Periarticular osteopenia in adjuvant induced arthritis: role of interleukin-1 in decreased osteogenic and increased resorptive potential of bone marrow cells

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Abstract

Objective—To clarify the local osteogenic and bone resorptive potential of periarticular bone in adjuvant induced arthritis (AIA).

Methods—Formation of fibroblast colony forming units (FCFU; osteogenic precursor cells) and osteoclast-like cells in bone marrow culture was studied in AIA rats. Osteoclast-inducing activity in the AIA rat bone marrow was assayed by the addition of the marrow supernatant from rats with AIA to control cultures. Bone mineral density was determined by dual x ray absorptiometry.

Results—Marrow from AIA rats and that from animals receiving recombinant human interleukin-1 (IL-1) β for seven days grew significantly fewer FCFU than control marrow. Formation of osteoclast-like cells was increased in bone marrow cultures from rats with AIA, especially when bone marrow cells were cultured in the presence of marrow supernatant. Formation of resorption lacunae on ivory slices was increased in the marrow cultures from rats with AIA, especially from the right (adjuvant inoculated) tibia. AIA rat marrow supernatant promoted osteoclast-like cell formation in control culture, and this was significantly suppressed by an anti-IL-1 antibody. Rats with AIA showed a significant decrease in the bone mineral density of the periarticular regions of the tibia and femur.

Conclusion—An uncoupled state in bone resorption-formation linkage, possibly mediated through an increase of IL-1 in the bone marrow, may contribute to the development of periarticular osteopenia in inflammatory arthritis.

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Development of osteopenia in the vicinity of inflamed joints is a common clinical feature of rheumatoid arthritis. This local osteopenia occurs early in the course of the disease, and is evident in some patients only a few months after the onset of symptoms.^{1,2} The pathogenesis of rheumatoid periarticular osteopenia is still not well understood, although the following factors have been implicated: immobilisation of the affected joints by pain;

local release of factors such as inflammatory cytokines and prostaglandins from adjacent sites of synovial inflammation;^{3,4} reflex sympathetic dystrophy.⁵ Previous histological studies of periarticular bone have demonstrated an increase in both osteoblastic and osteoclastic activity, suggesting increased bone turnover.^{6,7} However, these studies were performed on periarticular bone specimens obtained from patients with chronic rheumatoid arthritis at the time of joint replacement surgery; local changes in bone metabolism early in the course of rheumatoid arthritis have not been investigated.

Bone marrow cells possess an osteogenic potential and the ability of cultured bone marrow fibroblast-like stromal cells to form bone in vivo has already been demonstrated.^{8,9} Cultured marrow stromal cells form clones known as fibroblast colony forming units (FCFU).¹⁰ These FCFU colonies contain cells that can become osteoblastic in the absence of any specific inducers, and it is suggested that FCFU are the in vitro equivalent of osteogenic precursor cells.¹⁰ A previous study also demonstrated that the number of FCFUs appeared to be closely associated with in vivo osteogenesis and could be used as an indirect bioassay of osteogenesis.¹¹ In addition, cultured bone marrow cells form osteoclast-like multinucleated cells in the presence of osteotropic hormones, prostaglandins, and cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumour necrosis factors (TNF).¹²⁻¹⁵ To clarify the early changes of osteogenic and bone resorptive potential in the juxta-articular region of inflamed joints, we investigated FCFU growth and bone resorbing activity in bone marrow cultures obtained from rats with adjuvant induced arthritis (AIA), which is an animal model of human rheumatoid arthritis.

