Gene expression relevant to osteoclastogenesis in the synovium and bone marrow of mature rats with collagen-induced arthritis

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Objectives. To investigate gene expression relevant to osteoclastogenesis in the synovium and bone marrow during the development of collagen-induced arthritis (CIA) in mature rats.

Methods. Total messenger RNAs (mRNAs) were obtained from CIA synovium and bone marrow after immunization. First, reverse transcriptase–polymerase chain reactions (RT-PCR) were carried out to detect the mRNA encoding receptor activator of NF-κB (RANK), RANK ligand (RANKL), osteoprotegerin (OPG), tumour necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6 and the osteoclast markers tartrate-resistant acid phosphatase (TRAP) and cathepsin K. Secondly, the genes detected clearly by RT-PCR were quantified using real-time PCR.

Results. In the synovium, expression of all genes was confirmed by specific single bands in RT-PCR. In real-time PCR, the expression levels of TNF-α, IL-1β, IL-6, RANKL, TRAP and cathepsin K mRNA increased, whereas the expression levels of RANK and OPG were unchanged and decreased respectively. RANKL expression was highly correlated with the two osteoclast markers. In the bone marrow, RT-PCR did not clearly detect the expression of IL-6, RANKL or OPG mRNA. Quantitative real-time PCR showed that TNF-α, RANK and TRAP mRNA expression did not change significantly with time, and that IL-1β and cathepsin K changed slightly compared with those in the synovium.

Conclusions. In the early stages of arthritis, synovial RANKL is closely involved in osteoclastogenesis, and various changes in synovial cytokines, including down-regulation of OPG, probably accelerate osteoclast formation. In contrast, cytokine mRNA in the bone marrow showed little fluctuation. We suggest that synovial cytokines affect osteoclastogenesis not only in the synovium but in the bone marrow.

Key words: Osteoclastogenesis, Collagen-induced arthritis, Synovium, Bone marrow, RANKL/RANK/OPG, Inflammatory cytokines.

Rheumatoid arthritis (RA) is a chronic inflammatory disease that manifests mainly as joint destruction accompanied with progressive synovitis. In RA, osteoclasts are considered to participate in bone destruction and juxta-articular osteopenia [1]. It has recently been clarified that various cytokines produced in the synovium and juxta-articular bone marrow contribute to osteoclast formation.

Receptor activator of NF-κB ligands (RANKL) were shown in 1998 to finally determine the differentiation of osteoclasts by binding to receptor activator of NF-κB (RANK) expressed on osteoclast precursors [2,3], and increased expression of RANKL in RA synovial tissues has been demonstrated [4,5]. On the other hand, osteoprotegerin (OPG), a decoy receptor of RANKL, suppresses bone resorption by inhibiting osteoclast formation, and is expected to act as a mediator to prevent bone destruction in RA [6].

Inflammatory cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6, act on osteoblasts and synovial fibroblasts to promote production of RANKL [7,8] and finally accelerate osteoclast formation. Current studies indicate that these cytokines are also able to induce differentiation and activation of osteoclasts via a pathway without RANKL [9-11].

We have previously performed histological and immunohistochemical investigations of time-course changes in bone destruction of the knee joint in mature rats with collagen-induced arthritis (CIA) [12]. In this study, 14 days after immunization, tartrate-resistant acid phosphatase (TRAP)-positive multinuclear cells (osteoclast-like cells) appeared in the proliferative synovium. However, 21 days after immunization osteoclast-like cells also appeared in the epiphyseal bone marrow which were morphologically unconnected to the synovium. Thus, we concluded that bone destruction in the early stages of CIA occurred in two anatomically different regions, the synovium and the bone marrow. The purpose of this study was to clarify the dynamics of the cytokines involved in osteoclastogenesis by comparing the synovium with the bone marrow in CIA rats. On days 3–21 after induction of CIA, the synovium of the knee joint and the bone marrow close to the knee joint was collected, and messenger RNA (mRNA) expression of TNF-α, IL-1β, IL-6, RANKL, RANK, OPG and the osteoclast markers TRAP and cathepsin K were investigated using reverse transcription–polymerase chain reaction (RT-PCR). In addition, to analyse the dynamics in detail, we performed real-time PCR to quantify mRNA expression. We found that gene expression showed various changes in the synovium. RANKL expression was especially highly correlated with the osteoclast markers, while OPG expression significantly decreased with the development of clinical
arthritis. In contrast, mRNA expression showed little fluctuation in the bone marrow.

