Interleukin 1 Receptor Antagonist Inhibits Localized Bone Formation *in Vivo*

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ABSTRACT. ***Objective.*** To test the *in vivo* effects of interleukin 1 receptor antagonist (IL-1ra) on bone formation and tissue ingrowth using an implantable bone ingrowth chamber that can be infused with test solutions.

Methods. The bone ingrowth chamber was implanted in the proximal tibia of 10 mature NZW rabbits unilaterally. After an initial osseointegration period, the chambers were emptied of tissue and infused with either 0.05% bovine serum albumin (BSA) in phosphate buffered saline (PBS) or an IL-1ra solution for 4-week periods, which were separated by 4-week periods of no infusion. Tissue samples harvested from each chamber were snap-frozen and examined by histology and immunohistochemistry.

Results. The chambers were filled with longitudinally-oriented woven bone in a fibrovascular stroma during periods of infusion of 0.05% BSA in PBS or during periods without infusion. In contrast, infusion of IL-1ra for 4 weeks prevented tissue ingrowth in 4 of 6 chambers, and in 2 chambers exhibiting tissue ingrowth, bone formation was decreased. Bone formation remained at a lower level during the subsequent two 4-week periods without infusion after IL-1ra was discontinued, compared to samples prior to the IL-1ra treatment.

Conclusion. The results showed that tissue ingrowth and bone formation were suppressed in an *in vivo* model by continuous infusion of IL-1ra at an early phase of tissue regeneration and differentiation. (J Rheumatol 2003;30:2547–52)

Key Indexing Terms:
INTERLEUKIN 1 RECEPTOR ANTAGONIST BONE FORMATION

Interleukin 1 (IL-1) is involved in a wide spectrum of immunological and inflammatory responses[1,2]. In the physiological and pathological processes of bone remodeling and homeostasis, IL-1 is an important mediator[3-6]. IL-1 regulates osteoclast activity either by stimulating hemopoietic progenitor cells to differentiate along an osteoclastic lineage, or by directly stimulating the resorptive capacity of existing osteoclasts[7]. IL-1 stimulates the production and activation of other mediators of bone lysis, including prostaglandin E_2 (PGE_2) and collagenase[8]. IL-1 is one of the pivotal inflammatory cytokines that initiates the processes of rheumatoid arthritis (RA) and subsequent joint destruction[9].

IL-1 receptor antagonist (IL-1ra) is a naturally occurring antiinflammatory molecule that belongs to the IL-1 gene family[1,2]. IL-1ra is produced by different cell types, including monocyte-macrophages, fibroblasts, and chondrocytes[1]. The endogenous production of IL-1ra is unique in that IL-1ra is the only known example of a naturally occurring protein that acts as a specific receptor antagonist of a cytokine or hormone-like molecule[2]. Because IL-1ra binds competitively with IL-1 receptors but does not stimulate IL-1-induced intracellular signaling[10,11], it has been extensively studied for its potential therapeutic applications on IL-1 associated inflammatory diseases, especially on RA. IL-1ra blocks IL-1-stimulated bone resorption[5,12], suppresses lymphocyte proliferation, inhibits synthesis of IL-1, tumor necrosis factor and IL-6 by monocytes[12-16], and inhibits the production of PGE_2 and collagenase by human synovial cells and fibroblasts[9,12-14]. Mice that overexpress IL-1ra have a significant reduction in the incidence and severity of collagen-induced arthritis[17]. Continuous infusion of IL-1ra to mice or Lewis rats with collagen-induced arthritis results in 90% inhibition of inflammation as well as lower levels of collagen-induced cartilage and bone destruction[9,18,19]. An imbalance between proinflammatory IL-1 and antiinflammatory IL-1ra is considered as a potential mechanism leading to severe arthritis and even to spontaneous development of the disease[20].

