Enhancement of lymphocyte migration and cytokine production by ephrinB1 system in rheumatoid arthritis

Takuya Kitamura, Yukihito Kabuyaama, Akihisa Kamataki, Miwako K. Homma, Hideo Kobayashi, Shigeo Aota, Shin-ichi Kikuchi, and Yoshimi Homma

Department of Biomolecular Science and Orthopedics, Fukushima Medical University School of Medicine, Fukushima, Japan

Submitted 22 July 2007; accepted in final form 15 October 2007

RHEUMATOID ARTHRITIS (RA) is a systemic, chronic inflammatory disease characterized by abnormal immune responses and synovial proliferation that ultimately leads to the progressive destruction of tendons, cartilage, and bone (13). Although the etiology of the early events in RA remains undefined, a number of hypotheses that are not mutually exclusive have been proposed. One of these hypotheses suggests that RA is an antigen-driven, T cell-dependent disease and that the inflammatory events are initiated by T cells recognizing antigens in synovial tissues (7, 11). An aberration of peripheral tolerance mechanisms, a dysregulation of lymphoproliferation, and an anomaly in T cell homeostasis are involved in the disease process. Alternatively, it has been revealed that the proliferation of synovial-lining cells and their invasive growth are also involved in the pathogenesis of RA. Much evidence has been accumulated to indicate that overexpression of cytokines including EGF and fibroblast growth factor plays an important role in the proliferation and activation of synovial cells (9, 14). We have previously reported that ErbB2, an intrinsic receptor for EGF, is activated in the rheumatoid synovium and synovial cells in primary cultures from RA patients. We further demonstrated that the growth of synovial cells is inhibited by a tyrosine kinase inhibitor and anti-ErbB2 monoclonal antibody (18, 26). These results suggested abnormal immune responses in concert with activation of synovial cells might account for the process of RA.

Erythropoietin-producing human hepatocellular carcinoma (Eph) receptors, consisting of A and B types, are the largest family of transmembrane receptor tyrosine kinases (19, 23). Eph receptor-interacting proteins (ephrins) are the ligands to Eph receptors. The ephrin ligands also have two types: the glycosylphosphatidylinositol (GPI)-anchored A type and transmembrane B type. The representative functions of the ephrin/Eph signaling pathway are the development of immune networks including T cell development and migration (26). Recent reports have suggested that ephrinB1 signaling is essential in T cell-T cell interactions during T cell activation, whereas ephrinB1 provokes T cell migration (1, 27). In addition, it has been reported that the ephrin/Eph receptor system plays important signaling roles in inflammation processes and the pathogenesis of RA (16, 25). Based on these findings, we attempted to examine ephrinB molecules in T cells and synovial cells derived from RA in this study. Here, we demonstrate that the expression level of ephrinB1 is significantly higher in synovial fibroblasts and CD3-positive exudate lymphocytes in synovial tissues derived from patients with RA compared with those in osteoarthritis (OA). Protein and mRNA levels of ephrinB1 were also higher in peripheral blood lymphocytes (PBLs) prepared from patients with RA than those from normal controls. Similar results were obtained from an animal model of human RA, collagen antibody-induced arthritis mice. Moreover, a recombinant ephrinB1/Fc fusion protein stimulated normal PBLs to exhibit enhanced migration and production of TNF-α. EphrinB1/Fc also activated synovial cells established from patients with RA to produce IL-6. Tyrosine phosphorylation of EphB1 was induced in these cells by ephrinB1/Fc. The CpG islands in the 5′ upstream regulatory region of the ephrinB1 gene were hypomethylated in RA patients compared with those of normal donors. These results suggest that ephrinB1 and EphB1 receptors play an important role in the inflammatory states of RA, especially by affecting the population and function of T cells. Inhibition of the ephrinB/EphB system might be a novel target for the treatment of RA.