Materials and methods

Animals

Seven-month-old female Sprague-Dawley rats (retired breeding animals, body weight 250–350 g; Shimizu Laboratory Supply Co., Kyoto, Japan) were used in the experiment. This experiment was carried out in accordance with the Guidelines for Animal Experimentation of the Faculty of Medicine, Tottori University.

Preparation of collagen-induced arthritis models

After acclimatization for approximately 2 weeks, 1 ml of emulsion containing 500 μg of bovine type II collagen (0.3% acetic acid solution; K-41 Cosmo-Bio, Tokyo, Japan) and 500 μg of incomplete adjuvant (521-00021; Difco Laboratories, Detroit, MI, USA) were intracutaneously injected at three sites on the back of each rat anaesthetized intraperitoneally with 1 mg/kg of ketamine hydrochloride (Ketalar; Sankyo, Tokyo, Japan) plus xylazine (Celactal; Bayer, Leverkusen, Germany) (2:1). As a control, 1 ml physiological saline solution was injected by the same method instead of type II collagen.

Animal groups

A total of 30 rats were immunized, and five immunized and control rats were killed on each of days 3, 5, 7, 10, 15 and 21 after immunization.

Evaluation of arthritis

Rat paws were scored for arthritis, as previously described [13]. Severity of arthritis was graded in each toe, hind paw, ankle joint and knee joint from 0 to 3 (0 = normal, 1 = detectable, 2 = moderate, 3 = severe) based on increasing level of erythema and oedema. The maximum possible score was 24.

Tissue collection and RNA isolation

Synovium and bone marrow were obtained from the knee. We studied the knee because (i) the incidence of knee arthritis was high; (ii) arthritis of knee tended to be severe (mean arthritis score of the knee on day 21 was 3.2 ± 1.1); and (iii) our previous study investigated the knee joints of CIA histologically. After the animals had been killed, the synovial tissue was dissected from the bilateral knee joint space immediately, and was homogenized in appropriate volumes of Isogen Kit (Nippon Gene Co., Tokyo, Japan). Total RNA was extracted from the tissue homogenates according to the manufacturer’s instructions. Moreover, the femur was collected aseptically and all connective tissue, including the periosteum, was completely removed. The distal epiphysis, including the growth plate, was also removed. The synovial capsule adhered to the epiphysis, so it was removed with the epiphysial bone. After cutting off the middle third of the femur, the distal diaphyseal and metaphyseal bone marrow was collected, and total RNA was extracted by the same method as that described above. RNA concentrations were determined by spectrophotometry.

RT-PCR

One microgram of total RNA derived from the synovial tissue and bone marrow was reverse-transcribed into cDNA with random hexamers and subjected to PCR using a RT-PCR kit (Promega, Tokyo, Japan). Specific primers were used as shown in Table 1. Rat primers were used for the analysis of TNF-α, IL-1β, IL-6, RANKL, RANK, OPG, TRAP, cathepsin K and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, housekeeping gene), respectively. PCR conditions were as follows: denaturation at 95°C for 30 s, annealing at 60°C for 1 minute (TRAP at 64°C, OPG at 62°C), extension at 72°C for 1 min for 30 cycles, and final extension at 72°C for 7 min. PCR products were separated on 1.5% agarose gel and stained with ethidium bromide.

