RA-NLCs demonstrate pseudoemperipolesis with T and B lymphocytes and interact with them. RA-NLCs promote the survival of T and B cells in vitro, activate them to produce cytokines, and induce production of immunoglobulin by B cells.[4,6,7] RA-NLCs are believed to contribute to the pathogenesis and persistence of inflammation in RA.[4]

Multiple inflammatory cytokines are known to be produced by various types of cells such as infiltrating T and B lymphocytes and monocytes/macrophages in the inflammatory synovial tissues.[8] Several studies have reported that interaction between synoviocytes and T lymphocytes promoted cytokine production.

Bombara et al.[9] reported that cell contact between fibroblast-like synoviocytes (FLSs) and T lymphocytes induced the expression of adhesion molecules, VCAM-1 (vascular cell adhesion molecule 1, CD106) and ICAM-1 (intercellular adhesion molecule-1, CD54) on FLSs and the production of tumor necrosis factor (TNF), interferon (IFN)-γ, and interleukin (IL)-6. Min et al.[10] reported that coculture of rheumatoid synovial fibroblasts and type II collagen-reactive T cells induced the expression of IL-8, monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein 1α (MIP-1α) mainly by cell–cell contact through CD40 ligand–CD40 engagement.

Interaction between RA synoviocytes and B lymphocytes has also been reported. Shimaoka et al.[7] reported that NLCs from bone marrow and synovium of RA patients promoted the survival of human B cells and enhanced the function. Takeuchi et al.[4] demonstrated that coculture of RA-NLCs and B cells induced the production of IL-1β and TNF-α, and enhanced the production of IL-6, IL-8, and granulocyte-colony stimulating factor (G-CSF), the proliferation of B cells, and Ig production. Reparon-Schuijt et al.[11] reported that survival of synovial B cells was regulated by VCAM-1 expressed on FLSs in RA patients. Takeuchi et al.[12] demonstrated VLA-4-dependent and -independent pathways in the proinflammatory cytokine production by synovial NLCs from RA patients through cell–cell contact with MC/car, a human B-cell line. Recently our group reported that B-cell clones, obtained when RA-NLCs were established, proliferated depending on the presence of RA-NLCs and that each clone produced immunoglobulin, which recognizes human stromal cell lines from various tissues.[13]

Rheumatoid arthritis synoviocytes and monocytes/macrophages also interact. Our group reported that monocytes cultured with RA-NLCs differentiated into osteoclast precursors, which became multinucleated bone resorbing cells, i.e., osteoclasts, when supplemented with IL-3, IL-5, IL-7, granulocyte macrophage-colony stimulating factor (GM-CSF), or a combination of receptor activator of nuclear factor-κ B ligand (RANKL) and macrophage-colony stimulating factor (M-CSF).[14]

In the present study, we analyzed the interaction between RA-NLCs and monocytes/macrophages. Interaction between RA-NLCs and monocytes/macrophages requires direct cell–cell contact and induces inflammatory cytokines probably via membrane-bound TNF-α. The results suggest that this interaction plays an important role not only in destruction of joints but in induction and persistence of inflammation in RA patients.

Patients and methods

Patients

Synovial tissues were collected with informed consent from patients with RA or osteoarthritis (OA) who had undergone arthroplasty at the National Hospital Organization Sagamihara National Hospital. All patients with RA satisfied the 1987 revised diagnostic criteria of the American College of Rheumatology (formerly the American Rheumatism Association).[15] The patients with OA were diagnosed according to the ACR clinical and radiographic criteria for OA of the knee.[16]

Establishment of NLCs and FLSs from synovial tissues

RA-NLCs were established from the synovium of RA patients according to the procedure previously reported.[4] Fibroblast-like synoviocytes were similarly established from synovium of OA patients and named as OA-FLSs. Briefly, tissue specimens were finely minced and digested with a cocktail of enzymes consisting of 0.1% hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA), 0.1% type II collagenase (Sigma-Aldrich), and 0.01% DNase (Sigma-Aldrich) for 1 h in a shaking water bath at 37°C. The digested tissue specimens were filtered with a 100-μm-diameter nylon filter (Cell Strainer; BD Biosciences Discovery Labware, MA, USA) and washed twice with Hanks' Balanced Salt Solution (HBSS; Invitrogen, Tokyo, Japan). The cell pellets were resuspended in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS; Thermo Trace, Melbourne, Australia), 100 units/ml penicillin G sodium, 100μg/ml streptomycin sulfate, and 2mM L-glutamine (Invitrogen), and seeded into a tissue-culture flask (Asahi Techno Glass, Tokyo, Japan). Nonadherent cells were removed and adherent cells were maintained in humidified air containing 7.5% CO_2 at 37°C. The medium was changed twice a week and the cells were passaged when they became confluent. Homogeneous populations of stromal cells were obtained during several passages. Twelve RA-NLC and five OA-FLS lines were established from the synovium from RA and OA patients, respectively. Two RA-NLC and three OA-FLS lines were selected and used after 3–6 passages in the experiments.

To examine the ability of pseudoemperipolesis, 1×10^4 RA-NLCs or OA-FLSs were cocultured with 4×10^5 MOLT17 cells, a human lymphoma cell line (American Type Culture Collection, Rockville, MD, USA) or MC/car cells, a human B-cell line (American Type Culture Collection). After 6 h of coculture, the medium was changed gently to remove nonadherent cells. Pseudoemperipolesis was determined to be positive when more than three lymphoma cells were detected under one RA-NLC or OA-FLS.