Although IL-1ra is a key mediator in the chronic inflammation associated with arthritis, inflammatory bone loss, and bone remodeling, little attention has been paid to the

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role of IL-1ra in the *in vivo* biological process of bone ingrowth/fracture healing. Immediately after skeletal injury, an inflammatory response is induced by a sequence of biochemical and cellular events[21]. The local inflammation may play an important role in the recruitment, proliferation, and differentiation of mesenchymal stem cells that lead to both endochondral and intramembranous bone formation[21,22]. Given the important role of IL-1 in inflammatory responses, we hypothesize that administration of exogenous recombinant IL-1ra may interfere with bone formation. In this study, the *in vivo* effects of IL-1ra on bone ingrowth and tissue differentiation were examined in the rabbit implanted with a drug test chamber (DTC) that allows infusion of test solutions to the bone formation site and repeated tissue harvest.

MATERIALS AND METHODS

The drug test chamber (DTC). The DTC is a titanium device that is implanted in the proximal medial tibial metaphysis of rabbits at the level of the cortex[23] (Figure 1). The removable core of the DTC provides a 10 µl cavity acting as a fluid reservoir and a 1 × 1 × 5 mm tissue ingrowth canal. The fluid reservoir is linked with the ingrowth canal via a diffusion capillary 1 mm long, 0.25 mm diameter. For continuous infusion, 2 medical grade vinyl tubes (size v/3A; Scientific Commodities Inc., Lake Havasu City, AZ, USA) are inserted into the reservoir. One tube is connected to an osmotic minipump (Durect Corp., Cupertino, CA, USA) implanted subcutaneously. The other tube is open to the subcutis, thereby functioning as a drain for the reservoir. The percolation of the solvent through the diffusion capillary to the ingrowing tissue is minimal due to the reservoir being drained to the subcutaneous tissue[23,24]. Thus the ingrowing or remodeling tissue can be treated locally and continuously with a solution without disturbance of solvent flow. After each treatment, a 1 cm incision is made, the chamber is exposed and disassembled, and the tissue in the chamber is harvested for subsequent analysis. After sample removal, the minipump and tubing (containing the new solution to be infused) are replaced through the same incision. At intervals when only pump replacement is performed, a small incision is made on the posterior flank where the pump was implanted. The tubing is cut at the end to release the old pump, and a new pump loaded with new solution is connected to the existing tubing.

IL-1ra and diffusion pump. Human recombinant IL-1ra, 100 mg/ml in a solution containing sodium citrate, sodium chloride, disodium EDTA, and polysorbate, was provided in sterile bottles by Amgen Inc. (Thousand Oaks, CA, USA) and deemed endotoxin-free after being tested with a high sensitivity limulus amebocyte lysate assay (QCL-1000, BioWhittaker Inc., Walkersville, MD, USA). The Alzet model 2ML2 osmotic pumps were provided by Durect Corporation (Cupertino, CA, USA). The pump has a mean fill volume of 2.0 ml and mean pumping rate of 5.0 µl/h.

Surgical procedure. Institutional guidelines for the care and use of laboratory animals were strictly followed. 10 DTC were implanted unilaterally in 10 mature male New Zealand white rabbits aged 6–12 mo and weighing 3.5–4.2 kg following general endotracheal anesthesia. One dose of cephalosporin antibiotic was given as prophylaxis for infection before each operation. After a 5-week osseointegration period, the tissues in the chambers were harvested and discarded. The animals received no treatment for 4 weeks and the contents were harvested as the first control. The DTC was then connected, via polyvinyl tubing, to an Alzet osmotic diffusion pump containing 2 ml of 0.05% BSA in PBS for 4 weeks in total, followed by a harvest. The animals received no treatment for another 4 weeks, followed by a harvest. IL-1ra was then infused continuously for a 4-week period in total, followed by a harvest. To ensure full activity of the IL-1ra over the entire 4-week period, each pump was replaced with a new one loaded with fresh IL-1ra solution every 2 weeks. The animals received no treatment for 2 additional 4-week periods and samples were harvested as post-IL-1ra samples.

