Original Article

Etanercept Promotes Bone Formation via Suppression of Dickkopf-1 Expression in Rats with Collagen-Induced Arthritis

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ABSTRACT

Background   Various clinical reports suggest etanercept (ETN) has some efficacy in bone formation in rheumatoid arthritis (RA). To examine this effect, we investigated the gene expression of cytokines relevant to osteoblast/osteoclast differentiation, and evaluated histomorphometric findings in mature rats with collagen-induced arthritis (CIA).

Methods   Total RNA was extracted from knee joints with CIA after ETN or placebo administration. Subsequently, realtime-PCR was carried out to quantify the mRNAs encoding Wnt-1, Dickkopf-1 (DKK-1), receptor activator of nuclear factor kappa-B ligand (RANKL), osteoprotegelin (OPG) and TNF (tumor necrosis factor)-alpha. In histomorphometric analysis, the infiltrating pannus volume and pannus surface, and the following items in contact with pannus surface were measured: osteoclast number, osteoid surface, osteoid volume and labeling surface. These were evaluated in the distal femur with CIA with or without ETN administration.

Results   TNF-alpha, RANKL and OPG mRNA expressions, linked to osteoclastogenesis, were not significantly different with or without ETN administration. ETN administration significantly increased Wnt-1 mRNA expression, the osteoblast promoter, and decreased DKK-1 mRNA expression, the Wnt signal inhibitor. In histomorphometric analysis, pannus volume, pannus surface and osteoclast number, parameters of bone destruction, were not significantly different among groups. Osteoid volume, osteoid surface and labeling surface, parameters of bone formation, increased significantly with ETN administration.

Conclusion   Our results suggest that ETN suppresses DDK-1 expression, and, as a result, Wnt expression is promoted and osteoblastogenesis becomes more active, independent of the regulation of osteoclast activity. Marked bone formation is attributed to the fact that ETN directly promotes osteoblastogenesis, not as a result of suppressing osteoclastogenesis.

Key words   bone formation; Dickkopf-1; etanercept; rheumatoid arthritis; Wnt

Rheumatoid arthritis (RA) is a chronic inflammatory disease, accompanied by synovitis, that causes joint destruction. Various cytokines, including tumor necrosis factor (TNF)-alpha, have been implicated as important mediators of inflammation and joint destruction in RA.[1–3] The scientific consensus is that the receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegelin (OPG) system is pivotal to the pathogenesis of bone erosions in RA.[4] Also, TNF-alpha promotes the expression of RANKL, and that is associated with up-regulation of osteoclast activity.[5]

From the point of view of bone metabolism, it has also become clear that TNF-alpha regulates the activity of osteoblasts, which play a role in bone metabolism with osteoclasts.[6–8] Recently, it was reported that Dickkopf-1 (DKK-1), an inhibitor of the Wnt signaling pathway, regulated the balance between osteoclastogenesis and osteoblastogenesis in an inflammatory joint RA model. TNF-alpha may suppress osteoblast differentiation via promoting the expression of DKK-1, inhibiting Wnt the signaling pathway.[9–11]

In recent years, the development of TNF-alpha-targeted biologic agents has provided an excellent curative effect.[12–15] As previously reported, a number of clinical reports have described radiographic evidence of the repair of erosion in RA.[16–19] The administration of etanercept (ETN), an anti TNF-alpha agent, reduced the total Sharp score from the baseline established before the initiation of therapy in a large clinical study.[15] ETN may promote bone formation, as well as inhibiting bone destruction via suppression of TNF-alpha function. Although there are some indications that destructive bone lesions can be repaired in RA joints, the mechanism of this repair remains unknown and histologically unproven. To examine the effects of ETN on bone formation,

we i) investigated the gene expression of cytokines relevant to osteoblast differentiation within bone infiltrated by pannus (Experiment A), and ii) performed histological evaluation of bone repair (Experiment B) in mature rats with collagen-induced arthritis (CIA), an animal experimental model of RA.[20]

MATERIALS AND METHODS
Animals
Seven-month-old female Sprague-Dawley rats (retired breeding animals, body weight 266–381 g, Shimizu Laboratory Supply, Kyoto, Japan) were used in this experiment. This experiment was carried out in accordance with the Guidelines for Animal Experimentation of the Faculty of Medicine, Tottori University. During this experiment, animals were freely fed with tap water and food (CE-2; CLEA, Tokyo, Japan; calcium content 1.18 g/100 g, phosphorus content 1.09 g/100 g, vitamin D3 content 250 IU/100 g). The room temperature was set to 24 ˚C, and after about 2 weeks of preliminary breeding, the animals were used for the experiments.