Cells located beneath a synovial cell (an RA-NLC or an OA-FLS) (pseudoemperipolesis) looked like dark round cells inside of the outline of the synovial cell body, whereas cells which attached only to a cell body or a dendritic process of a synoviocyte looked like bright round cells and were easily washed out by a pipetting medium. Two hundred synoviocytes were counted in each experiment.

Coculture of RA-NLCs and peripheral blood cells

Peripheral blood samples were collected from RA patients and healthy adults with informed consent. The specimens were immediately heparinized, overlaid on 5 ml of Lymphocyte Separating Medium (LSM; ICN Biomedicals, Aurora, OH, USA), and centrifuged at 3000 rpm for 30 min at room temperature. Peripheral blood mononuclear cells (PBMCs) were collected and washed twice with HBSS. Monocytes, CD14-negative cells, and T and B lymphocytes were isolated from PBMCs using anti-CD14, -CD3, and -CD19 antibody-conjugated MACS beads (MACS; Miltenyi Biotech, Bergisch Gladbach, Germany), respectively, according to the manufacturer's instructions. The purity of each fraction was examined using FACSCalibur (Nippon Becton Dickinson, Tokyo, Japan) after staining with respective antibodies conjugated with phycoerythrin (PE) or fluorescein isothiocyanate (FITC) (CD14-PE, CD3-FITC, and CD19-PE; Nippon Becton Dickinson). Briefly, 5×10^5 cells of each fraction was resuspended in 100 µl of the medium, and 4 µl of respective fluorescent antibody was added and incubated on ice for 30 min. After centrifugation at 11 000 rpm for 10 s, the supernatant was removed. The cells were resuspended in 500 µl of medium for examination with FACSCalibur. The purities were greater than 95%.

To examine cytokine production, 1×10^3 RA-NLCs and 4×10^4 monocytes, T or B lymphocytes, or CD14-negative cells in 200 µl of the medium were dispensed to each well of a 96-well plate. In addition, to investigate if TNF-α was involved in the induction of cytokines, 1×10^3 of RA-NLCs and 4×10^4 monocytes were cultured in 200 µl of the medium in each well of a 96-well plate, with or without anti-TNF-α neutralizing monoclonal antibody at 0.01, 0.1, or 1 µg/ml (R&D Systems, Minneapolis, MN, USA). Furthermore, 1×10^4 RA-NLCs were cultured with or without 4×10^5 monocytes on a Millicell culture plate insert (Nihon Millipore, 0.45-µm pore; Kogyo, Yonezawa, Japan) or cocultured with the same number of monocytes without Millicell in each well of a 24-well plate. Cells were cultured for 72 h at 37°C in humidified air containing 7.5% CO_2 and the supernatant fluids were collected and stored at −20°C until use.

To quantitate the mRNA of cytokines, 3×10^5 RA-NLCs and 7×10^6 monocytes were dispensed into each well of a 6-well plate. Cells were cocultured or cultured alone for 24 h. The cells were collected after trypsin/EDTA treatment (Cambrex Bio Science Walkersville, Walkersville, MD, USA) and separated into two populations, monocytes and RA-NLCs, using CD14 antibody-conjugated MACS beads.

Quantification of cytokines

Levels of inflammatory cytokines, IL-6, IL-8, IL-1β, and TNF-α were determined in culture supernatant, using an enzyme-linked immunosorbent assay (ELISA) kit (BioSource International, Camarillo, CA, USA).

The levels of mRNA of IL-6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were assessed in RA-NLCs, monocytes (cultured alone, respectively), and a mixture of these cells after a coculture. A conventional reverse transcription–polymerase chain reaction (RT-PCR) procedure was performed using Premix Tag (TaKaRa, Shiga, Japan) and LightCycler Primer Set (search-LC, Heidelberg, Germany) of human IL-6 and human GAPDH with an annealing temperature of 60°C and amplification by 25 cycles for IL-6 and 20 cycles for GAPDH.

Moreover, the levels of mRNA of IL-6, IL-8, and GAPDH were assessed in RA-NLCs and monocytes by quantitative RT-PCR (LightCycler, Roche Diagnostics, Tokyo, Japan) using LightCycler Primer Set of human IL-6 and human GAPDH, LightCycler FastStart DNA Master^{PLUS} SYBR Green I (Roche Diagnostics), according to the manufacturer's instructions. GAPDH was used as an internal control.

Statistical analysis

The difference in the percentage of pseudoemperipolesis-demonstrating cells was compared between RA-NLCs and OA-FLSs by analysis of variance (ANOVA) and Bonferroni test. These statistical methods were also used to compare the levels of cytokine production among the RA-NLCs cultured alone and those cocultured with PBMCs or a fraction of PBMCs. The levels of IL-6 and IL-8 production were compared between cocultured RA-NLCs and RA-NLCs cultured alone, and between cultures with and without anti-TNF-α monoclonal antibody (mAb) by ANOVA and Bonferroni test. The levels of IL-6 production were compared between RA-NLCs and OA-FLSs by unpaired *t*-test. The cytokine levels were compared between coculture of RA-NLCs and monocytes with and without Millicell by ANOVA and Bonferroni test. A P value of less than 0.05 was considered statistically significant.

Results

Twelve RA-NLCs and five OA-FLSs were established from synovium from patients with RA and those with OA, respectively. RA-NLC lines demonstrated a higher percentage of pseudoemperipolesis (76% ± 12% with MOLT-17, 84% ± 19% with MC/car) than OA-FLS lines (5% ± 3% with MOLT-17, 7% ± 4% with MC/car) (Table 1).