Address for reprint requests and other correspondence: Y. Homma, Dept. of Biomolecular Science, Fukushima Medical Univ. School of Medicine, Fukushima 960-1295, Japan (e-mail: yoshihom@fmu.ac.jp).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

http://www.ajpcell.org 0363-6143/08 $8.00 Copyright © 2008 the American Physiological Society

C189
patients fulfilled the American Rheumatism Association 1987 revised criteria for the classification of RA (2) and classified into functional classes II or III (28). RA patients underwent surgery during remission. Synovium samples were obtained from three patients with OA (3 women, mean age: 70.7 yr, range: 67–73 yr) at the time of knee arthroplasty, and blood samples were from eight healthy volunteers (2 men and 6 women, mean age: 50.8 yr, range: 40–79 yr).

**Histological analysis.** Synovial tissues were fixed with 4% paraformaldehyde in PBS for 24 h at 4°C. After being blocked with 3% normal goat serum, 6-μm-thick sections were incubated with rabbit anti-ephrinB1 (Santa Cruz Biotechnology, Santa Cruz, CA) overnight in a moist chamber at 4°C. After being rinsed with PBS, sections were incubated with biotin-labeled goat anti-rabbit IgG followed by avidin-biotin peroxidase complex. Positive signals were visualized by an enhanced chemiluminescence system (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The intensity of each band was quantified by counting five random fields. The numbers of cells that had passed through the membrane into the lower chamber were quantified by counting five random fields.

**Bioassays for ephrinB1.** For the preparation of the ephrinB1/Fc fusion protein, the extracellular domain of ephrinB1 was subcloned into a human immunoglobulin Fc fusion protein expression vector (kindly provided by Drs. Takashi Saito and Arata Takeuchi) and transfected into COS7 cells. The protein was purified from the culture supernatant using protein A-agarose (Upstate). PBL migration was determined in 24-well plates with 5-μm-pore size cell culture inserts (Corning, Corning, NY). PBLs from normal donors suspended in RPMI-1640 at 2.5 × 10⁵ cells/ml were treated with various amounts of ephrinB1/Fc or control Fc fragment for 1 h, and 100-μl aliquots of cell suspension were added to the upper inserts; 600-μl aliquots of medium containing stromal cell-derived factor (SDF)-1α, a typical chemotactic factor for lymphocytes, were added to the lower chambers. After an incubation for 3 h, the numbers of cells that had passed through the membrane into the lower chamber were quantified by counting five random fields.

**Bisulfite sequencing analysis.** Bisulfite modification of genomic DNA was performed as previously described (24). Briefly, 5 μg genomic DNA suspended in 50 μl water was incubated for 10 min at 95°C and then for 30 min at 37°C with 1.5 μl of 10 M NaOH. The solution was further incubated overnight at 55°C after the addition of 310 μl of 5 M sodium bisulfite, 2.5 μl of 0.1 M hydroquinone, and 136 μl water in a final volume of 500 μl. After modification, DNA was purified using a Wizard clean-up kit (Promega, Madison, WI) and desulfonated with 0.3 M NaOH for 15 min at 37°C. Bisulfite-treated DNA was amplified using the following primers: human ephrinB1 sense (−205 to −181), 5'-GGATTGAGAGGGATTTAATTTTAAT-3'; human ephrinB1 antisense (−25 to −48), 5'-CAAAAACCAACCTACTCCTTAC-3'; mouse ephrinB1 sense (−332 to −308) 5'-GGTTGTGTTTTGTTAGGGTAGGTT-3'; and mouse ephrinB1 antisense (−48 to −69), 5'-ACAAACAAACCCTTTCTCTCCTT-3'. PCR amplification was performed for 35 cycles of 30 s at 95°C, 1 min at 50°C, and 3 min at 72°C, with a final extension at 72°C for 10 min. PCR products were cloned into the pGEM-T-easy vector (Promega), and at least eight clones for each independent sample were sequenced.

