Rheumatoid synovial endothelial cells produce macrophage colony-stimulating factor leading to osteoclastogenesis in rheumatoid arthritis

K. Nakano, Y. Okada, K. Saito, R. Tanikawa, N. Sawamukai, Y. Sasaguri1, T. Kohro2, Y. Wada3, T. Kodama4 and Y. Tanaka

Objectives. Periarticular osteoporosis and joint destruction are major complications in rheumatoid arthritis (RA), caused by osteoclast-mediated bone resorption. However, the mechanisms of monocyte/osteoclast maturation and role of RA endothelial cells (RAECs) in the control of osteoclastogenesis remain unclear. The present study was designed to determine the most important factors that influence monocyte accumulation and osteoclast formation among the many factors produced by RAEC.

Methods. We analysed the expression profiles of various genes in human endothelial cells from various organs (RA synovium, umbilical vein, skin, liver sinusoid, renal glomerulus and brain) using oligonucleotide microarrays. Specifically, up-regulated gene in RAECs was assessed by real-time quantitative polymerase chain reaction, enzyme-linked immunosorbent assay and immunostaining of RA synovia. Migration of monocytes was assessed by the chemotactic chamber EZ-TAXIScan™. Tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cell (MNC) formation was observed by microscopy.

Results. Among many epithelial-expressed factors, macrophage colony-stimulating factor (M-CSF) gene was abundantly expressed specifically in RAECs. Genes of fibroblast growth factor-2, interleukin-6 and osteoprotegerin were also overexpressed in RAECs. Migration of monocytes and osteoclast formation in co-cultures promoted by culture supernatants of RAECs were inhibited by M-CSF neutralizing antibody.

Conclusions. M-CSF produced by RAECs is involved in osteoclastogenesis from monocytes, migration and TRAP-positive MNC formation.

Key words: Rheumatoid arthritis, Osteoclasts, Pathogenesis, Endothelial cell, Macrophage colony-stimulating factor, Oligonucleotide microarray.

Rheumatoid arthritis (RA) is an autoimmune disease characterized by progressive joint destruction resulting from chronic inflammation of multiple synovial joints. Bone and cartilage destruction is one of the most serious problems in RA. Cells at the site of bone erosion in RA display phenotypic features of osteoclasts [1]. Osteoclasts, which are responsible for bone resorption in bone metabolism, are multinucleated cells (MNCs) formed by the fusion of circulating haematopoietic precursor cells of the monocyte/macrophage lineage [2]. Osteoclast precursors express the receptor activator for nuclear factor-κ B (RANK) and differentiate into fully functional osteoclasts in the presence of macrophage colony-stimulating factor (M-CSF) and RANK ligand (RANKL) [3–5].

Synovial lining cells mainly consist of synovial macrophages, which are derived from monocyte precursors in the bone marrow, and synovial fibroblasts [6]. Macrophages isolated from RA synovial tissues differentiate into osteoclasts in the presence of M-CSF and RANKL [7]. This osteoclastogenesis can be supported by synovial fibroblasts through the expression of RANKL [8]. Furthermore, RA synovial cells release proinflammatory prostaglandins and cytokines, such as interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α) and IL-6, all of which are known to promote osteoclastic bone resorption [9–11]. Taken together, these studies suggest that RA synovial tissue environment regulates osteoclastogenesis resulting in joint destruction. However, it is not clear why many monocytes/macrophage lineage cells, containing osteoclast precursors, are recruited in only RA synovial tissue among many chronic inflammatory diseases and result in bone destruction.

Blood vessels in various tissues have specialized functions, and endothelial cells (ECs) are therefore equipped with distinct properties—there might even be as many different EC types as there are organs in the body [12]. In addition to morphological heterogeneity, ECs also exhibit functional heterogeneity, including roles in the control of vasoconstriction and vasodilatation, blood coagulation and anti-coagulation, fibrinolysis, leucocyte trafficking and diapedesis, acute inflammation and wound healing, atherogenesis, antigen presentation and catabolism of lipoproteins [13].