Real-time PCR

Quantitative real-time PCR was performed to compare expression levels of each mRNA in non-immunized vs immunized CIA developing rats. Because real-time PCR is very sensitive method there is a high risk of contamination. To quantify the specific products correctly, we applied only the samples which could detect specific single bands in conventional RT-PCR to real-time PCR. Real-time PCR on the LightCycler® (Roche Diagnostics, Mannheim, Germany) was performed in the presence of 2 μl FastStart DNA master SYBR Green I® (Roche Diagnostics) and

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Table 1. Primers used for RT-PCR and real-time PCR

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Sense primer</th>
<th>Antisense primer</th>
<th>Product (base pairs)</th>
<th>Sequence reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α [38]</td>
<td>5'-TACTGAACTTCCGGGGTGATCG-3'</td>
<td>5'-CCTTGTCCCTGGAAAGAACC-3'</td>
<td>292</td>
<td>L00981</td>
</tr>
<tr>
<td>IL-1β [38]</td>
<td>5'-CACAACAAATGCCTCGTGCG-3'</td>
<td>5'-TGCTATGGATCAAGTTGGG-3'</td>
<td>331</td>
<td>M98820</td>
</tr>
<tr>
<td>IL-6 [38]</td>
<td>5'-AAATCTGCTTCGTTCTTCTGG-3'</td>
<td>5'-TTAGATACCCATCGACAGG-3'</td>
<td>380</td>
<td>M26745</td>
</tr>
<tr>
<td>RANKL [39]</td>
<td>5'-ACGCAGATTTGCCAGACTCGAC-3'</td>
<td>5'-TTCGTCCTACCTCTTCCATC-3'</td>
<td>493</td>
<td>AF019048</td>
</tr>
<tr>
<td>RANK [39]</td>
<td>5'-TTAACGACGTCTTCAGCGGG-3'</td>
<td>5'-ACGTAGAACCACAGGATGTCGCC-3'</td>
<td>497</td>
<td>AF018253</td>
</tr>
<tr>
<td>OPG [39]</td>
<td>5'-TGCCACACAGTGAATGCGA-3'</td>
<td>5'-GCTGGAAAGTGGTCTTGGG-3'</td>
<td>538</td>
<td>U94330</td>
</tr>
<tr>
<td>TRAP [40]</td>
<td>5'-CGCCAGAACGTCAGAACAGA-3'</td>
<td>5'-GCTGGAAAGTGGTCTTGGG-3'</td>
<td>357</td>
<td>M76110</td>
</tr>
<tr>
<td>Cathepsin K</td>
<td>5'-CCCCAGACTTCCATCGACTATGCA-3'</td>
<td>5'-CTGTACCCTCTCGACTTACGCC-3'</td>
<td>330</td>
<td>AF010306</td>
</tr>
<tr>
<td>GAPDH [42]</td>
<td>5'-ACCACATCTGGCCATAC-3'</td>
<td>5'-TCCACACCCCTTGGCTGTA-3'</td>
<td>452</td>
<td>NM017008</td>
</tr>
</tbody>
</table>
2 μl cDNA. MgCl₂ was added to a final concentration of 2–5 mM, and 2 μl of primer mix for each forward and reverse primer and H₂O up to 20 μl were added. Real-time PCR was performed with an initial denaturation step of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 5 s at an annealing temperature equal to the RT-PCR protocol and [product length (base pairs)/25] s at 72°C. Direct detection of PCR products was monitored in real time by measuring the increase in fluorescence caused by the binding of SYBR Green I Dye to dsDNA. Subsequently, the threshold cycle (Ct), the cycle number at which the amount of amplified gene of interest reached a fixed threshold, was determined. Relative quantification analysis was performed by the 2⁻ⁿCt method described by Livak and Schmittgen [14] after confirming that GAPDH and other cDNAs were amplified with the same efficiency (i.e. the absolute values of the slope of log input amount were within <0.05 of each other). The relative quantification value of the target, normalized to an endogenous control and relative to a calibrator, was expressed as 2⁻ⁿCt of the target gene – Ct of the endogenous control gene (GAPDH), and ΔCt = Ct of the samples for target gene – Ct of the calibrator for the target gene.

Statistical analysis

Statistical significance was analysed using the software package StatView 5.0 (Abacus Concepts, Berkeley, CA, USA). Data are expressed as the mean ± S.E.M. The Mann–Whitney U test was used to compare mean values. A value of P<0.05 was considered significant.

Ethical approval for this study was given by the guidelines for animal experimentation of the Faculty of Medicine, Tottori University.