**Luciferase assay.** The luciferase reporter plasmid containing the human ephrinB1 promoter sequence from −1000 to +1 relative to the start site of transcription was generated using pGL3-Basic (Promega). COS7 cells (5 × 10⁴) plated on 24-well plates were transfected with 0.4 μg of reporter construct and 5 μg of pRL-TK vector (Promega) as an internal control using Polyfect Transfection Reagent (QLIAGEN, Hilden, Germany). After an incubation for 48 h, relative luciferase activities were determined by a dual-luciferase reporter assay system (Promega). For the preparation of methylated and mock methylated vector, reporter vector (1.2 μg) was incubated with or without 4 units of SsI methylase (New England Biolabs, Ipswich, MA) and 160 μM S-adenosylmethionine for 2 h at 37°C, followed by an incubation for 20 min at 65°C to inactivate the methylase.
RESULTS

High expression of ephrinB1 in tissues and PBLs from RA patients. We examined the expression of ephrinB1 in RA and OA synovial tissues by immunohistochemistry. In the RA synovium, ephrinB1-positive mononuclear cells were observed in the sublining layer. Mononuclear cells that formed lymphoid follicles did not react with the anti-ephrinB1 antibody. In the OA synovium, some ephrinB1-positive cells were observed, but their numbers and expression levels in the OA synovium were quite low compared with those in RA tissues (Fig. 1A). To characterize ephrinB1-positive mononuclear cells, double immunofluorohistochemistry using antibodies against ephrinB1 and CD3 was carried out. The results shown in Fig. 1B clearly demonstrate that ephrinB1 and CD3 coexpress on mononuclear cells in the RA synovium, indicating an aberrant accumulation of ephrinB1-positive T cells in sublining layers of the RA synovium.

We examined the expression of ephrinB1 in PBLs of five RA patients and three age-matched normal controls. Protein levels of ephrinB1 in PBLs derived from RA patients were relatively high compared with those of controls (Fig. 2A); the mean level was 2.3 times more in RA than in control subjects. Enhanced mRNA levels were also observed in RA; the mean level of ephrinB1 mRNA was 1.4 times higher in RA patients than in healthy donors (Fig. 2B). Similar results were obtained from an animal model of human RA, CAIA mice, which were generated by injecting mice with a mixture of four kinds of anti-collagen type II monoclonal antibodies and LPS. The mean level of ephrinB1 protein in PBLs derived from four CAIA mice was 2.5 times higher than that of PBLs from three control mice (Fig. 2C).

Activation of lymphocytes and synovial cells by ephrinB1. Recent studies have demonstrated that the ephrin/Eph signaling system controls lymphocyte migration (1, 31). Based on this information, we examined the effect of ephrinB1 on lymphocyte migration using PBLs derived from healthy donors and a recombinant ephrinB1/Fc fusion protein. When PBLs treated with various amounts of ephrinB1/Fc were in the upper cham-

---

Fig. 1. Immunohistochemical detection of ephrinB1 expression in synovial tissues. A: paraffin-embedded synovium sections derived from patients with rheumatoid arthritis (RA; n = 5) and osteoarthritis (OA; n = 3) were examined by staining with an anti-ephrinB1 antibody. Representative results are shown. Bar = 50 μm. B: sections derived from the same samples were double stained with rabbit anti-ephrinB1 and mouse anti-CD3 antibodies, followed by an incubation with Alexa fluor 488-labeled anti-rabbit IgG or Alexa fluor 555-labeled anti-mouse IgG. Bars = 100 μm for RA sections or 50 μm for OA sections.
ber, ephrinB1 activated lymphocytes in a dose-dependent manner to respond to SDF-1α added to the lower chamber, whereas control Fc had no effect on the migration of PBLs (Fig. 3A). EphrinB1 itself possessed no chemotactic activity against PBLs, because PBLs in the upper chamber did not respond to ephrinB1/Fc added to the lower chamber. This activity of ephrinB1 was attenuated by genistein, a selective inhibitor of protein tyrosine kinases. It has been reported that EphB1 is a receptor of ephrinB1, so that tyrosine phosphorylation of EphB1 was analyzed using normal PBLs. As shown in Fig. 3C, tyrosine phosphorylation was enhanced by ephrinB1/Fc, and genistein inhibited this phosphorylation. In addition, we also detected a 60-kDa band of EphB1, which was produced upon stimulation with ephrinB1. These results are consistent with recent findings (6) suggesting that the ephrinB1/EphB1 signaling system might be involved in the stimulation of the lymphocytes.