Collagen arthritis models
According to the method described by Trentham et al.,[20] 1 mL of emulsion containing 0.5 mg bovine type II collagen (0.3% acetic acid solution; K-41 Cosmo-Bio, Tokyo) and 0.5 mg of incomplete Freund's adjuvant (521-00021; Difco Laboratories, Detroit, MI) were injected intracutaneously at three sites on the back of CIA group rats, and 1 week later, a half dosage of the same emulsions was injected. In the non-immunized group (N group), physiological saline was injected in the same manner. The development of arthritis was judged by the redness and swelling in the ankle by same observer. The onset of arthritis was observed about 2 weeks after immunization, and arthritis became severe until 4 weeks.

Administration of biologic agents
In the ETN administration group, rats were subcutaneously administered 3 mg/kg ETN 3 times per week (E group). In the placebo-administered group, physiological saline was injected in the same manner (P group).

Experiment A: Analysis of mRNA expression
Animal grouping and ETN administration
The animals were divided randomly into 3 groups: E group (n = 21), P group (n = 21) and non-immunized group (N group) (n = 5). Administrations of ETN (E group) and placebo (P group) were started 1 day after immunization, and 7 animals were killed 7, 14 and 21 days after immunization in each group.

Tissue collection and RNA isolation
After the animals were killed, the bilateral distal femur and proximal tibia (cut off 5 mm from the joint line) were collected and cooled with liquid nitrogen immediately. The bone tissues were collected aseptically and all connective tissue, including the periosteum, was completely removed. Frozen bones were homogenized in appropriate volumes in an Isogen Kit (Nippon Gene, Tokyo) using POLYTRON (KINEMATICA AG, Tokyo). Total RNA was extracted from the tissue homogenates according to the manufacturer's instructions. Additionally, we defecated the extracted total RNA using an RNeasy plus mini kit (Quiagen, Hilden, Germany). RNA concentrations were determined by spectrophotometry.

Reverse transcription
The total RNA was reverse-transcribed into cDNA using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA). The conditions were as follows: incubation 60 min at 37 ˚C, reaction inactivation by heating at 95 ˚C for 5 min, and maintenance at 4 ˚C.

Real-time PCR
Rat primers were used to analyze DKK-1, Wnt-1, RANKL, OPG, TNF-alpha and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, housekeeping gene), respectively. Specific primers designed with reference to the Universal ProbeLibrary Assay Design Center and Universal ProbeLibrary Probe (Roche Applied Science, Madison, WI) were used as shown in Table 1. A quantitative real-time PCR was performed to compare expression levels of each mRNA. EXPRESS qPCR Supermix with Premixed ROX (Invitrogen) was used, with an initial denaturation step of 20 s at 95 ˚C, followed by 45 cycles of 1 s at 95 ˚C, and 20 s at 60 ˚C as the annealing temperature. Relative quantification of mRNA expression was calculated by a 7900HT Fast Real-Time PCR System with SDS2.3 software (Applied Biosystems, Carsbad, CA).

Experiment B: Histological findings
Animal grouping and ETN administration
Twenty rats were immunized and divided into the E group (n = 10) and P group (n = 10), and killed 8 weeks after CIA immunization. ETN administration was started 3 weeks after immunization because bone destruction was observed histologically 3 weeks after immunization.[21]

Preparation of hard tissue specimens
Histological findings in hard tissue specimens were taken from the medial condyle of the femur in the mid-sagittal plane then compared between the groups. Bone

