest when assessing the potential influence of these drugs on bone health.

In our experimental model of RA, periarticular BMD loss occurred early in the course of disease activity (Day 3) in parallel with increasing joint inflammation. However, despite early antiinflammatory effects of simvastatin, bone protective effects of simvastatin did not occur until much later (Day 29). This finding is consistent with the postulate that the bone-protective effect of simvastatin was bone-specific and not simply an indirect consequence of antiinflammatory activity.

During the late, bone-destructive phase of SCW-induced arthritis when bone-protective effects of simvastatin were documented, the erosive, catabolic effect of increased bone resorption was further exacerbated by a decrease in bone formation. Simvastatin treatment partially reversed bone loss at this late timepoint in arthritic animals by preventing bone resorption while having no protective anabolic effect. Indeed, osteocalcin levels were actually lower in simvastatin-treated normal and SCW-injected animals, although this effect did not achieve statistical significance. Therefore, there was no evidence of an anabolic effect of simvastatin in preserving periarticular bone in arthritic joints; only an antiresorptive effect was documented in these studies. Simvastatin had no effect on the periarticular decrease in BMD documented in arthritic animals as early as Day 3, a time prior to the onset of synovial proliferation and bone erosions[19,21] when serum osteocalcin levels are decreased and pyridinoline levels are unchanged (Funk, *et al*, unpublished data). Therefore, in SCW-induced arthritis, the bone-protective effect of simvastatin appears to be limited to an inhibition of the bone resorption that occurs later in the course of disease.

Clear *in vitro* evidence of statin blockade of RANK-mediated osteoclast formation exists, as demonstrated here for simvastatin and in previous studies for other statins[11,12,38]. In particular, inhibition of HMG-CoA reductase has been demonstrated by Woo, *et al* to prevent RANK-induced fusion of osteoclast precursors[11]. However, in our arthritis experiments, *in vivo* simvastatin treatment had no effect on the response of resident bone marrow cells to *ex vivo* RANK pathway stimulation, nor did it appear to suppress the production of inflammatory cytokines known to synergize with RANKL[41]. This finding suggests that an inhibitory effect of simvastatin on osteoclast differentiation either (1) does not persist *ex vivo* following *in vivo* dosing; or (2) that the suppressive effect of simvastatin on *in vivo* osteoclast formation and activation may be mediated further upstream in the RANK pathway, including potential effects on local increases in RANKL expression that drive resorption in RA[22].

One limitation of these studies, which were conducted at a time when rat-specific RANKL reagents were not readily available, is that *in vivo* effects of simvastatin on RANKL production were not directly assessed. Recently, we documented a 5-fold increase in articular RANKL expression and a decrease in RANK decoy receptor (OPG) expression in the SCW model occurring concomitantly with joint destruction at late timepoints (Day 28)[25]. In RA, RANKL expression in exuberantly proliferating synoviocytes and activated T cells is thought to drive periarticular bone resorption[22,23]. While articular RANKL expression was not directly measured in the simvastatin-treatment experiments, the number of synoviocytes in the talo-tibial joints of simvastatin-treated SCW-injected animals (vs untreated SCW-

injected animals) was clearly decreased on histological examination. *In vitro* evidence of statin induction of apoptosis[42,43] and inhibition of proliferation (simvastatin IC_{50} = 100 μM; Funk, *et al*, unpublished data) in human rheumatoid synoviocytes are consistent with the postulate that statins may limit the drive toward bone resorption in RA, at least in part, by suppressing the tumor-like growth of the RANKL-producing synovium.

The antiinflammatory effect of simvastatin we observed in the SCW model is in agreement with the results of Leung, *et al*[2], who were the first to demonstrate a joint-protective effect of statins in RA, evaluating hydrolyzed simvastatin (40 mg/kg/day intraperitoneally) in murine collagen-induced arthritis (CIA). In the CIA model, an inhibitory effect on T cell activation and Th-1 cytokine production was postulated to mediate simvastatin's antiarthritic effect, a conclusion consistent with earlier *in vitro* evidence of simvastatin inhibition of leukocyte function antigen-1 (LFA-1)-mediated T cell activation[44]. Indeed, statin inhibition of T cell activation could also prevent bone resorption by further limiting local RANKL production in the rheumatic joint[22]. However, results obtained here with the SCW-induced arthritis model are not consistent with simvastatin suppression of T cell activation. In this model, elimination of T cells or their activation inhibits chronic (but not acute) arthritis and ablates granuloma formation at sites of SCW deposition in the liver[45,46]. However, in these experiments, simvastatin treatment had absolutely no effect on T cell-mediated-hepatic granuloma formation, while inhibiting both acute and chronic joint swelling. Thus, it is unlikely that simvastatin treatment blocked T cell activation in these animals. Closer examination of the *in vitro* effects of simvastatin on T cell activation also supports this conclusion, as the dihydroxy form of statin we used (vs the lactone) is much less potent in blocking LFA-1-mediated T cell activation[47].