Materials and methods

RAT MODEL OF AIA

Six week old female Lewis rats (Charles River Japan, Kanagawa, Japan) were used in this study. Experimental arthritis was induced by a single intradermal injection of a suspension of killed *Mycobacterium butyricum* in mineral oil (Sigma Chemical Co, St Louis, MO). The bacterial suspension was injected into the right hindpaw at a dose of 0.05-0.1 mg per rat. Inoculated rats and age matched controls were maintained under the same conditions, with

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free access to food and water. The development of arthritis was assessed by measuring the hindpaw volume distal to the ankle joint using a plethysmograph (Volume-meter, MK-500, Muromachi Kikai Co, Kanagawa). Polyarthritis developed eight to 10 days after bacterial inoculation. When the hindpaw volume reached a plateau (about 10 days after the development of arthritis), rats with arthritis and control rats were killed for study in the following experiments.

BIOCHEMICAL MEASUREMENTS

Serum concentrations of calcium, phosphorus, and alkaline phosphatase were measured using an autoanalyser (RX-40 Model, Nihon Denshi Inc Ltd). The serum osteocalcin concentration was determined by radioimmunoassay using a specific antibody against rat osteocalcin (Biochemical Technologies Inc, Stoughton, MA).

BONE MARROW PREPARATION

All procedures were performed under sterile conditions. Bilateral femora, tibiae, and humeri were resected and the epiphyseal ends of the bones removed. Bone marrow was flushed out into 15 ml conical culture tubes using 1 ml of α -minimum essential medium (α MEM) (Gibco BRL, Life Technologies Inc, Grand Island, NY) for each bone. The harvested marrow was filtered through a stainless steel mesh (50 μ m pore size) and centrifuged at 250 *g* for five minutes. The resulting supernatant was passed through a filter with 0.22 μ m pores and stored at -80°C . The cell pellet was resuspended in fresh α MEM and the number of viable cells counted in a haemocytometer using the trypan blue dye exclusion technique. The total number of bone marrow cells from AIA rat femur was similar to that from control rat femur ($3.7 \times 10^7/\text{bone}$ *v* $3.6 \times 10^7/\text{bone}$). The total number of tibial bone marrow cells was slightly increased in AIA rats (right tibia $3.1 \times 10^7/\text{bone}$, left tibia $2.6 \times 10^7/\text{bone}$) compared with the control group ($2.4 \times 10^7/\text{bone}$), though not significantly.

FCFU IN BONE MARROW CULTURE

Bone marrow cells were plated out in 25 cm^2 culture flasks at a density of $10^6/\text{ml}$ ($10^7/\text{flask}$). Cells were grown in α MEM supplemented with 15% fetal bovine serum (FBS) and a 1% penicillin-streptomycin mixture under an atmosphere of 5% carbon dioxide. The medium was changed completely after the first 24 hours of culture and every three days thereafter. The cells attached and grew to form FCFU colonies, were fixed in 2% glutaraldehyde or citrate-acetone-formaldehyde, and were stained with Wright-Giemsa stain or for alkaline phosphatase activity (Sigma kit, Sigma Chemical Co). The total number of FCFUs which contained a minimum of 50 cells were counted, as were the number of alkaline phosphatase positive FCFU with 50 or more cells.

OSTEOCLAST-LIKE CELLS IN BONE MARROW CULTURE

Bone marrow cells were plated in four well Lab Tek chamber slides (Nunc Inc, Naperville, IL) at a density of $1 \times 10^6/\text{ml}$, and were incubated in α MEM with 15% FBS and antibiotics. Culture was performed in the presence or absence of marrow supernatant to test the effect of marrow humoral factors on the induction of osteoclast-like cells. The medium was changed every three days and marrow supernatant was added at the time of medium change. Cells which showed attachment were fixed in citrate-acetone-formaldehyde and stained for tartrate resistant acid phosphatase (TRAP) activity (Sigma Kit, Sigma Chemical Co). TRAP positive cells which contained three or more nuclei were counted microscopically as osteoclast-like cells.

PIT FORMATION ASSAY

Circular slices of ivory (6 mm diameter) were prepared using a bone slicer and an office paper punch. The slices were sonicated and sterilised in 70% ethanol, washed with α MEM, and each one placed in a well of a 96 well culture plate. Bone marrow cells were cultured in the presence of marrow supernatant for 12 days as described above, then plated onto the slices at a density of $2 \times 10^6/\text{well}$. The marrow cells were scraped with a rubber-policeman in 2 mol/l NaOH and the slices then stained with Coomassie Brilliant Blue. The pits (resorption lacunae) were counted microscopically.