Two RA-NLCs and three OA-FLSs were selected based on the average ability of pseudoemperipolesis and used after 3–6 passages in the experiments. RA-NLCs were cultured with PBMCs for 72 h, and the levels of IL-6 in the

culture supernatant were assessed. The levels of IL-6 were 10 times higher ($P < 0.01$) in the supernatant from coculture of RA-NLCs (RA275SY) and PBMCs than in those from cultures of RA-NLCs or PBMCs cultured alone (Fig. 1).

When RA-NLCs were cultured with CD14-negative cells, CD3-positive cells and CD19-positive cells, the levels of IL-6 in the culture supernatants were 10, 23, and 31 times higher ($P < 0.01$), respectively, than that in the culture supernatants from RA-NLCs alone (Fig. 1). When RA-NLCs were cultured with CD14-positive cells, the levels of IL-6 were 200–660 times higher than the culture of RA-NLCs alone (Figs. 1 and 2) and 12000–48000 times higher than the culture of monocytes alone (data not shown). Similar results were obtained using the PBMC fractions from four

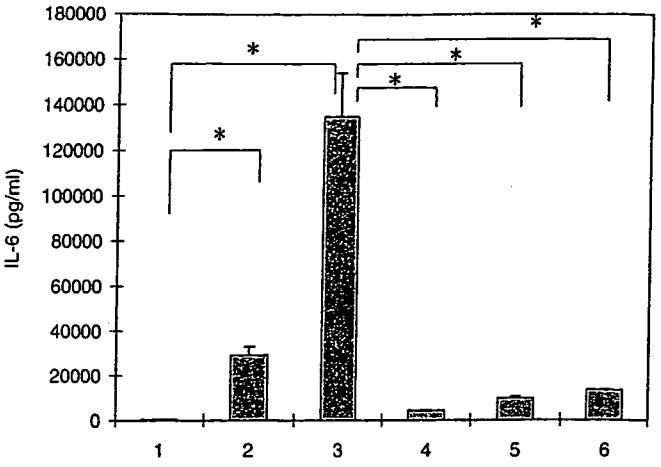


Fig. 1. Interleukin-6 (*IL-6*) production by nurse-like cells derived from rheumatoid arthritis synovium (RA-NLCs) cocultured with peripheral blood mononuclear cells (PBMCs) or fractionated cells. *1*, production of IL-6 by RA-NLCs cultured alone; *2–6*, production of IL-6 by RA-NLCs cocultured with PBMCs, CD14-positive cells, CD14-negative cells, CD3-positive cells, and CD19-positive cells, respectively. Levels of IL-6 are expressed as the mean ± SE ($n = 3$). RA275SY, one of the established cell lines, was used. 1×10^4 RA-NLCs and 2.5×10^5 cells isolated from PBMCs were cocultured or cultured alone in wells of a 24-well plate for 72 h. Levels of IL-6 were assessed by an enzyme-linked immunosorbent assay (ELISA) kit. Data were statistically analyzed by analysis of variance (ANOVA) and Bonferroni test. $^*P < 0.01$

Table 1. Pseudoemperipolesis of synoviocytes and lymphoma cell lines

Origin of synoviocytes	Cell lines	Pseudoemperipolesis (%) (mean ± SE)
RA ($n = 12$)	MOLT-17	76 ± 12*
OA ($n = 5$)	MOLT-17	5 ± 3
RA ($n = 12$)	MC/car	84 ± 19*
OA ($n = 5$)	MC/car	7 ± 4

1×10^4 synoviocytes established from rheumatoid arthritis (RA) and osteoarthritis (OA) as described in the text were cocultured with 4×10^5 human lymphoma cell line MOLT-17 or human B-cell line MC/car for 6 h. The number of the synoviocytes was counted having more than three lymphoma cells per one synoviocyte beneath themselves. The data were examined using analysis of variance (ANOVA) and Bonferroni test