Table 1. Primers used for real-time PCR

Molecule	Left primer Right primer	Used probe number	Product (base pairs)	Accession number
TNF-alpha	5'-agttggggagggagacctt-3' 5'-catccacccaaggatgtttag-3'	71	60	L00981
RANKL	5'-agacacagaagcactacctgactc-3' 5'-ggccccacaatgtgttgta-3'	2	90	AF187319
OPG	5'-gaagcaactcaaaattgtggaa-3' 5'-tcagccaattcggtataatcttg-3'	98	94	U94330
TRAP	5'-gcctcttgcgtcctctatga-3' 5'-agcaccatccacgtatcca-3'	65	60	M76110
DKK-1	5'-cgggaattactgcaaaaacg-3' 5'-caatgatgccttcctcgatt-3'	76	83	NM001106350
Wnt-1	5'-tcggcaagatcgtcaacc-3' 5'-gtagtcgcaggtgcaggatt-3'	15	128	NM001105714
GAPDH	5'-acaactttggcatcgtgga-3' 5'-cttctgagtggcagtgatgg-3'	114	62	NM017008

DKK-1, Dickkopf-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OPG, osteoprotegelin; RANKL, receptor activator of nuclear factor kappa-B ligand; TNF, tumor necrosis factor; TRAP, tartrate-resistant acid phosphatase.

labeling by subctaneous injection of calcein at 10 mg/kg was performed twice at 17 days and 1 day before the animals were killed, and tetracycline at 20 mg/kg was given once at 9 days before the animals were killed [schedule 1/7/1/7/1 (number of days of the 1st labeling/number of days between 1st and 2nd labeling/number of days of the 2nd labeling/number of days between 2nd labeling and 3rd labeling/number of days between 3rd labeling and killing)]. The labeling schedule was decided as the result of trial and error of preliminary experiment. The knee joint with larger swelling in each rat was collected with the joint capsule and synovial membranes preserved, and fixed in 70% alcohol. The remaining whole knee joint was stained with Villaneuva bone stain for 7 days, dehydrated in graded concentrations of ethanol, and embedded in methyl-methacrylate without decalcification. The resulting blocks of knee joint specimens were sectioned in the sagittal plane at a thickness of 5 μm with a microtome (Leica Biosystems, Solms, Germany).

Bone histomorphometry

Bone histomorphometric measurements of the femur were made using a semiautomatic image analyzing system (System Supply, Nagano, Japan) and a fluorescent microscope (eclipse 80i; Nikon, Tokyo) set at a magnification power of 400. Because it was difficult to quantify bone formation after definite destructive action, we regarded osteoid production and bone calcification seen on the surface of the pannus as evidence of the repair of erosion. Twenty sections closest to the mid-sagittal plane of the medial femoral condyle were sampled for each group, 2 sections per rat, each parameter was measured and the average was compared among the different groups. A line connecting the intersection of the epiphy-
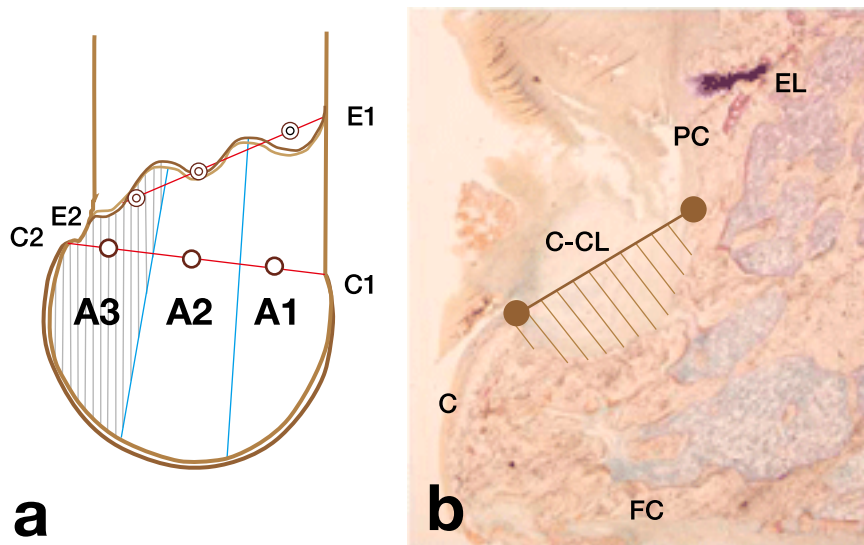

Fig. 1. Schematic presentations of unit area **(a)** and infiltrating pannus **(b).**

a: Epiphyseal bone marrow is divided into 3 regions (A1-A3) by 2 lines connecting 2 points, each equally dividing lines E1-E2 and C1-C2 into 3 portions.

b: We defined the shaded area as the pannus infiltrating the epiphyseal medulla of the posteromedial femoral condyle.