Results

Changes in arthritis

The first signs of arthritis were observed on day 15 after immunization, and the maximal arthritis score was obtained on day 21 (the mean arthritis score was 5.8 ± 2.9).

The incidence of arthritis was 90% (9 of 10) on day 15 and 100% (5 of 5) on day 21, so the animals were considered suitable for investigation to compare pre-arthritis findings with findings after the onset of arthritis.

No erythema or oedema was found in rats injected with physiological saline solution as controls.

Gene expression in the synovium

To investigate the expression of genes relevant to osteoclastogenesis in the synovium, mRNA expression was investigated using RT-PCR. Expression of all genes was confirmed by the specific single bands (Fig. 1a).

Since the expression of these genes tended to change with time during the course of development and progression of clinical arthritis, we attempted relative quantification using real-time PCR. The expression levels of TNF-α, IL-1β, IL-6, RANKL, TRAP and cathepsin K increased in CIA rats compared with non-immunized rats, while the expression of RANK and OPG was unchanged and decreased respectively (Fig. 2). TNF-α peaked (2.3 ± 0.3-fold) before other cytokines on day 7 after immunization, then slowly decreased. IL-1β and IL-6 reached their peaks (6.0 ± 2.0-fold and 49.7 ± 18.0-fold respectively) concurrently with occurrence of clinical arthritis on day 15. The mRNA expression levels of RANKL, cathepsin K and TRAP increased with time, and RANKL exhibited high correlation with these two osteoclast
markers (Fig. 3). In contrast, OPG mRNA significantly decreased at the time of onset of clinical arthritis (day 15, 0.45±0.09-fold; day 21, 0.30±0.05-fold). RANK mRNA was expressed constantly, and there were no significant differences in its time-course changes. There were no time-course changes in any gene expression levels in the control group (data not shown).

Gene expression in the bone marrow

To compare the gene expression levels with those in the synovium, femoral bone marrow was collected and the time-course expression of the genes was investigated. RT-PCR detected the specific single bands of the TNF-α, IL-1β, IL-6, RANKL, TRAP and cathepsin K genes, but did not distinctly detect the expression of IL-6, RANKL or OPG (Fig. 1b). Since real-time PCR is a very sensitive method, only the genes detected as clear single bands by RT-PCR were used in the relative quantification (Fig. 4). The mRNA expression levels of IL-1β peaked (2.2±0.6-fold) on day 7 after immunization, and on day 5 and 15 they were significantly high compared with levels in the bone marrow of non-immunized rats. The average expression of cathepsin K and TRAP was higher than in non-immunized rats on day 21 (3.5±0.6-fold and 1.7±0.28

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**Osteoclastogenesis in CIA synovium and bone marrow**

Fig. 2. Time course of mRNA expression in the synovium during collagen-induced arthritis. Relative mRNA expression normalized with an internal control gene (GAPDH) compared with non-immunized rats was determined by real-time PCR (five separate experiments). The expression levels of TNF-α, IL-1β, IL-6, RANKL, TRAP and cathepsin K mRNA increased, while RANK and OPG was unchanged and decreased respectively. N, non-immunized rats; d, days after immunization. Values are mean±s.e.m. *P<0.01 vs non-immunized rats; **P<0.05 vs non-immunized rats. d, days.
respectively), but the difference was statistically significant only for cathepsin K. There were no significant changes in TNF-α or RANK mRNA expression.

There were no time-course changes in any gene expression levels in the control group (data not shown).

**Discussion**

RANKL has recently been identified as a factor responsible for the differentiation and activation of osteoclasts, and the role of RANKL and the roles of its receptor and decoy receptor, RANK and OPG, in bone and cartilage destruction have been attracting attention [4–6]. Inflammatory cytokines such as TNF-α, IL-1β and IL-6 are considered to be involved in the differentiation and activation of osteoclasts in a RANKL-dependent or -independent manner [7–11]. Although there are many reports on the relationship between these various factors and RA, most investigators have performed cross-sectional studies. Considering that cross-sectional studies are not sufficient for the clarification of the dynamics of various factors that interfere with each other in a complex manner during the developmental process of arthritis, we limited the study to RANKL/RANK/OPG and inflammatory cytokines from the aspect of the induction of differentiation.
of osteoclasts in inflammatory arthritis, and attempted to trace time-course changes in these factors in vivo. Time-course investigation requires a superior animal model, so we immunized mature female rats to induce CIA as a postmenopausal female RA model. The CIA model develops arthritis very similarly to RA pathologically, histologically and immunologically [15, 16], and shares many features of the expression of RANKL/RANK/OPG and inflammatory cytokines in human RA [15, 17, 18].