We next examined the effect of ephrinB1 on the production of cytokines using PBLs derived from normal donors. As shown in Fig. 3B, ephrinB1/Fc stimulated PBLs to produce TNF-α in a dose-dependent manner. Production levels in ephrinB1/Fc-stimulated PBLs were significantly higher than those in Fc-treated PBLs. The stimulation of the TNF-α production was suppressed by genistein. A moderate stimulation was observed in PBLs treated with Fc at high concentrations, although the underlying mechanism was unclear. On the other hand, no stimulation in the production of IL-4 was observed by ephrinB1/Fc at any dose examined (data not shown). It is well known that the activation of T helper cell type 1 (Th1), rather than T helper cell type 2 (Th2), is predominant in patients with RA. Since TNF-α is typical of Th1-type T cells, and IL-4 of Th2-type T cells, the ephrinB1/EphB1 system should play an important role in the activation of Th1-type T cells.

We also investigated the effect of ephrinB1 on the production of cytokines in synovial cells. As shown in Fig. 4A, ephrinB1/Fc, but not Fc, stimulated the production of IL-6 in synovial cells derived from RA patients in a dose-dependent manner. The production of IL-6 was significantly suppressed by genistein, suggesting an important role of receptor tyrosine kinase EphB1 for the production of IL-6. Indeed, EphB1 of synovial cells was phosphorylated at tyrosine residues by ephrinB1/Fc, and genistein inhibited the phosphorylation of EphB1 (Fig. 4B). These results suggest that ephrinB1 expressed on the T cell surface might be involved in the activation of synovial cells via EphB1 stimulation, which per se induces an expansion of inflammation in RA.

EphrinB1 gene is differentially methylated in RA. To understand genetic and epigenetic conditions underlying the high expression of ephrinB1 in RA, we analyzed the methylation status of the 5′ upstream region of the ephrinB1 gene in genome samples derived from seven patients with RA and five healthy donors. Although no disease-associated single-nucleotide polymorphism was detected in the 5′ upstream region of the ephrinB1 gene, a significant difference in methylation...
status of this region was detected between RA and control. There are 11 CpG sites between positions −179 and −21 in the promoter region of the ephrinB1 gene (Fig. 5A), and the methylation states of each clone are shown in Fig. 5B. The results indicate that the methylation levels of this region in the RA genome are lower than in the normal control. This tendency was more evident in the region from −79 to −140 compared with the downstream region (Fig. 5C); the methyl-C content was 25% in RA samples and 72% in control samples at position −148 and 21% in RA samples and 65% in control samples at position −142.

![Graph A](image1.png)

**Graph A:** Number of Cells/field

- EphrinB1-Fc
- Fc
- Genistein

![Graph B](image2.png)

**Graph B:** IL-6 production (pg/ml)

- EphrinB1-Fc
- Fc
- Genistein

![Graph C](image3.png)

**Graph C:** TNF-α production (pg/ml)

- EphrinB1-Fc
- Fc
- Genistein

Fig. 4. Stimulation of cytokine production by ephrinB1/Fc. A: synovial cells derived from RA patients (18) were treated with ephrinB1/Fc or control Fc in the absence or presence of genistein for 48 h. Levels of IL-6 produced in the culture supernatant were determined (means and SD). The examination was repeated 3 times, and representative results are shown. Statistical significance of the differences between normal and RA groups was calculated by Student’s t-test (*P < 0.05; **P < 0.01). B: synovial cells derived from RA patients were stimulated by the indicate doses of ephrinB1/Fc in the absence or presence of genistein (10 μg/ml) for 15 min. Lysates were incubated with 4G10, and immunoprecipitates were analyzed by immunoblot analysis with an anti-EphB1 antibody. GAPDH was used as an internal control.

To confirm the effect of the methylation of the detected CpG motif on ephrinB1 expression, we made luciferase reporter constructs harboring the 5′ upstream region (−1000 to +1) of the human ephrinB1 gene. Reporter constructs were incubated...