The persistence of an inflammatory infiltrate within tissues requires an imbalance between those factors that enhance cellularity (recruitment and proliferation) and those that decrease...
cellularity (emigration and apoptosis). It is now well-established that the differential expression of cytokines, chemokines, chemokine receptors and adhesion molecules play an important role in determining tissue-specific trafficking and the positioning of leucocyte subsets within both normal and inflamed tissues [14–16].

Angiogenesis, the formation of new blood vessels, is one of the most striking features in RA. A number of studies have shown that persistent angiogenesis is a crucial support to continuous proliferation of the synovium, through delivery of nutrients and recruitment of inflammatory leucocytes into the synovium [17]. Therefore, to identify the specific molecules expressed on RA endothelial cells (RAECs) is very important to clarify the pathogenesis of RA, but the mechanisms of osteoclast maturation of precursor monocytes and efficient control of osteoclastogenesis by RAECs remain unclear. In order to clarify these mechanisms, the present study was designed to determine the influential factors on accumulation of monocytes and formation of osteoclasts among many factors produced by RAECs.

Materials and methods

Cell cultures and treatments

Synovial tissues were obtained from patients with active RA, diagnosed according to the criteria of the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) [18], who were treated by joint replacement surgery or synovectomy. This study was approved by the Human Subjects Research Committee of our university. Informed consent was obtained from all subjects enrolled in the study. Synovial membrane samples were perfused with 10% paraformaldehyde, and paraffin sections (3 μm thick) were prepared for immunochemical studies. Samples were also dissected under sterile conditions in phosphate buffered saline (PBS), and immediately prepared for culture of synovial endothelial cells. Briefly, the tissue sample was minced into small pieces and digested with collagenase (Sigma Aldrich) for 4 h. After duplicate washing in PBS, the cells were analysed with a FACSCalibur (Dil-Ac-LDL; Sigma Aldrich) for 4 h. After washing and staining with 5.0 μg/ml streptavidin/phycoerythrin (Molecular Probes, Eugene, OR, USA) and 2.0 mg/ml acetylated BSA (Sigma) using a FluidicsStation (Affymetrix), the GeneChip was scanned with a Hewlett-Packard GeneArray scanner (Affymetrix). As the RNA sample of RAECs, ECs obtained from an active RA patient were used.

Quantitative analysis

The intensity for each feature of the array was captured with GeneChip software (Affymetrix), and a single raw expression level for each gene was derived from the 20 probe pairs representing each gene by means of a trimmed mean algorithm. A threshold of 20 units was assigned to any gene with a calculated expression level <20, because discrimination of expression below this level cannot be performed with confidence. The expression level of each gene and the fold change between the two experiments was calculated using GeneChip software (Affymetrix). The average difference of each experiment was normalized to 100.

Real-time PCR analysis for M-CSF mRNA expression

Gene expression of the components of the M-CSF was assessed by reverse transcription followed by real-time polymerase chain reaction (PCR; TaqMan®; PE Applied Biosystems, Foster City, CA, USA) according to published methods. In brief, total RNAs from these cells were isolated with ISOGEN and were added to the master mixture. To detect the amount of the M-CSF mRNA RT-PCR amplification, target (M-CSF) and control [glyceraldehyde 3-phosphate dehydrogenase (GAPDH)] hybridization probes were mixed with the target and control PCR primers, respectively. This mixture was transferred to a set of thermocycler tubes and transcribed at 42°C for 30 min, followed by 40 cycles of amplification at 95°C for 15 s and 60°C for 1.5 min, and analysed using an ABI PRISM 7000 sequence detector (Applied Biosystems). M-CSF mRNA expression was estimated from the ratio of fluorescence intensity to GAPDH.

Measurement of M-CSF levels in culture supernatants by enzyme-linked immunosorbent assay (ELISA)

The ECs were cultured for 48 h in DMEM containing 10% FCS in 6-well culture plates at 37°C in 5% CO2 and cell-free supernatants were harvested. The cultured ECs were spun down at 800 rpm for 5 min at 4°C, and the supernatant was collected and immediately frozen at -80°C until measurement by M-CSF ELISA kit (R&D systems, Minneapolis, MN, USA).
**Immunohistochemistry**

The 3 μm-thick sections prepared from RA synovial tissues were incubated with goat anti-human M-CSF antibody (dilution 1:25, R&D) and then incubated with secondary antibody (Envision system, Dako) [22].