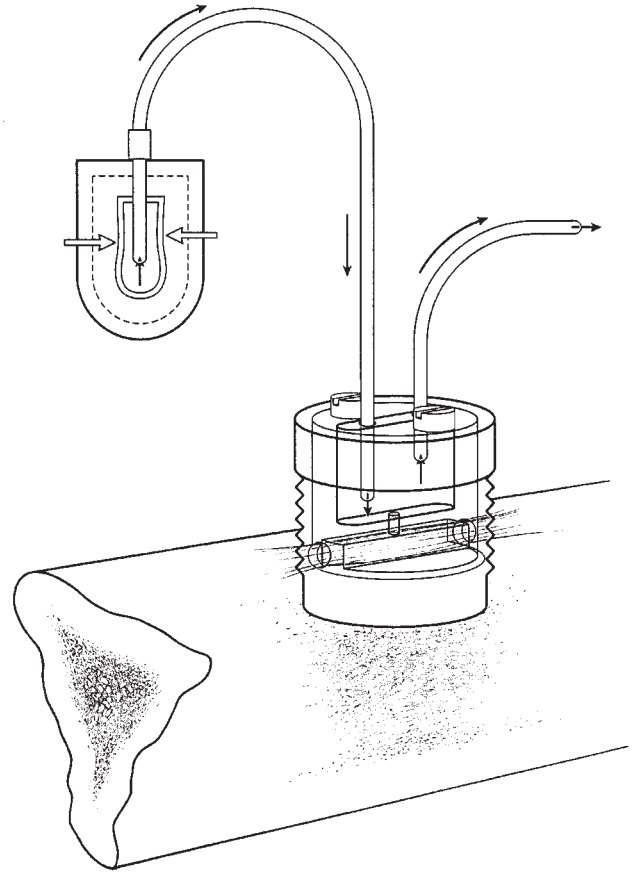

Figure 1. The drug test chamber. The commercial pure-titanium device is implanted in the proximal medial tibial metaphysis of rabbits at the level of the cortex. The removable core of the DTC provides a 10 µl cavity as a fluid reservoir and a 1 × 1 × 5 mm tissue ingrowth canal. The fluid reservoir is linked with the ingrowth canal via a diffusion capillary 1 mm long, 0.25 mm diameter. For continuous infusion of IL-1ra, 2 medical-grade vinyl tubes are inserted into the reservoir: one is connected to an osmotic minipump implanted subcutaneously; the other tube is open to the subcutis, functioning as a drain for the reservoir. The device allows slow continuous flow of solution from the pump through the reservoir to the ingrowth canal, creating a concentration gradient along the diffusion capillary.

Tissue processing. Harvested specimens were embedded in optimal cutting temperature medium (OCT), snap-frozen in liquid nitrogen, and stored at –70°C until processed. Then 4 µm sections were obtained using a cryostat (International Equipment Company, Needham Heights, MA, USA) and mounted on microscope slides. Five or more sections from the center of the specimen were stained with hematoxylin and eosin for general morphological characterization. Cells expressing the vitronectin receptors (osteoclast-like cells) were identified by immunoperoxidase staining[25-27].

Immunohistochemistry. Frozen sections were fixed in absolute acetone at 4°C for 10 min, air dried, and fixed in the acetone for an additional 10 min. The slides were then dried for 45 min, placed in an airtight container, and stored at –70°C until processed. After thawing, the sections were incubated at room temperature in 3% H_2O_2 in distilled water for 5 min, washed in PBS

for 15 min, and incubated in mouse anti-human integrin αVß3 monoclonal antibody (Chemicon, Temecula, CA, USA) for 30 min. After washing in PBS for 25 min, the sections were incubated in biotinylated goat anti-mouse IgG (H+L) (Vector, Burlingame, CA, USA) for 30 min, washed in PBS for 20 min, and incubated in avidin and biotinylated horseradish peroxidase macromolecular complex (ABC Kit, Vector) for 30 min. Positively labeled cells were visualized with diaminobenzidine (Sigma Fast DAB tablet, Sigma, St. Louis, MO, USA) dissolved in 5 ml of deionized water. The slides were counterstained with hematoxylin, dehydrated, and mounted. Positively stained cells correlate strongly with tartrate resistant acid phosphatase staining[25] and reveal more concentrated staining than cells stained with tartrate resistant acid phosphatase.