* $P < 0.05$ vs OA

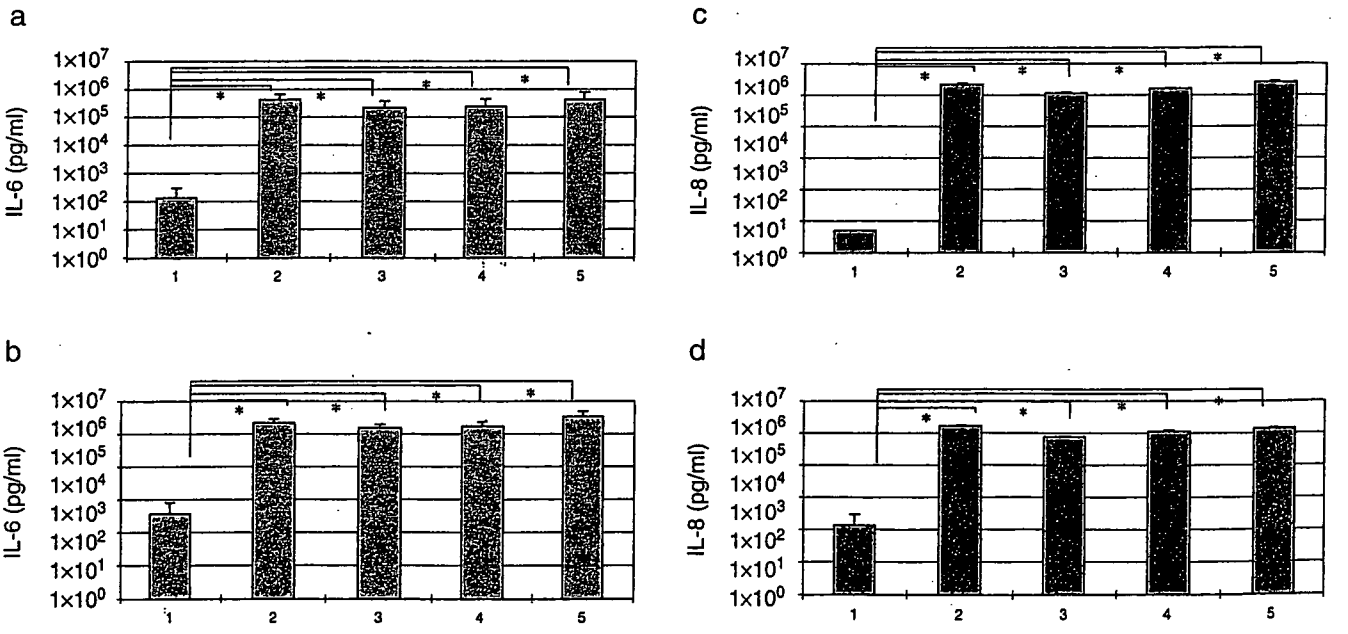


Fig. 2a–d. Production of interleukin-6 (*IL-6*) and interleukin-8 (*IL-8*) by nurse-like cells derived from rheumatoid arthritis synovium (RA-NLCs) cultured with monocytes (CD14-positive cells) from healthy donors. Levels of cytokines are expressed as the mean ± SE ($n = 3$). Data were statistically analyzed by analysis of variance (ANOVA) and Bonferroni test. **a** IL-6 production by RA275SY cultured alone (*1*) or by RA275SY cultured with monocytes from four healthy donors (*2–5*). **b** IL-6 production by RA615SY cultured alone (*1*) or RA615SY cultured with monocytes from four healthy donors (*2–5*). **c** IL-8 production by RA275SY cultured alone (*1*) or by RA275SY cultured with monocytes from four healthy donors (*2–5*). **d** IL-8 production by RA615SY cultured alone (*1*) or RA615SY cultured with monocytes from four healthy donors (*2–5*). $^*P < 0.01$

donors. The levels of IL-6 were significantly higher in the coculture of RA-NLCs and monocytes than in RA-NLC culture ($P < 0.01$, Fig. 2a,b) or in monocyte culture (data not shown).

The culture supernatant was also examined for IL-8. The levels of IL-8 were also much higher in the coculture of RA-NLCs and monocytes than in RA-NLC culture and in monocyte culture, respectively (Fig. 2c,d, data not shown). Neither IL-1β nor TNF-α was detected in any of the culture supernatant fluids (data not shown).

In the next series of experiments, OA-FLSs were cultured with monocytes and the induction of IL-6 was examined. The levels of IL-6 were significantly higher in the coculture of OA-FLSs and monocytes than in OA-FLS culture ($P < 0.05$) and in monocyte culture ($P < 0.05$), respectively (Table 2). However, the levels were not as high as those in the coculture of RA-NLCs and monocytes (Table 2).

To elucidate the mechanism of cytokine production, RA-NLCs were cultured with monocytes in the presence of anti-human TNF-α mAb at 0.01, 0.1, or 1 μg/ml. Induction of IL-6 was inhibited by the mAb at 0.1 and 1 μg/ml mAb by 44% ($P < 0.05$) and 58% ($P < 0.01$), respectively. Interleukin-8 induction was also inhibited by the mAb at 0.01, 0.1 and 1 μg/ml by 44% ($P < 0.01$), 62% ($P < 0.001$), and 74% ($P < 0.001$), respectively. These results suggest that TNF-α plays a role in the induction of IL-6 and IL-8.

To examine whether direct contact is required for the interaction between RA-NLCs and monocytes, RA-NLCs and monocytes were cocultured with Millicells to inhibit contact. The level of IL-6 in the coculture of RA-NLCs and monocytes without direct contact was 285 ± 19 pg/ml, while the level in the supernatant from RA-NLCs alone was 255 ± 21 pg/ml ($P = 1.000$) (Table 3). The level of IL-8 in the

Table 2. Comparison of interleukin-6 (IL-6) production levels

Synovial cells	Monocytes	IL-6	
		pg/ml	mean ± SE
None	Healthy donor 1	5 90 5	33 ± 35
RA-NLCs			
RA615SY	None	181 280 253	238 ± 36*
	Healthy donor 1	126900 203400 331900	220733 ± 73252
OA-FLSs			
OA2823	None	63 70 71	68 ± 3*
	Healthy donor 1	451 784 814	683 ± 142
OA4615	None	5 42 5	17 ± 15*
	Healthy donor 1	588 1182 1888	1219 ± 460
OA8491	None	5 17 5	9 ± 5*
	Healthy donor 1	179 284 314	259 ± 50

Nurse-like cells derived from RA synovium (RA-NLCs) and fibroblast-like cells derived from OA synovium (OA-FLSs) were cultured without monocytes and with monocytes from healthy donors. Data were statistically analyzed by unpaired *t*-test
* $P < 0.05$ vs healthy donor 1

Table 3. Induction of interleukin-6 (IL-6) production by nurse-like cells derived from rheumatoid arthritis synovium (RA-NLCs) with or without direct interaction with monocytes