NEUTRALISATION OF MARROW OSTEOCLAST-INDUCING ACTIVITY

To clarify the involvement of inflammatory cytokine(s) in bone marrow osteoclast-inducing activity, we used anti-IL-1 and anti-TNF antibodies in a neutralisation study. These antibodies were a polyclonal rabbit antimurine IL-1 antibody (Genzyme C, Cambridge, MA) and a monoclonal hamster antimurine TNF antibody (2 mg/ml) (Genzyme), which could detect rat and mouse IL-1 α , and TNF α and TNF β , respectively. The anti-IL-1 antibody inhibited concanavalin A stimulated rat spleen cell proliferation completely at a dilution of 1:50 and was used in the same dilution for the neutralisation study. The concentration of the anti-TNF antibody was 5 $\mu\text{g}/\text{ml}$. Rabbit IgG (Organon Teknica Co, Durham, NC) was used as a control. Bone marrow cells from normal rats were cultured in the presence of marrow supernatant from rats with AIA, under the conditions described above.

EFFECT OF INFLAMMATORY CYTOKINES ON FCFU

Five week old female DDY mice were used for this study. Recombinant human (rh) IL-1 β (Otsuka Pharmaceutical Co, Osaka, Japan), rhTNF α (Dainippon Pharmaceutical Co, Osaka, Japan) and rhIL-6 (Genzyme) were given subcutaneously at a dose of 400–2000 U/day for seven days. The animals were then sacrificed,

the femoral and tibial bone marrow cultured for seven days, and the number of FCFU counted.

MEASUREMENT OF BONE MINERAL DENSITY
The femoral and tibial bone mineral densities were determined by dual energy x ray absorptiometry (DXA) using an Aloka DCS-600 (Aloka Co, Mitaka, Japan). The bone mineral density was measured at the proximal, mid, and distal portions of each bone.

STATISTICAL ANALYSIS
Differences between the mean values of two groups were determined by Student's unpaired *t* test; *p* < 0.05 was taken to indicate significance.

Results
BIOCHEMICAL DATA AND SERUM OSTEOCALCIN CONCENTRATION
Rats with AIA showed significant weight loss (*p* < 0.001) (table 1). The serum concentration of phosphorus of these rats was increased significantly compared with control animals, but serum calcium and alkaline phosphatase concentrations were unchanged. The serum osteocalcin concentration—a marker of osteoblastic activity—was significantly decreased in the rats with arthritis (table 1).

GROWTH OF MARROW FCFU
The number of FCFUs which developed in marrow cell cultures from rats with AIA was significantly fewer than those developing in control cultures (figs 1, 2), though the deficit of FCFU growth was less severe in marrow from the humerus compared with tibial and femoral marrow. In all groups, about 80% of the cells inoculated initially failed to adhere to the flasks after 24 hours. The size of the FCFUs was similar in all cultures, but differences in FCFU numbers were apparent as early as day 7 of culture. About 50% of the colonies became alkaline phosphatase positive on day 7 in both the control and arthritis groups, but the number of positive FCFU was fewer in the rats with arthritis (fig 1).

FORMATION OF OSTEOCLAST-LIKE CELLS
Formation of TRAP positive multinucleated cells was increased in the bone marrow cultures

Table 1 Body weight, hindpaw volume, and serum concentrations of calcium, phosphorus, alkaline phosphatase, and osteocalcin

	Control rats	AIA rats
Body weight (g)	184.9 (11.6)	146.3 (12.3)***
Hindpaw volume (ml)		
Right (inoculated)	1.42 (0.03)	3.52 (0.28)***
Left (uninoculated)	1.41 (0.03)	2.56 (0.45)***
Serum calcium (mg/ml)	9.9 (0.3)	9.8 (0.3)
Phosphorus (mg/ml)	7.8 (0.2)	8.5 (0.8)*
Alkaline phosphatase (IU/l)	495 (78)	452 (56)
Osteocalcin (ng/ml)	57.6 (6.7)	50.1 (3.8)**

Values are mean (SD) for 25 rats. AIA = Adjuvant induced arthritis. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 v control.