C, cartilage;
C-CL, the line connecting the remaining edge of the posterior cortex to the posterior cartilage;
EL, epiphyseal line;
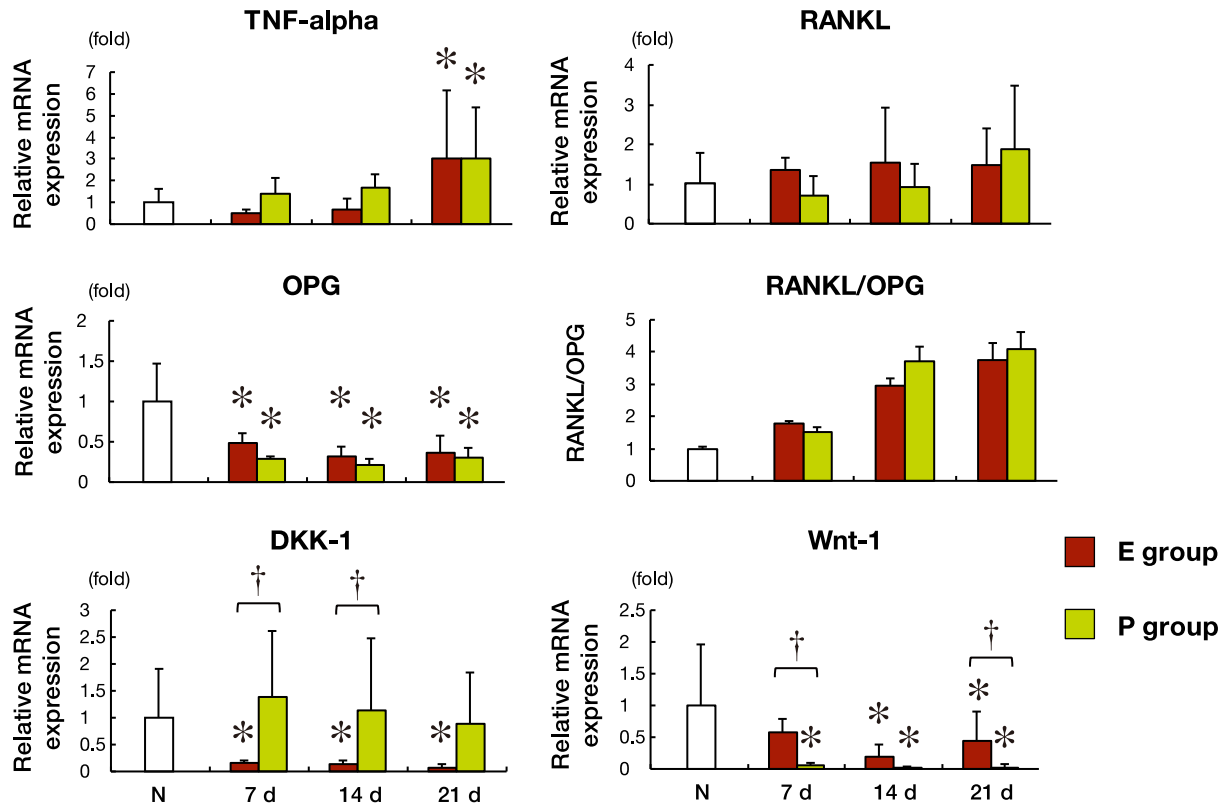FC, femoral condyle;
PC, posterior cortex.

Fig. 2. Relative quantification of mRNA expression in bone. mRNA expressions of tumor necrosis factor (TNF)-alpha, receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegelin (OPG) do not change with etanercept (ETN) administration. Treatment with ETN tends to reduce the RANKL/OPG ratio. DKK-1 mRNA expression in the E group is lower than the P group. Wnt-1 mRNA expression is increased with ETN administration. N, non-immunized group; d, days after immunization. Values are the mean ± SD ($n = 7$). *$P < 0.05$ versus the N group. †$P < 0.05$ versus the P group.