Synovial cells	Additional cells	IL-6, pg/ml (mean ± SE)
RA-NLCs	None	255 ± 21
RA-NLCs	Monocytes (separated)	285 ± 19*
RA-NLCs	Monocytes (mixed)	217000 ± 11800

1×10^4 RA-NLCs were cultured with medium in a 24-well plate with or without 4×10^5 monocytes on a Millicell culture plate insert or cocultured with the same number of monocytes without Millicell for 72 h. The data were analyzed with analysis of variance (ANOVA) and Bonferroni test
* $P < 0.05$ vs monocytes (mixed)

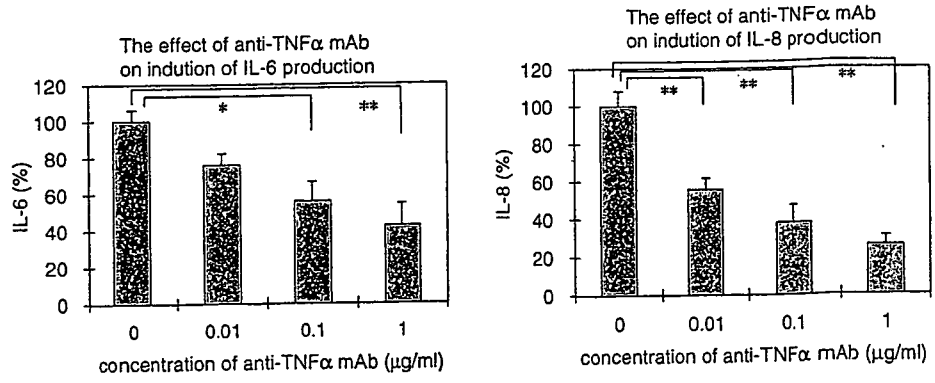


Fig. 3. Nurse-like cells derived from rheumatoid arthritis synovium RA615SY (1×10^3) and monocytes from peripheral blood mononuclear cells (PBMCs) of a healthy donor (4×10^4) were cocultured for 72 h in the presence of anti-human tumor necrosis factor α (*TNFα*) monoclonal antibody (*mAb*) at 0, 0.01, 0.1, or 1 μg/ml. Supernatant fluids were analyzed for the levels of IL-6 and IL-8 by an enzyme-linked immunosorbent assay (ELISA) kit. The levels of IL-6 and IL-8 were compared with those in the supernatant of the coculture without mAb. Data were analyzed using ANOVA and Bonferroni test. * $P < 0.05$, ** $P < 0.01$

Table 4. Levels of interleukin (IL)-6 and interleukin (IL)-8 mRNAs in nurse-like cells derived from rheumatoid arthritis synovium (RA-NLCs) and monocytes that were fractionated after 24-h coculture

Cytokines	Cells	Ratio of mRNA	
		mRNA/GAPDH mRNA	Ratio
IL-6	RA-NLCs cultured alone	0.016	1
	RA-NLCs cultured with monocytes	1.189	76.5
	Monocytes cultured alone	0.025	1
	Monocytes cultured with RA-NLCs	0.131	5.2
IL-8	RA-NLCs cultured alone	0.025	1
	RA-NLCs cultured with monocytes	15.627	616
	Monocytes cultured alone	0.021	1
	Monocytes cultured with RA-NLCs	0.076	3.7

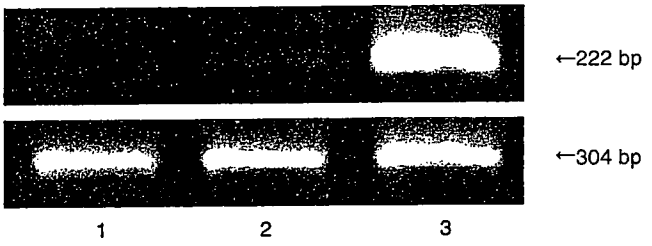


Fig. 4. Electrophoresis of polymerase chain reaction products of interleukin-6 (IL-6) (*top lane*) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*bottom lane*). The conventional revere transcription–polymerase chain reaction procedure was performed using Premix Tag (TaKaRa, Shiga, Japan) and LightCycler Primer Set (search-LC, Heidelberg, Germany) of human IL-6 and human GAPDH with annealing temperature at 60°C and amplification by 25 cycles for IL-6 and 20 cycles for GAPDH. Polymerase chain reaction products from monocytes cultured alone (*1*), nurse-like cells derived from rheumatoid arthritis synovium (RA-NLCs) cultured alone (*2*), and cocultured RA-NLCs and monocytes (*3*) are shown

coculture without direct contact was 347 ± 36 pg/ml, while that in the supernatant from RA-NLCs alone was 320 ± 25 pg/ml ($P = 1.000$) (Table 3). These results indicate that direct contact between RA-NLCs and monocytes is required for induction of cytokines.

Levels of mRNA were examined after RA-NLCs and monocytes were cocultured for 24 h by conventional RT-PCR (Fig. 4) and by quantitative RT-PCR (Table 4). For quantitative RT-PCR, cytokine mRNA levels were normalized using GAPDH mRNA as an internal control (Table 4). Relative levels of IL-6 and IL-8 mRNA in RA-NLCs cocultured with monocytes were approximately 80 and 620 times higher than those in RA-NLCs cultured alone, respectively (Table 4). Levels of IL-6 and IL-8 mRNA in monocytes cocultured with RA-NLCs were approximately 5 and 4 times higher, respectively, than those in monocytes cultured alone (Table 4).