Assessment and statistical analysis. Histomorphometric analysis was performed on multiple serial, longitudinal sections using a computerized, interactive image analysis system utilizing National Institutes of Health "Image" interfaced with a personal computer and a real-time video-light microscope system. One section stained with hematoxylin and eosin was used for the general morphometric analysis. All measurements were performed blindly, without knowledge of the treatment.

Histomorphometric analysis was based on the measurement of total tissue section area, total bone area, and the total number of vitronectin-receptor positive cells per unit bone area.

Statistical analysis was performed using paired t tests and Fisher's exact test in the StatView software program. P values < 0.05 were considered significant.

RESULTS

Number of animals. The experiment started with 10 rabbits receiving a series of treatments for 4-week periods (Table 1). Four animals were lost due to infection and attrition.

Harvests prior to IL-1ra infusion. All the animals exhibited tissue ingrowth and bone formation. Tissue explants from each of the 3 harvests contained longitudinally oriented woven bone in a fibrovascular stroma (Figure 2). Percentages of bone areas in tissue sections averaged 29.8 ± 3.0% for harvest 1, 27.4 ± 4.1% for harvest 2, and 27.2 ± 9.4% for harvest 3. Vitronectin-receptor positive cells were found dispersed through the tissues, adjacent to or away from the bone surface (Figure 2). The number of osteoclast-like cells per unit bone area remained consistent among all the harvests (Table 2).

Response to infusion of IL-1ra. Four out of 6 animals that underwent IL-1ra treatment yielded no tissue sample for harvesting. Among the 2 animals (R3, R4) that yielded a tissue sample during the treatment of IL-1ra, one (R4) exhibited markedly reduced bone area, the other (R3) also