![Graph D](image4.png)

**Graph D:** Stimulation of cytokine production by ephrinB1/Fc.

- EphrinB1-Fc
- Fc
- Genistein

![Graph E](image5.png)

**Graph E:** Stimulation of PBLs derived from normal donors by ephrinB1.

A: PBLs from normal donors suspended in RPMI-1640 (2.5 × 10^5 cells/ml) were pretreated with the indicated doses of ephrinB1/Fc or control Fc in the absence or presence of genistein for 1 h. Migration was determined in 24-well plates with 5-μm-pore size cell culture inserts; a 100-μl aliquot of the cell suspension was added to the upper insert, and a 600-μl aliquot of medium containing stromal cell-derived factor-1 (100 ng/ml) was added to the lower chamber. After an incubation for 3 h, numbers of cells that had passed through the membrane into the lower chamber were quantified by counting 5 random fields (means and SE). The examination was repeated five times, and representative results are shown. Statistical significance of the differences between normal and RA groups was calculated by Student’s t-test (*P < 0.05; **P < 0.01). B: PBLs prepared from normal donors were treated with ephrinB1/Fc or Fc in the absence or presence of genistein for 48 h. Levels of TNF-α produced in the culture supernatant were determined using ELISA kits (means and SE). The examination was repeated 5 times, and representative results are shown. Statistical significance of the differences between ephrinB1 and Fc was calculated by Student’s t-test (*P < 0.05; **P < 0.01). C: PBLs from normal donors were stimulated by an indicate dose of ephrinB1/Fc in the absence or presence of genistein (10 μg/ml) for 15 min. Lysates were incubated with anti-phosphotyrosine antibody 4G10, and immunoprecipitates were analyzed by immunoblot analysis with an anti-EphB1 antibody and an anti-GAPDH antibody. GAPDH was used as an internal control.
in the presence or absence of Ss1 methylase, and their luciferase activities were measured. High levels of relative activity were observed in cells transfected with the unmethylated construct, whereas the activity was strongly diminished by methylation of the construct (Fig. 5D).

**DISCUSSION**

Ephrin ligands on the cell surface transmit signals through interactions with their receptors, Eph receptors, on adjacent cells, and there is growing evidence that ephrins and Eph receptors play an important role in the regulation of cell migration and activation in the neuronal system (10). EphrinB1 activates EphB receptors to generate adhesive or repulsive signals through the modulation of integrin activation and actin cytoskeleton rearrangement. In the immunological sphere, various types of ephrinB and EphB receptors have been detected in immune cells (31), and the activation of EphB receptors might be involved in the regulation of the immune system (12, 21, 25, 27, 29). In fact, we demonstrate here using an ephrinB1/Fc fusion protein that ephrinB1 itself has no chemotactic activity but effectively stimulates the SDF-1 receptor on PBLs in a dose-dependent manner (Fig. 3A). Furthermore, the production of TNF-α in PBLs and that of IL-6 in RA synovial cells were also stimulated by ephrinB1/Fc (Fig. 4, A and B). These biological effects, named, enhanced migration and cytokine production, were suppressed by genistein. EphB1 receptor on PBLs and RA synovial cells was actually phosphorylated by ephrinB1/Fc, and this phosphorylation was inhibited by genistein (Figs. 3B and 4B). Since EphB1 is highly expressed on some T cells and synovial cells in RA synovial tissues (data not shown), it is conceivable that ephrinB1 expressed on T cells derived from patients with RA interacts with EphB1 on neighboring T cells or other types of cells, such as synovial cells. 