Discussion

Coculture of RA-NLCs established from the synovial tissues of RA patients and monocytes freshly isolated from PBMCs of healthy donors resulted in the induction of high levels of IL-6 and IL-8. The levels of IL-6 and IL-8 were much higher when RA-NLCs were cocultured with CD14-positive cells (i.e., monocytes)[17] than when cocultured with CD14-negative cells, CD3-positive cells (i.e., T lymphocytes),[18] or CD19-positive cells (i.e., B lymphocytes).[19] The levels of IL-6 and IL-8 mRNA in RA-NLCs were also increased when cocultured with monocytes. When cultured together, RA-NLCs were more activated than monocytes, determined by the levels of IL-6 and IL-8 mRNAs in respective fractions. These results suggest that monocytes are more potent stimulators to RA-NLCs, than they are to monocytes.

Multiple inflammatory cytokines are known to be produced by various types of cells such as infiltrating T and B lymphocytes and monocytes/macrophages in the inflammatory synovial tissues.[8] Several studies have reported that interaction between synoviocytes and T and B lymphocytes promoted cytokine production.

RA-NLCs also interact with monocytes/macrophages. Our group[14] reported that monocytes differentiated into osteoclasts in two steps: cultured in the presence of RA-NLCs and then supplemented with IL-3 and distorted bones. Recently we also induced osteoclasts from CD14-positive cells in synovial fluids (SFs) from RA patients and OA patients by culturing whole cells in each SF and then with supplement of IL-3, and found that osteoclasts derived from RA-SF were larger, had more nuclei, and had more capacity of resorption pit formation on dentine slice and of resorption area formation on osteologic discs than those induced from OA-SF.[20] Chomarat et al.[21] reported that interaction of monocytes and synoviocytes from RA patients induced the expression of adhesion molecules, VCAM-1 and ICAM-1. There were reports of IL-6 production in the coculture of synoviocytes from RA patients and monocytes.[21,22] One study demonstrated that coculture of U937, monocytic cell line, and FLSs leads to enhanced production of IL-6.[23] The levels of IL-6 were, however, only three times higher in the supernatant fluids from coculture of RA synoviocytes and U937 cells than in those from cultures of RA synoviocytes alone. As U937 is an established cell line, use of monocytes freshly isolated from PBMCs is more appropriate and will provide more physiological information. Chomarat et al.[24] reported that coculture of monocytes from healthy donors and synoviocytes from RA patients resulted in IL-6 production; the levels of produced IL-6 were, however, only 15–25 times higher than the sum of those produced by monocytes and synoviocytes cultured alone. Moreover, they compared the effect of coculture of monocytes from healthy donors and synoviocytes from RA patients and that of coculture of monocytes and synoviocytes obtained from patients with knee ligament symptoms. There was no difference in the amount of IL-6 production.

The present study demonstrated that coculture of NLCs from RA patients (RA-NLCs), not FLSs from OA patients (OA-FLSs), and monocytes resulted in production of high levels of IL-6 and IL-8. The results suggest that NLCs from RA patients may have a unique property to be activated more easily than OA-FLSs and that, for RA-NLCs, monocytes are more potent stimulators than T or B lymphocytes.

Our results also indicate that direct cell–cell contact is required for the interaction between RA-NLCs and monocytes. Cytokine induction through coculture of RA-NLCs and monocytes was inhibited by anti-human TNF-α mAb. No supernatant sample contained detectable levels of TNF-α by ELISA. Monocytes/macrophages are known to be a major producer of TNF-α.[25] Tumor necrosis factor α is produced as a membrane-bound, 26-kDa proform,[26] and the mature, 17-kDa TNF subunit is released from the proform by proteolytic cleavage.[27–31] The membrane-bound TNF-α has biological activities as soluble TNF-α: inducing apoptosis, proliferation, or cytokine induction.[32] Together, it is likely that interaction between RA-NLCs and monocytes is mediated by the membrane-bound TNF-α.

The present study also demonstrated that monocytes are more potent stimulators for RA-NLCs than T or B lymphocytes. The results suggest that production of a large amount of cytokines through the interaction between RA-NLCs and monocytes may be one mechanism in the pathogenesis and maintenance of arthritis in RA. Recently, infliximab,[33] a chimeric anti-TNF-α mAb, and etanercept,[34] a soluble TNF-α receptor conjugated to Fc fragment of IgG, have been clinically applied as therapeutic reagents to RA. It is expected that these will effectively inhibit the interaction between RA-NLCs and monocytes/macrophages in inflammatory synovium in RA.

Acknowledgments We thank Dr. Ichiro Kurane, National Institute of Infectious Diseases, Tokyo, Japan, and Professor Tsunetoshi Itoh, Division of Immunology and Embryology, Department of Cell Biology, Tohoku University School of Medicine, Sendai, Japan, for critical evaluation of the manuscript. This study was supported by a research grant for rheumatoid arthritis from the Ministry of Health, Labour and Welfare of Japan.