There are two types of quantitative PCR: absolute quantification and relative quantification. Although absolute quantification measures the input copy number of the target gene, relative quantification is desirable for time-course studies because it is superior for comparison with untreated controls [14]. Normalization of target genes with an internal control gene is essential for relative quantification [19]. However, no ideal control gene is available for diverse tissues [20], so we used GAPDH, which is widely accepted as a housekeeping gene [21]. Schmittgen et al. measured mRNA using four methods of two types: endpoint (band densitometry and probe hybridization) and real-time (SYBR Green and TaqMan) PCR methods, and found that the method using SYBR Green was the most effective [22].

We had previously performed histological and immunohistochemical investigations of time-course changes in bone destruction of the knee joint in CIA rats [12]. In the previous study, osteoclast-like cells appeared in the proliferative synovium, and only a few were observed in the epiphyseal bone marrow on day 14 after immunization. In contrast, 21 days after immunization, osteoclast-like cells were observed also in the bone marrow; nevertheless, the synovium and bone marrow had not connected. Therefore, we concluded that osteoclast formation occurred in the synovium and bone marrow separately from a morphological aspect. Our next concern was the dynamics of various cytokines which affect osteoclastogenesis in the synovium and bone marrow during the development of arthritis.

The main feature of RA is abnormal growth of the joint synovium. Proliferative synovium produces large amounts of inflammatory cytokines [23, 24] as well as RANKL [4, 5]. In this study, the mRNA expression of osteoclast markers, TRAP and cathepsin K, increased with time in the synovium. These two osteoclast markers were highly correlated with RANKL mRNA expression, suggesting that synovial RANKL is closely involved in osteoclast formation in vivo. Interestingly, the OPG mRNA expression in the synovium significantly decreased at the same time as the onset of clinical arthritis. The ratio of RANKL mRNA/OPG mRNA in synovial membranes of RA patients was positively proportional to the number of functional osteoclasts in the in vitro study [25]. Our findings show that, in vivo as well as in vitro, up-regulation of RANKL and down-regulation of OPG may lead to osteoclast differentiation and activation.

RANK mRNA expression did not increase in this study. Caulfield et al. observed time-course histological changes of the knee joint of CIA rats. They demonstrated that infiltration of mononuclear cells was seen after day 18–20 [26]. Meanwhile, Fujikawa et al. and Takayanagi et al. assumed that the origin of osteoclasts was synovial membrane A cells, based on culturing the cells in the presence of osteoclast differentiation factor. On the basis of these reports, we speculate that the synovial type A cells which probably expressed RANK fused to osteoclasts in the presence of RANKL. As a result, TRAP and cathepsin K mRNA expression increased. However, since the appearance of additional osteoclast precursors, such as mononuclear cells, requires 18–20 days, RANK mRNA expression may not have significantly changed within 21 days of immunization.

Inflammatory cytokines such as TNF-α, IL-1β and IL-6 produced by the proliferative synovium play central roles in the pathogenesis of RA [23]. In this study, the expression of TNF-α mRNA in the synovium preceded expression of the other cytokines, and this finding was consistent with other reports in which TNF-α was an initiator of arthritis development [17, 29]. The mRNA expressions of IL-1β and IL-6 peaked at the same time as the onset of clinical arthritis, indicating the importance of these cytokines in the development of arthritis. It is generally agreed that inflammatory cytokines contribute to osteoclast formation via the pathway with or without RANKL [7–11]. In the present study, all the inflammatory cytokines and RANKL, as well as osteoclast markers, showed a positive correlation (data not shown), suggesting that these cytokines contribute to the differentiation of osteoclasts. In contrast to previous reports [30], which demonstrated that inflammatory cytokines were stimulators of OPG in vitro, expression of OPG mRNA decreased significantly in spite of up-regulation of inflammatory cytokines. Yano et al. assumed that basic fibroblast growth factor in RA synovial fluid may lead to joint destruction by suppressing the production of OPG [31]. We therefore speculate that any cytokines we had not traced may inhibit the production of OPG.