Macrophages, rather than T cells, appeared to be the primary inflammatory cell targeted by HMG-CoA reductase inhibition in this model, as simvastatin prevented joint inflammation in association with a 60% decrease in the influx of articular macrophages. Thus, blockade of macrophage influx may be a critical antiinflammatory mechanism of statins in both atherosclerotic lesions[8,9] and arthritic joints, a conclusion also supported by a recent study examining pravastatin in murine CIA[5]. Moreover, while inhibitory effects of statins on leukocyte adhesion and transmigration may limit monocyte/macrophage influx into the arthritic joint[8,9,48], our finding of profound inhibition of monocytosis provides evidence of an additional novel mechanism by which simvastatin may block monocyte/macrophage influx at sites of inflammatory injury.

When discussing potential mechanisms of simvastatin's antiinflammatory effect in RA, it should be pointed out that the protective effects of simvastatin we observed and the CIA experiments of Leung, *et al*[2] were not replicated in one other report examining simvastatin in a preclinical model of RA[3]. However, in this third study, the extremely high (50%) and early mortality in simvastatin-treated animals and the description of the hydrolyzation method used suggest that the hydrolyzation procedure may have been inadequate and/or resulted in contamination of the test product.

This brings us to the question of how the joint-protective effects of simvastatin demonstrated here may translate into clinical use. While we are unaware of any clinical assessment of simvastatin and RA bone loss, clinical evidence of antiinflammatory effects of simvastatin in RA already exists. One small clinical study evaluating simvastatin in patients with RA who had failed to respond to methotrexate showed a remarkable American College of Rheumatology 50% response in 9 of 10 patients[6]. Simvastatin inhibition of the endothelial cell dysfunction associated with a high rate of premature coronary artery disease mortality in this population has also been documented in one clinical trial[49]. In contrast, in the first double-blinded, randomized, placebo-controlled clinical trial of statins in RA, atorvastatin, the most potent cholesterol-lowering statin, decreased C-reactive protein levels by 50% while only modestly decreasing joint inflammation[7]. This highlights the complex pharmacology of statins, whose antiinflammatory potency does not appear to correlate with their cholesterol-lowering efficacy[50].

The human equivalent dose of simvastatin studied here is 180 mg/kg/day[28,29], which closely approximates the highest dose (160 mg/kg/day) tested in clinical cholesterol-lowering trials[51]. In most preclinical trials examining noncholesterol-lowering effects of simvastatin (e.g., ovariectomized-induced bone loss or stroke infarct size[10,27]), this dosage level is required for efficacy. Additionally, as simvastatin was synthetically designed to target the liver (i.e., a prodrug with high hepatic first-pass clearance that is activated to the hydrolyzed form while in the liver[1]), it must be hydrolyzed for use in preclinical trials targeting nonhepatic organs[2,3,27]. Clearly, existing statins have not been optimized for targeting bone or other nonhepatic tissues, as evidenced, for example, by the reported 50-fold higher potency of transdermal (vs oral) lovastatin in targeting bone[52]. Additionally, the pharmokinetics of simvastatin and other existing statins, which have 50% higher peak plasma levels in normal women compared to men[53], have not been determined in patients with RA, many of whom are women whose livers are chronically secreting acute-phase proteins. Therefore, additional preclinical and clinical trials will be required to identify an optimal dose of simvastatin, or any other HMG-CoA reductase inhibitor, for possible use in RA.

The results of these studies support the use of simvastatin as both an antiinflammatory and an antiresorptive joint-protective agent in RA. Moreover, given the possibility of additional therapeutic effects of simvastatin in this crippling disease, including a decrease in cardiovascular mortality, the potential for a multifaceted and clinically significant protec-

tive effect of simvastatin in RA should not be underestimated[54]. At the same time, because currently marketed statins have been optimized for their effects on hepatic cholesterol synthesis, the most effective statin molecule or formulation for the treatment of arthritis or other nonhepatic inflammatory processes remains to be developed.

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