**Horizontal chemotaxis assay**

The EZ-TAXIScan™ (Effector Cell Institute, Tokyo) was used to detect real-time horizontal chemotaxis of monocytes. The EZ-TAXIScan consists of an etched silicon substrate and a flat glass plate, both of which form two compartments with a 5 μm deep microchannel [23]. In some experiments, Thermanox coverslips (Nalogen Nunc International, Naperville, IL, USA) were placed onto the glass plates. Purified peripheral blood CD14+ monocytes from healthy donors (1 μl of 10⁶ cells/ml) were placed into the single hole with which the device is held together with a stainless steel holder, and 1 μl of cell-free supernatants from the culture of RAECs with or without 1 ng/ml of neutralizing anti-M-CSF antibody (R&D) were placed into the contra-hole. A charge-coupled device (CCD) camera was used to record the migration of monocytes toward the supernatants of RAECs with or without anti-M-CSF antibody on the microchannel. Moving cells in a fixed gate were counted using TAXIScan Analyzer (Effector Cell Institute).

**Transendothelial migration and subsequent osteoclast formation in co-culture system of synovial endothelial cells and monocytes**

Transendothelial migration of monocytes was assessed using 5 μm pore 24-well microchemotaxis chambers (Transwell; Costar, Cambridge, MA, USA). The inner wells were seeded with RAECs (1 x 10⁶ cells/well) in 100 μl EC medium. As a control, HUVECs were seeded. The EC monolayers were cultured for 24 h at 37°C and rinsed with assay medium before performing the assays. The inner wells were placed in microchemotaxis chambers, with 500 μl of α-MEM containing 10% FCS and 10⁻² M 1,25(OH)₂D₃ in the presence or absence of 10 ng/ml of anti-M-CSF antibody (R&D). Purified peripheral blood CD14+ monocytes from healthy donors (5 x 10⁶ cells/well) were added to the EC monolayers, which were incubated at 37°C in 5% CO₂. After 24 h, cells that migrated into the lower wells were counted by light microscopy. Furthermore, 3 days later, soluble RANKL (sRANKL, PeproTech, London, UK) was added in the culture medium, all dishes were stained for tartrate-resistant acid phosphatase (TRAP, Sigma) after 9 days of co-culture as described previously [24]. The number of TRAP-positive MNCs that contained more than three nuclei were identified as osteoclasts and counted by light microscopy.

**Statistical analysis**

Data are expressed as mean ± s.d. Comparisons between two groups were performed with Student’s two-tailed t-test, and more than two groups were compared by analysis of variance (ANOVA). P-values <0.05 were considered statistically significant.

**Results**

**Oligonucleotide microarray analysis of endothelial cells from multiple organs**

We compared the gene expression levels of various cytokines, containing colony-stimulating factors and growth factors, in human ECs from various organs, including RA synovial tissue (RAECs), umbilical vein (HUVECs), skin (HMECs), liver sinusoid (LSECs), renal glomerulus (GECs) and brain (BMECs) using oligonucleotide microarrays (Table 1). Among the cytokines, the average difference values of M-CSF gene and fibroblast growth factor-2 (FGF-2) gene were >100 in RAECs, and <100 in