seal line and the anterior cortex (E1) to the intersection of the epiphyseal line and the posterior cortex (E2) was equally divided into 3 portions, and the 2 division points were connected to 2 points equally dividing into 3 the line connecting the anterior end (C1) with the posterior end (C2) of the articular cartilage; the bone marrow was thus divided into 3 regions: A1 to A3. We defined the A3 region as a unit area (tissue volume) (Fig. 1A).[21] To measure the pannus volume and pannus surface infiltrating the epiphyseal medulla of the posteromedial femoral condyle, we chose the line connecting the remaining edge of the posterior cortex to the posterior cartilage (C-CL) (Fig. 1B). We defined the shaded area as the infiltrating pannus. The pannus surface was measured in this area exclusive of the surface on the C-CL. Then, the following items in contact with the pannus surface were measured: osteoclast number, osteoid surface, osteoid volume and labeling surface. We also counted the number of plasma cells, macrophages, and synovial blood vessels per high power field (HPF) in the same area to assess the activity of the infiltrating pannus. The field was inclusive of the pannus close to the bone surface, but exclusive of bone tissue.

Statistical analysis

Statistical significance was analysed using the software package Dr. SPSS 2 for Windows 11.0.1J (SPSS, Tokyo). In experiment A, Fisher's protected least significant difference procedure was performed after repeated-measures analysis of variance. In experiment B, the Mann-Whitney U test was used to compare median values. $P < 0.05$ was considered significant in both experiments.

RESULTS
Experiment A: Analysis of mRNA expression

Expression of TNF-alpha increased significantly with time in the E group ($P = 0.005$), and tended to increase over the course of time in the P group ($P = 0.065$) (Fig. 2). Compared with the N group, significant differences were shown on day 21 in both the E and P groups ($P = 0.033, 0.035$, respectively). Expression of RANKL in the P group tended to increase over time, whereas in the E group it showed no marked change. In all groups and at all times, there were no significant differences compared to the N group. OPG mRNA expression on day 7 tended

Table 2. Bone histomorphometry of the posteromedial femoral condyle in the mid-sagittal plane

Group	PV/TV (%)	PS/TV ($\mu m/\mu m^2$)	Oc.N/PS (/mm)	OV/PV (%)	OS/PS (%)	LS/PS (%)
E	34.3 ± 10.9	0.0014 ± 0.0005	3.13 ± 8.09	1.88 ± 1.48*	27.6 ± 12.9*	41.6 ± 14.1*
P	30.8 ± 12.8	0.0015 ± 0.0006	3.31 ± 6.77	0.21 ± 0.19	8.3 ± 5.7	11.7 ± 6.9

Values are the mean ± SD ($n = 10$).
LS, labeling surface; Oc.N, osteoclast number; OS, osteoid surface; OV, osteoid volume; PS, pannus surface; PV, pannus volume; TV, tissue volume.
PV/unit area (TV), PS/TV and Oc.N/PS are not significantly different between the groups. OV/PV, OS/PS and LS/PS increase significantly with etanercept administration.
*$P < 0.05$ versus the P group.

to be higher in the E group than in the P group ($P = 0.053$), and significantly decreased in all groups and at all times compared with the N group, independent of ETN administration. TNF-alpha, RANKL and OPG mRNA expressions were not significantly different between E and P groups at all times. Treatment with ETN tended to reduce the RANKL/OPG ratio on day 14 ($P = 0.072$), but the difference did not reach statistical significance at any time. DKK-1 mRNA expression in the E group decreased significantly on days 7 and 14 ($P = 0.009, 0.033$, respectively) compared to the P group, and tended to be lower than the P group on day 21 ($P = 0.085$). With the exception of day 7 in the E group, expression of Wnt-1 mRNA significantly decreased in all groups and at all times compared with the N group, independent of ETN administration. However, ETN administration significantly increased Wnt-1 mRNA expression on days 7 and 21 ($P = 0.014, 0.047$, respectively) compared with the placebo administration.

Experiment B: Histological findings
Bone histomorphometric analysis of the posteromedial condyle of the femur in the mid-sagittal plane was performed. The pannus volume and pannus surface were not significantly different between the groups ($P = 0.478$, 0.718, respectively) (Table 2). The average number of osteoclasts on the pannus surface did not change with ETN administration ($P = 0.398$). Osteoid volume, osteoid surface and labeling surface on the surface of the pannus increased significantly with ETN administration ($P < 0.001$). The number of HPF, which met the above con-

Table 3. The number of plasma cells, macrophages and blood vessels per high power field (HPF) in the infiltrating pannus