RA is the most common form of inflammatory arthritis and is characterized by a disordered synovial microenvironment in which there is hyperplasia of resident stromal cells and a heavy infiltration of hematopoietic cells (5). The inflammatory process is usually tightly regulated, involving both mediators that initiate and maintain inflammation and mediators that shut the process down. In the states of chronic inflammation, an imbalance between the two mediators leaves inflammation unchecked, resulting in cellular damage and synovial tissue destruction. Thus, ephrinB1/EphB signaling might be involved in the imbalance of immune regulation and the aberrant accumulation of inflammatory lymphocytes in synovial tissues of patients with RA (25). In this context, it is quite interesting that the production of TNF-α, but not IL-4, in PBLs derived from normal donors (Fig. 4A). The TNF-α production seemed to be totally dependent on ephrinB1. TNF-α is a cytokine that plays a central role...
in the pathogenesis of RA, and anti-TNF-α antibodies strongly suppress inflammatory reactions in RA joints. Taken together, it is possible that ephrinB1-EphB interactions occur in RA synovial tissues, resulting in situ in activating Th1-type T cells to exhibit enhanced production of inflammatory cytokines such as TNF-α.

It is noteworthy that DNA hypomethylation is involved in the high expression of ephrinB1 in PBLs derived from patients with RA. Both human (Fig. 5A) and mouse (Suppl. Fig. A) ephrinB1 genes possess a CpG island 200–400 bp upstream of the transcription start site. The results clearly demonstrate that the methylation status of the CpG island of ephrinB1 promoters was lower in RA patients (Fig. 5, B and C) and CAIA mice (Suppl. Figs. B and C) than in normal controls. DNA methylation of this promoter region affects transcription activity (Fig. 5D). Indeed, high expression levels of ephrinB1 were observed in PBL samples derived from RA patients (Fig. 2, A and B), and ephrinB1-positive CD3 cells were abundant in the synovial tissues of RA patients (Fig. 1B). These changes were detected in all PBL and tissue samples from patients with RA examined and were significant compared with samples derived from normal controls and patients with OA. In addition, similar results were also obtained using an experimental RA model, CAIA mice. These findings suggest that methylation states are closely associated with expression levels of Th1-type T cells.

There are several binding sites in this CpG region for methyl-CpG-sensitive transcription factors such as activator protein-2, STAT, c-Myb, and Ets. Methylation of CpG in binding sites for these factors prevents transcription activity (4, 8). A cluster of Sp1-binding sites is also located in this region. Sp1 family members act as positive regulators of TATA-less promoters (3). The Sp1 protein can bind methylated CpG sites but not activate transcription when the sites are covered with methyl-CpG-binding proteins (20). Thus, methylation of the promoter region of the ephrinB1 gene affects the expression level of ephrinB1, which might be associated with controlling the mechanism of inflammation and, per se, an imbalance in T cell function. The mechanism underlying the hypomethylation of the ephrinB1 gene in RA or CAIA lymphocytes is not clearly understood. Our results reveal that the stimulation of PBLs prepared from normal donors with ephrinB1/Fc did not affect the methylation status (unpublished data). On the other hand, it has been reported that some ephrins in rats are induced in circulating lymphocytes by the administration of LPS (17). Thus, it is quite natural to hypothesize that some environmental factors, such as LPS, induce an ephrinB1-positive subset of T cells, which frequently interact with EphB receptor-positive stroma or Th1-type T cells. This interaction activates EphB receptor-expressing cells to produce excess amounts of inflammatory cytokines. Further careful studies are required to identify ephrinB1-positive T cells and to gain an understanding of their function in the pathogenesis of RA.

In conclusion, ephrinB1-positive T cells are abundant in synovial tissues of patients with RA, and high expression levels of ephrinB1 were observed in blood lymphocytes derived from RA patients, probably due to hypomethylation in the promoter region of the ephrinB1 gene. EphrinB1 is involved in enhancing the migration and production of TNF-α of lymphocytes and production of IL-6 of synovial cells. These results suggest that ephrinB1-positive T cells play an important role in the pathogenesis of RA. Further studies on the function and regulation of ephrinB1 may lead to an understanding of the etiology of RA and to the development of effective therapies for RA.

ACKNOWLEDGMENTS

We thank Dr. Margaret Dooly Ohto for comments on the manuscript. Present address of A. Kamataki: Dept. of Pathology, Iwate Medical Univ. School of Medicine, Iwate, Japan.

GRANTS

This work was supported by grant from the Fukushima Foundation for the Promotion of the Medicine.

REFERENCES


1. Supplemental material for this article is available online at the American Journal of Physiology-Cell Physiology website.