<table>
<thead>
<tr>
<th>GenBank accession #</th>
<th>Gene</th>
<th>RAECs</th>
<th>HUVECs</th>
<th>GECs</th>
<th>HMECs</th>
<th>LSECs</th>
<th>BMECs</th>
</tr>
</thead>
<tbody>
<tr>
<td>M28983</td>
<td>IL-1α</td>
<td>7</td>
<td>16</td>
<td>28</td>
<td>25</td>
<td>8</td>
<td>28</td>
</tr>
<tr>
<td>M22005</td>
<td>IL-2</td>
<td>–25</td>
<td>–16</td>
<td>–52</td>
<td>–25</td>
<td>–11</td>
<td>2</td>
</tr>
<tr>
<td>M20137</td>
<td>IL-3</td>
<td>15</td>
<td>10</td>
<td>8</td>
<td>1</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>X04688</td>
<td>IL-5</td>
<td>–2</td>
<td>0</td>
<td>17</td>
<td>4</td>
<td>3</td>
<td>–2</td>
</tr>
<tr>
<td>X04602</td>
<td>IL-6</td>
<td>120</td>
<td>35</td>
<td>4</td>
<td>1</td>
<td>870</td>
<td>13</td>
</tr>
<tr>
<td>J04156</td>
<td>IL-7</td>
<td>–1</td>
<td>–2</td>
<td>–4</td>
<td>–4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>M28130</td>
<td>IL-8</td>
<td>12</td>
<td>21</td>
<td>–90</td>
<td>–17</td>
<td>193</td>
<td>2</td>
</tr>
<tr>
<td>U16720</td>
<td>IL-10</td>
<td>39</td>
<td>25</td>
<td>227</td>
<td>41</td>
<td>45</td>
<td>–1</td>
</tr>
<tr>
<td>U14407</td>
<td>IL-15</td>
<td>3</td>
<td>2</td>
<td>–13</td>
<td>2</td>
<td>–2</td>
<td>3</td>
</tr>
<tr>
<td>X02910</td>
<td>TNF-α</td>
<td>–7</td>
<td>–10</td>
<td>–58</td>
<td>–10</td>
<td>–17</td>
<td>–9</td>
</tr>
<tr>
<td>J00219</td>
<td>IFN-γ</td>
<td>–2</td>
<td>–13</td>
<td>8</td>
<td>–3</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>U63717</td>
<td>RANKL</td>
<td>1</td>
<td>47</td>
<td>708</td>
<td>9</td>
<td>1</td>
<td>80</td>
</tr>
<tr>
<td>U94332</td>
<td>OPG</td>
<td>111</td>
<td>8</td>
<td>0</td>
<td>7</td>
<td>694</td>
<td>19</td>
</tr>
</tbody>
</table>

**Table 1. Cytokine genes expressed in various endothelial cell types**

RAECs, rheumatoid arthritis endothelial cells; HUVECs, human umbilical vein-derived endothelial cells; GECs, renal glomerular endothelial cells; HMECs, human microvascular endothelial cells isolated from skin; LSECs, liver sinusoid endothelial cells; BMECs, brain microvascular endothelial cells; IL, interleukin; TNF-α, tumour necrosis factor-α; IFN-γ, interferon-γ; RANKL, receptor activator for nuclear factor κB ligand; OPG, osteoprotegerin; GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; FGF-2, fibroblast growth factor-2; TGF-β, transforming growth factor-β.
other ECs. Notably, the average difference value of M-CSF gene exceeded ~400, and thus was regarded as abundantly expressed especially in RAECs. M-CSF, as well as RANKL, is essential for osteoclastogenesis in vitro [25]. This finding suggested that differentiated ECs in RA synovial tissue play an important role in the recruitment of monocyte/macrophage lineage cells and formation of osteoclasts by tissue-specific expression of M-CSF.

Complete data of the GeneChip results including the average difference of ECs can be downloaded from http://www.med.rcast.u-tokyo.ac.jp/data/NakanoTanaka/EC_chipdata.tab (last accessed on 16 October 2006).

**M-CSF production by RA synovial endothelial cells**

In order to validate the GeneChip results, real-time PCR assay was carried out to detect M-CSF mRNA expression (Fig. 1A). RAEC samples were obtained from three RA patients. The expression of M-CSF mRNA in RAECs was significantly up-regulated although the level of expression could be hardly detected in ECs from other tissues. The culture supernatants from ECs were assayed for M-CSF protein production using sensitive ELISA technique. As shown in Fig. 1B, the production of M-CSF was also high in RAEC samples while it was low or undetected in the other tissue samples. These data confirm the GeneChip results showing that RAECs are unique in their remarkable production of M-CSF among ECs from various other organs.