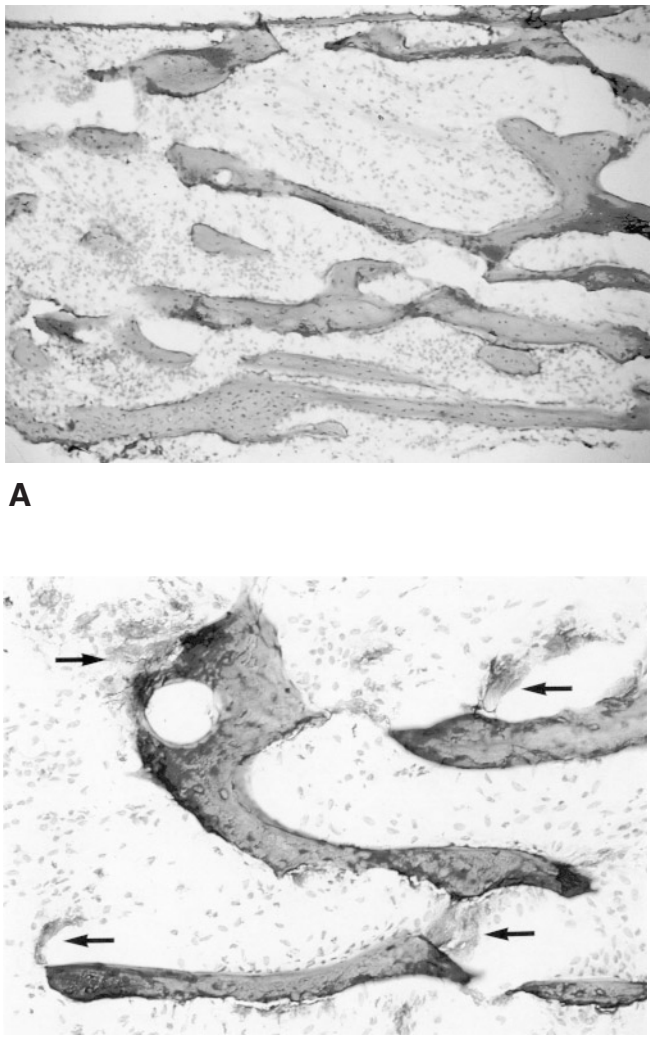

Figure 2. Tissue harvested from the drug test chamber in which no IL-1ra was given for 4 weeks. A. The tissue is composed of longitudinally aligned trabecular woven bone in a fibrovascular stroma. H&E stain; original magnification ×100. B. Immunohistochemistry stain for vitronectin receptors. Osteoclast-like cells (arrows) were dispersed through the tissue, adjacent to or away from bone surface. Original magnification ×200.

Table 1. Number of animals and tissue samples harvested from each treatment and percentage of bone*.

Harvest	Treatment	No. of Animals	No. of Tissue Ingrowth	Bone, %
1	None	10	10	29.8 ± 3.0
2	Infusion of 0.05% BSA	6	6	27.4 ± 4.1
3	None	6	6	27.2 ± 3.9
4	Infusion of IL-1ra	6	2	See Table 3
5	None	6	5	20.39 ± 4.8**
6	None	6	6	23.72 ± 2.4†

* (Bone area divided by tissue section area) x 100 (mean ± SEM). ** p = 0.023 compared with harvest 3. † p = 0.0067 compared with harvest 1. BSA: bovine serum albumin.

Table 2. Data regarding osteoclast. Values are means ± SEM.

Harvest	Treatment	Total VR+/BA
1	None	260.8 ± 25.9
2	Infusion of 0.05% BSA	238.2 ± 50.4
3	None	206.2 ± 30.1
4	Infusion of IL-1ra	—
5	None	412.3 ± 157.9
6	None	206.1 ± 25.0

Total VR+/BA: total number of vitronectin receptor positive cells divided by bone area.

showed lower levels of bone area when compared to the harvest immediately preceding the IL-1ra treatment (Table 3). When the number of animals that did not produce a tissue sample during the IL-1ra treatment versus other harvests are compared, Fisher's exact test p value is 0.06, indicating a strong trend.

Post-IL-1ra response. During the first 4 weeks when IL-1ra was discontinued and no infusion was given (harvest 5), tissue ingrowth including bone was seen in 5 of 6 animals; a sixth animal had a deficiently assembled chamber, making tissue ingrowth impossible. The average percentage of bone area was among the lowest level for all the harvests (Table 1). The number of osteoclast-like cells per unit bone area did not show significant change. During the last 4-week period with no infusion (harvest 6), tissue ingrowth was observed in all 6 animals. The mean bone area rebounded slightly, but was still lower than the level of pre-IL-1ra harvests (p = 0.0067).

DISCUSSION

The drug test chamber is designed as an *in vivo* device for testing the effects of biological, pharmacological, and biomaterial components on tissue ingrowth and bone formation in rabbits[23,24,26]. The device allows a slow continuous flow of the solution from the pump through the reservoir to the ingrowth canal, creating a concentration gradient along the diffusion capillary[23,24]. The test solution must pass through the bone ingrowth canal before dispersing in the rest of the body[23].

To ensure that the pump would work properly with the model, 0.05% BSA in PBS, the carrier solution in our previous study, was infused first. Infusion of the 0.05% BSA solution for 4 weeks had no significant effect on bone ingrowth, which validated the model. Based on prior experiments[28], the vehicle solution of the IL-1ra has no effect on animals. Therefore, we did not infuse this solution alone to avoid the risk of infection in animals associated with multiple operations.