References

1. Burmester GR, Dimitriu-Bona A, Waters SJ, Winchester RJ. Identification of three major synovial lining cell populations by monoclonal antibodies directed to Ia antigens and antigens associated with monocytes/macrophages and fibroblasts. Scand J Immunol 1983;17:69–82.
2. Weckerle H, Ketelsen UP, Ernst M. Thymic nurse cells. Lymphoepithelial cell complexes un murine thymuses: morphological and serological characterization. J Exp Med 1980;151:925–44.
3. Weckerle H, Ketelsen UP. Thymic nurse cells: Ia-bearing epithelium involved in T-lymphocyte differentiation? Nature 1980; 283:402–4.
4. Takeuchi E, Tomita T, Toyosaki-Maeda T, Kaneko M, Takano H, Hashimoto H, et al. Establishment and characterization of nurse cell-like stromal cell lines from synovial tissues of patients with rheumatoid arthritis. Arthritis Rheum 1999;42:221–8.
5. Iwagami S, Furue S, Toyosaki T, Horikawa T, Doi H, Satomi S, et al. Establishment and characterization of nurse cell-like clones from human skin. Nurse cell-like clones can stimulate autologous mixed lymphocyte reaction. J Immunol 1994;153:2927–38.
6. Tomita T, Takeuchi E, Toyosaki-Maeda T, Oku H, Kaneko M, Takano H, et al. Establishment of nurse-like stromal cells from bone marrow of patients with rheumatoid arthritis: indication of characteristic bone marrow microenvironment in patients with rheumatoid arthritis. Rheumatology (Oxford) 1999;38:854–63.
7. Shimaoka Y, Attrep JF, Hirano T, Ishihara K, Suzuki R, Toyosaki T, et al. Nurse-like cells from bone marrow and synovium of patients with rheumatoid arthritis promote survival and enhance function of human B cells. J Clin Invest 1998;102:606–18.
8. Sweeney SE, Firestein GS. Rheumatoid arthritis: regulation of synovial inflammation. Int J Biochem Cell Biol 2004;36:372–8.
9. Bombara MP, Webb DL, Conrad P, Marlor CW, Sarr T, Ranges GE, et al. Cell contact between T cells and synovial fibroblasts causes induction of adhesion molecules and cytokines. J Leukocyte Biol 1993;54:399–406.
10. Min DJ, Cho ML, Lee SH, Min SY, Kim WU, Min JK, et al. Augmented production of chemokines by the interaction of type II collagen-reactive T cells with rheumatoid synovial fibroblasts. Arthritis Rheum 2004;50:1146–55.
11. Reparon-Schuijt CC, van Esch WJ, van Kooten C, Schellekens GA, de Jong BA, van Venrooij WJ, et al. Secretion of anti-citrulline-containing peptide antibody by B lymphocytes in rheumatoid arthritis. Arthritis Rheum 2001;44:41–7.
12. Takeuchi E, Tanaka T, Umemoto E, Tomita T, Shi K, Takahi K, et al. VLA-4-dependent and -independent pathways in cell contact-induced proinflammatory cytokine production by synovial nurse-like cells from rheumatoid arthritis patients. Arthritis Res 2002;4:R10.
13. Nakamura-Kikuoka S, Takahi K, Tsuboi H, Toyosaki-Maeda T, Maeda-Tanimura M, Wakasa C, et al. Limited VH gene usage in B cell clones established with nurse-like cells from patients with rheumatoid arthritis. Rheumatology 2005; in press.
14. Toyosaki-Maeda T, Takano H, Tomita T, Tsuruta Y, Maeda-Tanimura M, Shimaoka Y, et al. Differentiation of monocytes into multinucleated giant bone-resorbing cells: two-step differentiation induced by nurse-like cells and cytokines. Arthritis Res 2001; 3(5):306–10.
15. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
16. Altman R, Asch E, Bloch D, Bole G, Borenstein D, Brandt K, et al. Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Diagnostic and Therapeutic Criteria Committee of the American Rheumatism Association. Arthritis Rheum 1986;29:1039–49.
17. Ziegler-Heitbrock HW, Ulevitch RJ. CD14: cell surface receptor and differentiation marker. Immunol Today 1993;14:121–5.
18. Klausner RD, Lippincott-Schwartz J, Bonifacino JS. The T cell antigen receptor: insights into organelle biology. Annu Rev Cell Biol 1990;6:403–31.
19. Clark EA, Lane PJ. Regulation of human B-cell activation and adhesion. Annu Rev Immunol 1991;9:97–127.
20. Takano H, Tomita T, Toyosaki-Maeda T, Maeda-Tanimura M, Tsuboi H, Takeuchi E, et al. Comparison of the activities of multinucleated bone-resorbing giant cells derived from CD14-positive cells in the synovial fluids of rheumatoid arthritis and osteoarthritis patients. Rheumatology (Oxford) 2004;43:435–41.
21. Chomarat P, Rissoan MC, Pin JJ, Banchereau J, Miossec P. Contribution of IL-1, CD14, and CD13 in the increased IL-6 production induced by in vitro monocyte-synoviocyte interactions. J Immunol 1995;155:3645–52.
22. Blue ML, Conrad P, Webb DL, Sarr T, Macaro M. Interacting monocytes and synoviocytes induce adhesion molecules by a cytokine-regulated process. Lymphokine Cytokine Res 1993;12: 213–8.
23. Chen V, Croft D, Purkis P, Kramer IM. Co-culture of synovial fibroblasts and differentiated U937 cells is sufficient for high interleukin-6 but not interleukin-1beta or tumour necrosis factor-alpha release. Br J Rheumatol 1998;37:148–56.
24. Chomarat P, Rissoan MC, Pin JJ, Banchereau J, Miossec P. Contribution of IL-1, CD14, and CD13 in the increased IL-6 production