Figure 1 Total number of fibroblast colony forming units (FCFU) (upper panel) and number of alkaline phosphatase (ALP) positive FCFU (lower panel) which developed in bone marrow cell cultures from rats with adjuvant induced arthritis (AIA). CF = Control femur; CT = control tibia; CH = control humerus; AF = AIA femur; rAT = right AIA tibia; lAT = left AIA tibia; AH = AIA humerus. Results are means + SEM of 25 animals. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 v control marrow cultures from the respective bones.

from rats with AIA, especially from the right tibia (inoculated side), compared with the control group (fig 3). When bone marrow cells were cultured in the presence of bone marrow supernatant, marked stimulation of osteoclast-like cell formation was observed in the cultures from rats with arthritis and more osteoclast-like cells were generated in cultures of tibial

Figure 2 Fibroblast-like cell colonies stained with Wright-Giemsa stain in seven day bone marrow cultures. Clonal growth of marrow stromal cells was markedly impaired in cultures from rats with adjuvant induced arthritis.

Figure 3 Formation of tartrate resistant acid phosphatase (TRAP) positive multinucleated cells in bone marrow cultures when the bone marrow cells (BMC) were incubated alone or with marrow supernatant from the same bone (BMS). Results are means $\pm$ SEM of 25 animals. CF = Control femur; AF = AIA femur; CT = control tibia; rAT = right AIA tibia; IAT = left AIA tibia. * p < 0.05, ** p < 0.01, *** p < 0.001 v the respective controls.

marrow compared with cultures of femoral marrow (fig 3). In contrast, the addition of control marrow supernatant to control bone marrow cultures only slightly stimulated osteoclast-like cell formation.

BONE RESORBING ACTIVITY

When marrow cells from control rats were cultured on ivory slices in the presence of control marrow supernatant, shallow resorption lacunae (pits) were observed on the surface of the slices. Formation of pits was increased in the marrow cultures from rats with AIA, especially from the right tibia (inoculated side). When the marrow supernatants from AIA rats were added to the control culture, a marked increase in the number of pits was observed (figs 4, 5). The number of pits on the ivory slices correlated significantly with the number of osteoclast-like cells generated on the chamber slides ($r = 0.563$, $p < 0.01$).

NEUTRALISATION OF OSTEOCLAST-INDUCING ACTIVITY

When bone marrow cells from control rats were cultured in the presence of femoral and tibial marrow supernatant from rats with arthritis, the formation of osteoclast-like cells increased markedly. This increase in osteoclast-like cell formation was suppressed significantly by addition of the anti-IL-1 antibody, compared with no antibody or addition of control rabbit IgG (fig 6). The increase in osteoclast-like cell formation associated with the presence of femoral bone marrow supernatant from rats with arthritis was suppressed in addition by

Figure 4 Coomassie Brilliant Blue stained resorption lacunae (pits) on the ivory slices, produced by: (A) control tibial bone marrow cells + control tibial marrow supernatant, (B) control tibial bone marrow cells + right tibial marrow supernatant from rats with adjuvant induced arthritis (AIA), (C) right tibial marrow cells + right tibial marrow supernatant from AIA rats, (D) left tibial marrow cells + left tibial marrow supernatant from AIA rats. A-D: Horizontal bars represents 500 μ m.

Figure 5 Formation of resorption pits on ivory slices, produced by rat bone marrow cells (BMC) cultured on the slices in the presence of marrow supernatants (BMS). Results are means + SEM from five cultures. CF = Control femur; AF = AIA femur; CT = control tibia; rAT = right AIA tibia; LAT = left AIA tibia. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 v the respective controls.