Furthermore, immunohistochemical studies demonstrated that microvascular RAECs were positive for M-CSF (Fig. 1C). This finding suggests a critical role for RAECs in promoting chemotaxis of monocytes, osteoclast formation and bone resorption in RA synovial tissues.

**Real-time records of monocyte migration by RA endothelial cell-produced M-CSF**

The chemotactic effects of M-CSF on monocytes in vitro were examined using the EZ-TAXIscan™, which can detect real-time horizontal migration of cells. Freshly isolated peripheral blood CD14+ monocytes from healthy donors (1 µl of 10⁶ cells/ml) were placed into the one hole and 1 µl of cell-free supernatants from the culture of RAECs with or without anti-M-CSF antibody (1 ng/ml) was placed into the other contra-hole, and then the migration of monocytes was recorded with a CCD camera (Fig. 2A). In the chamber lacking anti-M-CSF antibody, many monocytes started to migrate, with wide spreading lamellipodia, towards the supernatants of RAECs. In contrast, directional migration was significantly decreased in the chamber containing anti-M-CSF antibody (Fig. 2B). These results indicated that M-CSF produced by RAECs markedly induced the lamellipod formation and subsequent horizontal migration of monocytes.

**Effect of M-CSF on transendothelial migration and osteoclast formation in co-cultures of monocytes and RA endothelial cells**

To evaluate the effect of M-CSF produced by RAECs on osteoclastogenesis, we used a co-culture system of ECs and monocytes. As shown in Fig. 3A, RAEC monolayers induced significantly higher rate of transendothelial migration of monocytes than HUVEC monolayers. After a 9-day culture with sufficient recombinant RANKL, we were able to observe marked TRAP-positive MNC formation in co-cultures of transendothelial migrated cells and RAEC, whereas TRAP-positive MNCs were

---

**FIG. 1. Specific expression of M-CSF on RA endothelial cells (RAECs).** (A) M-CSF mRNA expression levels on ECs from each organ were examined by real-time PCR and results were normalized to GAPDH. Data are mean ± s.d. of three independent experiments, performed in triplicate. (B) M-CSF production levels in culture supernatants of ECs from each organ measured by ELISA. Data are mean ± s.d. of three independent experiments, performed in triplicate. (C) Immunostaining for M-CSF in RA synovial tissue (brown staining, magnification: 100×).
not induced in co-cultures of transendothelial migrated cells and HUVECs. Furthermore, such formation of TRAP-positive MNCs by co-cultures of transendothelial migrated cells and RAECs was strongly inhibited by the addition of neutralizing anti-M-CSF antibody, suggesting that RAECs are the source supply and play a pivotal role in osteoclastogenesis of RA synovial tissue (Fig. 3B and C).

**Discussion**

Tissue-specific accumulation of leucocyte subsets allows functional compartmentalization within the immune system [26].

ECs of various organs produce organ-specific mediators such as cytokines and chemokines, express organ-specific cellular adhesion molecules, and play a central role in tissue-specific accumulation of leucocyte subsets [27]. Therefore, large-scale differential expression analysis of ECs from various organs could clarify the pathogenesis of tissue-specific autoimmune disease such as RA and help design new therapeutic strategies for such conditions.

In the present study, gene expression profiling of ECs from various organs revealed that the ECs that differentiate in the RA synovium abundantly and specifically expressed M-CSF gene, an essential factor for osteoclastogenesis. Furthermore, we showed that M-CSF, produced by RAECs, contributed to chemotaxis and transendothelial migration of monocytes and osteoclast formation. These results suggest that RAECs directed the RA synovium towards osteoclast factory. Although it is necessary to compare RAECs with synovial ECs from normal volunteers or patients with osteoarthritis (OA), or ECs from another chronic inflammatory tissue, we could not obtain sufficient samples from such individuals because of poor vasculature. Based on the present findings and the results of previous study showing deficiency of synovial macrophages and bone osteoclasts in osteopetrosis (op) mutant mice, which is due to the lack of functional activity of M-CSF [28], it is conceivable that normal synovial ECs express M-CSF constitutively and recruit monocyte/macrophage lineage cells. There are still higher numbers of ECs in RA compared with normal and OA tissues, and this possibly implicates the high expression of M-CSF in the pathogenesis of RA.