Group	Plasma cells/HPF	Macrophages/HPF	Blood vessels/HPF
E	0.40 ± 0.33	0.26 ± 0.30	1.86 ± 0.95
P	0.56 ± 0.52	0.40 ± 0.43	2.33 ± 0.77

Values are the mean ± SD ($n = 10$).
There are no significant differences between the groups.

ditions, varied across sections (2–5 fields per section). Totals of 55 and 53 HPF were examined in the P group and E group, respectively. The number of plasma cells, macrophages and blood vessels per HPF were not significantly different between the groups ($P = 0.355, 0.355, 0.063$, respectively) (Table 3). Histologic sections of the knee joint stained with Villanueva bone stain are shown in Fig. 3. The pannus infiltrated the posteromedial femoral condyle (Fig. 3a). Although pannus formation and bone destruction were not different between the E group (Fig. 3b) and P group (Fig. 3d), more osteoid production (Fgs. 3b and d: black arrowheads) and bone calcification (Figs. 3c and e: white arrowheads) were seen on the pannus-contacting bone surface in the E group than in the P group.

DISCUSSION
Genovese et al. reported that 10% (20 of 201) patients treated with ETN had negative change in the total Sharp score from the baseline on 5-year radiographs.[22] Klareskog el al. also reported similar results of radiographic changes in RA patients treated with ENT and methotrexate combination therapy.[15] It is clear that development of several effective treatment options such as ETN has induced repair of bone erosions in RA, but the mechanism of "bone repair" is still unknown. To clarify this uncertain mechanism, we attempted to investigate "bone repair" using molecular biological and histological methods with CIA.

Trentham et al. reported that sensitization to type II collagen caused arthritis similar to RA.[20] CIA has been used as an RA model for studies on the pathology and treatment of RA. In the present study, we chose knee joints because i) the incidence of knee arthritis was high; ii) arthritis of the knee tended to be severe and iii) our previous study investigated knee joints of CIA histologically and with molecular biology.[21, 23]

ETN is a protein that intercepts or inhibits the work of TNF-alpha, but does not inhibit production.[3] Therefore, it is acceptable that expression of TNF-alpha
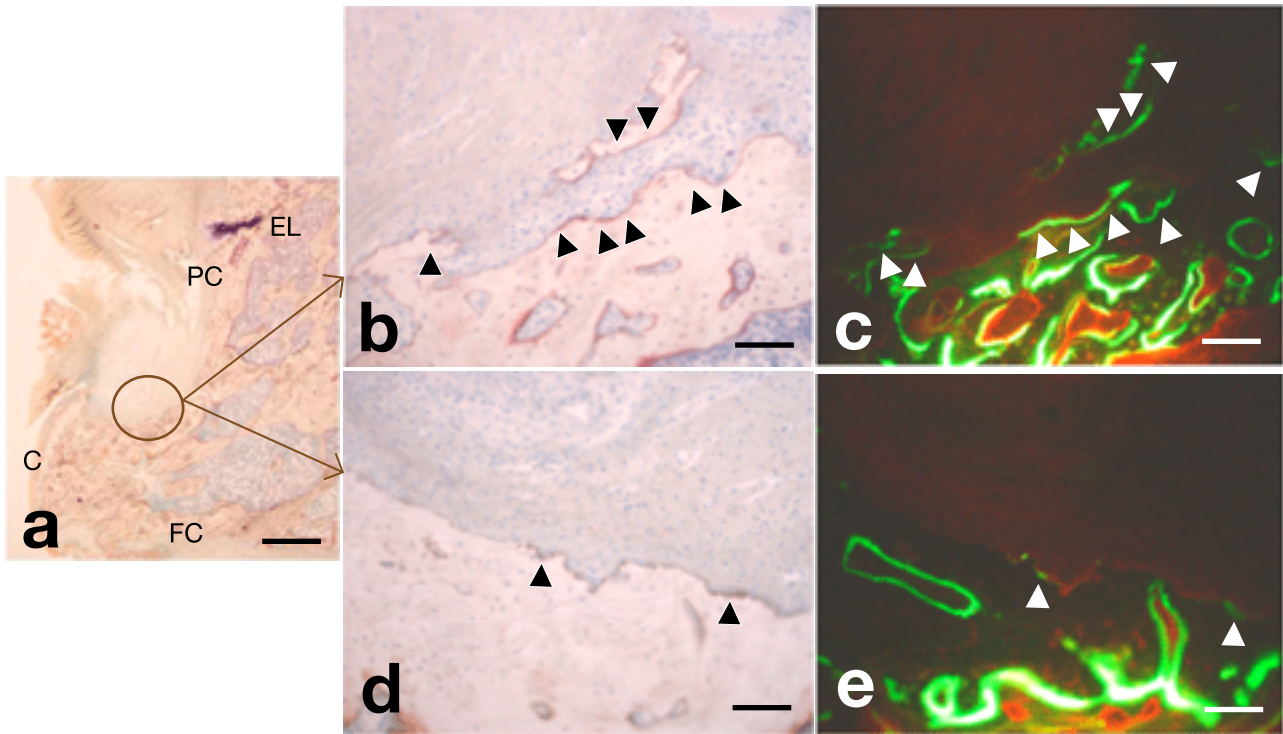
Fig. 3. Pannus infiltrated the posteromedial femoral condyle (**a**). Pannus formation and bone destruction are not different between the E group (**b**, **c**) and P group (**d**, **e**). More osteoid production (**b**, **d**: black arrowheads) and bone calcification (**c**, **e**: white arrowheads) are seen on the pannus-contacting bone surface in the E group than in the P group (Villaneuva bone stain). **a:** bar = 500 μm. **b, d:** bar = 100 μm. **c, e:** fluorescence, bar = 100 μm. C, cartilage; EL, epiphyseal line; FC, femoral condyle; PC, posterior cortex.