Figure 6 Effect of bone marrow supernatants on osteoclast induction, and neutralisation of osteoclast-inducing activity in the marrow from rats with adjuvant induced arthritis. Results are means + SEM from 10 experiments. CF = Control femur; AF = AIA femur; CT = control tibia; rAT = right AIA tibia; LAT = left AIA tibia; Ig = rabbit IgG; IL-1 α = antimurine IL-1 antibody; TNF α = antimurine TNF antibody. **p* < 0.05; ***p* < 0.01.

anti-TNF antibody, but that associated with the presence of tibial marrow supernatant from AIA rats was not significantly suppressed by that antibody.

Table 2 Bone mineral density of the femur and tibia in control and adjuvant induced arthritis (AIA) rats

	Control rats		AIA rats	
			Right	Left
Femur (mg/cm ²)				
Proximal	127.3 (3.3)		100.1 (10.1)**	102.2 (9.6)**
Mid portion	80.1 (3.0)		74.6 (4.4)	76.8 (2.1)
Distal	155.3 (3.3)		95.3 (4.0)***	102.2 (9.6)***
Tibia (mg/cm ²)				
Proximal	95.5 (7.5)		55.9 (7.5)***	72.2 (15.7)*
Mid portion	67.3 (3.3)		55.4 (5.0)*	58.9 (4.3)*
Distal	106.7 (7.8)		73.2 (8.1)***	84.5 (17.1)**

Values are mean (SEM) from 10 animals. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 v control.

Figure 7 Effect of vehicle (V), rhIL-1 β 400 or 2000 U/day, rhTNF α 400 or 2000 U/day, and rhIL-6 400 U/day on marrow FCFU growth. Each cytokine was given intraperitoneally to five week old female DDY mice for seven days. Results are means + SEM of six animals. **p* < 0.05, ***p* < 0.01 v vehicle injected control.

EFFECT OF CYTOKINES ON FCFU

Marrow from animals receiving 400 U/day of rhIL-1 β for seven days grew significantly fewer FCFU than control marrow. Even more marked inhibition of marrow FCFU formation was observed after administration of the highest dose of rhIL-1 β (2000 U/day) (fig 7). Administration of rhTNF α 400–2000 U/day also decreased marrow FCFU formation, but this change was neither significant nor dose dependent. No significant change in marrow FCFU growth was noted after the administration of rhIL-6 400 U/day.

CHANGES IN BONE MINERAL DENSITY

Rats with AIA showed a significant decrease in the bone mineral density of the proximal and distal portions of the femur and tibia compared with control animals. However, the mid portions of both bones showed no remarkable changes in rats with arthritis (table 2).

Discussion

Periarticular osteopenia is well known as the initial bone manifestation of rheumatoid arthritis. The acceleration of bone resorption has been suggested to be caused by cytokines or chemical mediators released from inflamed joints into the adjacent bone.^{2 16 17} Previous histological studies of bone specimens obtained from patients with chronic rheumatoid arthritis have demonstrated an increase of both bone resorption and bone formation, supporting this concept.^{6 7} However, periarticular osteopenia develops in the early stage of rheumatoid arthritis when erosive changes of the bone or thickening of the synovial membrane are not yet evident. In addition, a decrease in bone turnover has been reported in this disease.¹⁸ These findings suggest that the pathogenesis of systemic osteopenia may differ from that of localised osteopenia in the periarticular region.