Although various inflammatory cytokines seem to up-regulate M-CSF expression in RAECs, the exact regulatory mechanisms that control M-CSF expression are not clear at present. In the current study, IL-6 was found to be predominantly up-regulated in RAECs. Because, IL-6 is known to inhibit the differentiation of dendritic cells from CD34⁺ progenitors [29] and to skew monocyte differentiation towards macrophages by increasing functional M-CSF receptors on activated monocytes [30, 31], further studies are needed to investigate the interaction between IL-6 and M-CSF in the RA synovium.
Although RANKL gene expression was low, the expression of osteoprotegerin (OPG), a soluble decoy for RANKL receptor, was >100. In RA, although RANKL was not expressed on the synovial lining layers of the synovial membranes, including ECs, it was strongly expressed in T cell-rich areas of the synovial membrane and the expression increased by inflammatory cytokines, such as IL-1β and TNF-α [32]. In contrast, OPG was constitutively expressed on ECs in the synovial tissue, and its expression level correlated inversely with RA disease activity [33]. A recent study reported [34] that anti-TNF therapy reduced RANKL/OPG ratio, suggesting effective reduction of osteolytic lesions. There is also evidence that RANKL/OPG ratio is increased in severe inflammatory lesions and the ratio is influenced by inflammatory cytokines. Although the RANKL/OPG ratio was low in our study, this finding may be related to reduce levels of inflammatory cytokines in the in vitro culture system.

Angiogenesis, the formation of new blood vessels, is one of the most striking features in RA. FGF-2 seems to be the principal mediator of angiogenesis and can act cooperatively with vascular endothelial cell growth factor (VEGF) [35]. FGF-2 and VEGF are produced by both RA synovial fibroblasts (RAFLS) and RAECs that have been exposed to hypoxia, IL-1 or TNF-α [36, 37] and are involved in vascular proliferation. On the other hand, FGF-2 can induce RANKL expression on RAFLS and osteoclast maturation [38], and VEGF can substitute M-CSF in osteoclast differentiation [39]. These reports suggest that those molecules specifically expressed on RAECs contribute to both the maintenance of pannus and osteoclastogenesis. Furthermore, M-CSF as well as granulocyte colony-stimulating factor (G-CSF) can also mobilize endothelial progenitor cells from the bone marrow into the peripheral blood through VEGF, resulting in augmentation of neovascularization and blood flow in the ischaemia-induced limbs in mice [40]. Therefore, it is suggested that M-CSF itself is directly involved in pannus formation.

Treatment of RA patients with anti-TNF-α leads to marked clinical improvement and inhibit synovial hypervascularity [41, 42]. It is not certain if this is due to a direct effect of TNF-α, which has angiogenic activity, or a secondary action on the production of other angiogenesis factors like VEGF and FGF-2. In either case, specific targeting of pathological endothelial function could prevent joint destruction and benefit patients with inflammatory rheumatic condition.

In conclusion, M-CSF produced by RAECs is involved in sequential events of osteoclastogenesis from monocytes; migration and osteoclast formation. Specific targeting of pathological endothelial function might provide a novel strategy for the prevention of bone destruction and osteoporosis in RA.

The authors have declared no conflicts of interest.

References

Acknowledgements
This work was supported in part by a Research Grant-In-Aid for Scientific Research by the Ministry of Health, Labour and Welfare of Japan, the Ministry of Education, Culture, Sports, Science and Technology of Japan and University of Occupational and Environmental Health, Japan. We thank Akashi Izumi and Tomoko Adachi for the excellent technical assistance.

**Rheumatology**

<table>
<thead>
<tr>
<th>Key messages</th>
</tr>
</thead>
<tbody>
<tr>
<td>The gene expression profiles of ECs from various organs are very informative to clarify the pathogenesis of tissue-specific autoimmune diseases.</td>
</tr>
<tr>
<td>M-CSF produced by RA endothelium is involved in osteoclastogenesis from monocytes.</td>
</tr>
</tbody>
</table>