mRNA was not inhibited by ETN administration. Expression of RANKL and OPG were not different with or without ETN administration. However, treatment with ETN showed a tendency to reduce the RANKL/OPG ratio. Catrina et al. reported that anti-TNF therapy did not suppress synovial RANKL expression, but promoted OPG expression in RA patients. It was concluded that therapy with TNF antagonists in RA modulates the OPG/RANKL system, a potential mechanism that could explain the retardation of radiographic damage observed following anti-TNF therapy.[24] We think our findings from an animal model in which anti-TNF therapy reduced the RANKL/OPG ratio in synovial tissue support their conclusion.

Furthermore, in our histological study, the pannus volume, pannus surface and average number of osteoclasts on the pannus surface did not change with ETN administration. By the same token, the number of plasma cells, macrophages and synovial blood vessels were not significantly different among groups. This result is reasonable in the light of our analysis of mRNA expression that indicated ETN did not suppress RANKL expression. This implies ETN has limited influence on osteoclastogenesis.

In the meanwhile, from the standpoint of bone formation, more osteoid and bone calcification were seen on the pannus-contacting bone surface in the E group than in the P group. Recently, it was clarified that the Wnt family stimulated osteoblast formation via co-receptor LRP5/6, and DKK, an antagonist of LRP5/6, suppressed osteoblast formation by blocking the Wnt signaling pathway.[10, 25] Interestingly, Diarra et al. reported bone formation in RA was hampered by TNF-mediated expression of DKK-1.[9] These facts and our results suggest that ETN suppresses DDK-1 expression via the blocking effect of TNF-alpha and, as a result, Wnt expression is promoted and osteoblastogenesis becomes more active independent of regulation of osteoclast activity. Therefore, increase of bone formation is attributed to the fact that ETN directly promotes osteoblastogenesis and not as a result of suppressing osteoclastogenesis.

Our study has a few limitations. In experiment A, ETN administration was started 1 day after immunization because TNF-alpha expression began to decrease after peaking 7 days after immunization.[23] A previous study reported that ETN reduced the incidence of arthritis,[26] so one limitation is that ETN administration was started before the onset of arthritis. However, in our preliminary experiment, the incidence of arthritis in CIA rats was not significantly different between the E group and P group (85.7%, 89.9% respectively). In experiment B, ETN administration was started 3 weeks after im-

munization because bone destruction was observed histologically 3 weeks after immunization.[21] As described above, if we assume that expression of TNF-alpha may deteriorate soon after 3 weeks' post-initial immunization, ETN may be no longer involved in the regulation of osteoclastogenesis from that point onwards. As a result, bone resorption during histomorfometry was not downregulated. ETN administration was started less than 3 weeks after immunization, which may cause inhibition of pannus formation itself. This is the second limitation of this study.

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