The present study clearly demonstrated decreased colony formation by osteogenic precursor cells in cultures of bone marrow

obtained from sites adjacent to inflamed joints. This may reflect a decrease in the absolute number of FCFUs per bone in rats with arthritis, because the total numbers of bone marrow cells per bone were similar in both control and arthritis groups. As the number of FCFUs can be used as an indirect bioassay of osteogenic activity, our data suggest a local decrease in osteogenic potential in rats with adjuvant induced arthritis. The decreased serum concentration of osteocalcin in these rats is consistent with this conclusion. The systemic suppression of bone formation may occur by immobilisation and malnutrition in rats with arthritis. However, compared with the culture of bone marrow from humerus, the decrease in osteogenic precursor cell colonies was more evident in cultures of marrow from the tibia and femur, which were the bones adjacent to the site of arthritis. This finding suggests that the local effect of arthritis may be more important for the periarticular suppression of bone formation, though a systemic effect cannot be excluded. We also demonstrated that the systemic administration of rhIL-1 β induced a marked suppression of osteogenic precursor cell colony formation in marrow cultures from normal animals. These data are supported by the finding that systemic administration of IL-1 induced a decrease in serum osteocalcin concentration in rats.¹⁹ In addition, an in vitro inhibitory effect of IL-1 on bone formation has been reported.²⁰ As IL-1 induces other inflammatory cytokines, such as IL-6, TNF α , and IL-8, these cytokines could also be involved in the decrease in marrow osteogenic precursor cell colony formation. However, our administration of TNF α or IL-6 did not induce any significant suppression of colony formation, suggesting that IL-1 might be the most important cytokine, at least in our experimental system.

Osteoclast-like cells differentiate from bone marrow derived macrophages in the presence of osteotropic hormones in vitro. In previous studies, no significant formation of osteoclast-like cells from bone marrow cells was observed in the absence of inductive substances such as hormones, cytokines, or prostaglandins. In the present study, a very small number of osteoclast-like cells was generated in cultures of normal marrow without any inducers. However, bone marrow from rats with AIA formed a significantly larger number of osteoclast-like cells, although the number was still small. These findings might be explained by an increased number of osteoclast precursors in the bone marrow of rats with arthritis.

Several local factors have been suggested to have a role in the regulation of osteoclast maturation and activation. They include cytokines, prostaglandins, colony stimulating factors, and osteoblast derived soluble factor(s).^{11-14 21 22} In the present study, addition of normal bone marrow supernatant caused significant formation of osteoclast-like cells and resorption pits on the ivory slices in normal marrow cultures. This suggested the presence of some osteoclast inducing factor in the normal marrow supernatant. Increased formation of osteoclast-like

cells and resorption pits in the culture of tibial bone marrow from AIA rats indicates that bone resorbing activity was increased in the bone marrow adjacent to the site of arthritis. In addition, marked stimulation of osteoclast-like cell and pit formation was observed when marrow supernatant from AIA rats was added to the normal marrow culture. This suggested an increase in osteoclast inducing factor in AIA bone marrow. Although it is not clear if the same factors were responsible for the induction of osteoclast-like cells by normal and AIA marrow supernatant, the results of our study suggested that one of the factors present in arthritis bone marrow was IL-1. Other cytokines or growth factors may be involved in osteoclast induction by marrow supernatants, because anti-TNF antibody decreased the osteoclast inducing activity in the femoral bone marrow from AIA rats. As inflamed rheumatoid synovium produces IL-1, IL-6, and prostaglandins,²³⁻²⁵ the increase in IL-1 in the bone marrow might be the result of its diffusion from the adjacent inflamed joints. However, we cannot exclude the possibility that these bone marrow changes are a primary event.

Measurement of bone mineral density demonstrated localised bone loss in the periarticular regions of bilateral femur and tibia. However, the decrease in the distal portion of the tibia was rather small, possibly because of sclerotic changes induced by severe arthritis of the hind foot.

In conclusion, both a local decrease in osteogenic potential and accelerated bone resorption, partly mediated through an increase in IL-1 in the bone marrow, may be involved in the development of periarticular osteopenia associated with adjuvant induced arthritis. Accordingly, strategies aimed at decreasing IL-1 production may help to control such osteopenia. In addition, the role of other cytokines such as IL-6, and that of colony stimulating factors and chemical mediators in the pathogenesis of periarticular osteopenia requires clarification.

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