Hayashida et al. reported that IL-1β and IL-6 levels increased markedly in the bone marrow before the onset of histological synovitis and clinical arthritis in CIA rats [32], and Kamiya et al. reported that bone marrow transplantation into CIA mice inhibited the progression of arthritis [33]. These reports indicate the importance of the bone marrow as the causative lesion of RA. We looked at the bone marrow from the view of osteoclastogenesis, and in a previous study we concluded that osteoclast formation in the bone marrow was independent of the synovium morphologically. In this study, expression of IL-6, RANKL, and OPG in the bone marrow was weak, and the levels of TNF-α, RANK and TRAP expression did not change significantly during the development of arthritis. The expression of IL-1β and cathepsin K changed significantly, but the changes were slight compared with those in the synovium. These findings are different from the previous study, demonstrated by Hayashida et al. [32]. They collected the bone marrow from total femur and tibia, so their data means the whole bone marrow response. On the other hand, we collected the juxta-articular bone marrow to evaluate the local bone marrow response. Therefore, we suggest that this caused the discrepancies between two studies.

In the bone marrow, the expression levels of osteoclast markers tended to increase, although RANKL expression was not detectable. We can draw three inferences. First, osteoclastogenesis in the bone marrow is RANKL-independent. Secondly, osteoclasts in the bone marrow originate in the synovium. Thirdly, synovial cytokines affect osteoclastogenesis in the bone marrow. As for the first inference, it has been demonstrated that several cytokines such as TNF-α and IL-1 accelerate osteoclast formation independently of the RANKL/RANK pathway. However, in arthritic RANKL knockout mice, the degree of destruction in cortical and trabecular bone was dramatically reduced [34]. This finding shows that RANKL is pivotal in osteoclastogenesis in arthritis. As for the second inference, we demonstrated previously that osteoclastogenesis in the synovium and in the bone marrow were morphologically independent; consequently, we thought that the marrow osteoclasts did not originate in the synovium. Hence, we consider the third inference essential. It has not been clarified how synovial cytokines reach the marrow space. Caulfield et al. demonstrated that blood vessels penetrated from the soft tissue (joint space) to the bone marrow [26]. We speculate that these vessels allow the passage of synovial cytokines. Further studies are needed to examine this inference.

Metaphyseal bone marrow, but not epiphyseal bone marrow, was collected in this study because of the anatomical characteristic in rats that the epiphyseal line does not close. Possibly this difference may have brought about slight changes in cytokine mRNA in the bone marrow. This subject requires investigation in the future.

It is clear that RANKL/RANK/OPG is crucial to osteoclastogenesis. Moreover, inflammatory cytokines, which play central roles in the immunological responses of CIA, affect osteoclastogenesis, especially in the early stage of arthritis. It has been shown
that other cytokines, such as IL-7, IL-18 and interferons, affect osteoclastogenesis in human or experimental arthritis [35–37]. As these cytokines have not been studied, it is clear that so far we have comprehended the phenomenon of osteoclastogenesis only in part.

We investigated the time-course expression of genes relevant to osteoclastogenesis in the synovium and bone marrow in mature CIA rats. The cytokine mRNA levels in the synovium changed markedly, and RANKL was highly correlated with osteoclast formation. In contrast, in the bone marrow, lesser changes in mRNA expression were observed. It is possible that cytokines produced in the synovium have an affect on osteoclastogenesis not only in the synovium but in the bone marrow. The results of this study are expected to be of help in the analysis of the pathology of autoimmune rheumatic diseases, including RA.

### Key messages

- It is possible that RANKL up-regulation and OPG down-regulation in the synovium lead to osteoclast formation.
- Synovial cytokines may affect osteoclastogenesis not only in the synovium but also in the bone marrow.

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The authors have declared no conflicts of interest.

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