Human recombinant IL-1ra has been used in many animal studies because it has little species specificity[29]. In this rabbit study, continuous infusion of the IL-1ra at 5.0 µl/h, around 15 mg/kg/day, over a 4-week period resulted in marked suppression of tissue ingrowth. The dosing was based on previous *in vivo* studies using IL-1ra[18,30,31]. The number of animals not producing tissue samples (4 out of 6) following treatment of IL-1ra indicates a strong trend compared with controls using Fisher's exact test. We recognize that a small number of animals underwent the various treatments in this study. However, it is notable that even the 2 samples available for harvest from the IL-1ra treatment contained much less bone than the controls immediately preceding the treatment.

Infusion of IL-1ra appeared to have a sustained depressive effect on bone ingrowth, since lower levels of bone formation were observed in the subsequent 4-week controls after the IL-1ra treatment was discontinued. One explanation for the decreased bone formation during the last 2 control periods would be increased bone erosion by osteoclasts. However, an increase in the number of osteoclasts present in tissue sections (Table 2) was not confirmed as a mechanism to explain these results. Alternatively, IL-1ra concentrations may affect bone formation so that release kinetics or protein turnover may be important variables in the process.

It is well established that an influx of inflammatory cells to an injury site and the release of inflammatory mediators are important for tissue repair, bone ingrowth, and fracture healing[32]. In animal models and human studies, IL-1ra mitigates inflammation-induced cartilage and bone destruction when the inflammatory condition was established prior to the administration of IL-1ra[9]. In our study, IL-1ra was infused concurrent with the initial inflammatory response at the local site and the suppression of bone formation was observed. It is interesting that animals genetically deficient for the cyclooxygenase-2 gene, which is responsible for production of the inflammatory mediator PGE_2 [33], exhibit reduced osteoblastogenesis and skeletal repair[21]. Similarly, animals treated with specific cyclooxygenase-2 inhibitors immediately following bone injury also exhibit delayed

Table 3. Percentage of bone from the 2 animals that produced samples after infusion of IL-1ra.

Animal	Treatment					
	None 1	0.05% BSA	None 2	IL-1ra	None 3	None 4
R3	20.2	25.5	30.1	23.4	32.0	19.9
R4	44.1	46.4	40.2	25.1	29.3	32.8

* (Bone area divided by tissue section area) x 100.

bone formation and fracture healing[27,34]. Previous work using the bone harvest chamber showed much higher levels of IL-1ß expression in tissues harvested after 3 weeks versus 4 weeks[35]. Kon, *et al* demonstrated that IL-1α and IL-1ß showed peaks in expression within 24 h after the generation of simple transverse fracture in mouse tibias, and depressed levels until day 21 and day 28, when bone remodeling was initiated[7]. These findings suggest that local accumulation of IL-1 plays an important role in the early local response of bone to injury and repair. The marked suppression of tissue ingrowth into the DTC in this study may be caused by inhibition of IL-1 in the early stages when IL-1 is a crucial cytokine in the cascade of cellular interaction and tissue regeneration. The recruitment, proliferation, and differentiation of mesenchymal cells associated with the initial inflammation[36,37] may also be suppressed by the infusion of IL-1ra in the early phase.

IL-1 enhances alkaline phosphatase activity in osteoblastic cells *in vitro* by acting on DNA and collagen synthesis[38], and induces fibroblast proliferation and a neovascular response[39,40]. The absence of tissue ingrowth in DTC after continuous infusion with IL-1ra for 4 weeks may be due to the inhibition of such activities.

Local infusion of IL-10 has recently been shown to mitigate the suppression of bone ingrowth induced by ultra-high molecular weight polyethylene particles when IL-10 was infused 3 weeks after the onset of the particle-induced inflammatory phase[24]. When IL-10 was infused concurrent with placement of the particles, this effect could not be observed[24]. The results of our study with IL-1ra infusion provide further evidence supporting the important temporal relationships between cytokine modulation and tissue proliferation and differentiation.

In summary, we showed that local infusion of IL-1ra prevents tissue ingrowth and bone formation in an implantable chamber. The marked inhibition of tissue ingrowth may be linked to the early stages of inflammatory reaction and mesenchymal tissue differentiation essential for bone and fibrovascular tissue formation.

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