induced by in vitro monocyte-synoviocyte interactions. J Immunol 1995;155:3645–52.
25. Satomi N, Haranaka K, Kunii O. Research on the production site of tumor necrosis factor (TNF). Jpn J Exp Med 1981;51:317–22.
26. Kriegler M, Perez C, DeFay K, Albert I, Lu SD. A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. Cell 1988;53:45–53.
27. Scuderi P. Suppression of human leukocyte tumor necrosis factor secretion by the serine protease inhibitor p-toluenesulfonyl-L-arginine methyl ester (TAME). J Immunol 1989;143:168–73.
28. Kim KU, Kwon OJ, Jue DM. Pro-tumour necrosis factor cleavage enzyme in macrophage membrane/particulate. Immunology 1993; 80:134–9.
29. Mohler KM, Sleath PR, Fitzner JN, Cerretti DP, Alderson M, Kerwar SS, et al. Protection against a lethal dose of endotoxin by an inhibitor of tumour necrosis factor processing. Nature 1994;370: 218–20.
30. Gearing AJ, Beckett P, Christodoulou M, Churchill M, Clements J, Davidson AH, et al. Processing of tumour necrosis factor-alpha precursor by metalloproteinases. Nature 1994;370:555–7.
31. Moss ML, Jin SL, Milla ME, Bickett DM, Burkhart W, McGeehan G, et al. Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. Nature 1997;385:733–6.
32. Decoster E, Vanhaesebroeck B, Vandenabeele P, Grooten J, Fiers W. Generation and biological characterization of membrane-bound, uncleavable murine tumor necrosis factor. J Biol Chem 1995;270:18473–8.
33. Maini R, St Clair EW, Breedveld F, Furst D, Kalden J, Weisman M, et al. Infliximab (chimeric anti-tumour necrosis factor alpha monoclonal antibody) versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate: a randomised phase III trial. ATTRACT Study Group. Lancet 1999;354:1932–9.
34. Bathon JM, Martin RW, Fleischmann RM, Tesser JR, Schiff MH, Keystone EC, et al. A comparison of etanercept and methotrexate in patients with early rheumatoid arthritis. N Engl J Med 2000;343: 1586–93.

DNA RESEARCH 13, 169–183 (2006) doi:10.1093/dnares/dsl006

Isolation and Expression Profiling of Genes Upregulated in Bone Marrow-Derived Mononuclear Cells of Rheumatoid Arthritis Patients

Nobuo NAKAMURA,[1,†] Yasunori SHIMAOKA,[2,†] Takahiro TOUGAN,[3] Hiroaki ONDA,[3,4] Daisuke OKUZAKI,[3] Hanjun ZHAO,[3] Azumi FUJIMORI,[3] Norikazu YABUTA,[3] Ippei NAGAMORI,[3] Akie TANIGAWA,[4] Jun SATO,[3] Takenori ODA,[5] Kenji HAYASHIDA,[6] Ryuji SUZUKI,[7] Masao YUKIOKA,[2] Hiroshi NOJIMA,[3,4,*] and Takahiro OCHI[7]

Center of Arthroplasty, Kyowakai Hospital, Suita, Japan[1], Yukioka Hospital, Osaka, Japan[2], Department of Molecular Genetics, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 562-0031, Japan[3], Innovation Plaza Osaka, Izumi, Japan[4], Department of Rheumatology, NHO Osaka-Minami Medical Center, Kawachinagano, Japan[5], Hoshigaoka Kosei-Nenkin Hospital, Hirakata, Japan[6] and Clinical Research Center for Allergy and Rheumatology, National Sagamihara Hospital, 18-1 Sakura-dai, Sagamihara, Kanagawa 228-8522, Japan[7]

(Received 25 July 2006; revised 21 August 2006)

Abstract

We have comprehensively identified the genes whose expressions are augmented in bone marrow-derived mononuclear cells (BMMC) from patients with Rheumatoid Arthritis (RA) as compared with BMMCs from Osteoarthritis (OA) patients, and named them *AURA* after *au*gmented in *RA*. Both stepwise subtractive hybridization and microarray analyses were used to identify *AURA* genes, which were confirmed by northern blot analysis and/or reverse transcription polymerase chain reaction (RT–PCR). We also assessed their expression levels in individual patients by quantitative real-time RT–PCR. Of 103 *AURA* genes we have identified, the mRNA levels of the following 10 genes, which are somehow related to immune responses, were increased in many of the RA patients: *AREG* (=*AURA*9), FK506-binding protein 5 (FKBP5 = *AURA45*), C-type lectin superfamily member 9 (*CLECSF9* = *AURA24*), tyrosylprotein sulfotransferase 1 (*TPST1* = *AURA52*), lymphocyte G0/G1 switch gene (*G0S2* = *AURA*8), chemokine receptor 4 (CXCR4 = *AURA86*), nuclear factor-kappa B (NF-κB = *AURA25*) and two genes of unknown function (FLJ11106 = *AURA1*, BC022398 = *AURA*2 and XM_058513 = *AURA17*). Since *AREG* was most significantly increased in many of the RA patients, we subjected it to further analysis and found that AREG-epidermal growth factor receptor signaling is highly activated in synovial cells isolated from RA patients, but not in OA synoviocytes. We propose that the expression profiling of these *AURA* genes may improve our understanding of the pathogenesis of RA.

Key words: stepwise subtraction; microarray; RA; OA; amphiregulin; synoviolin

1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by arthritis that predominantly results in chronic inflammation of systemic joints associated with the overgrowth of synovial cells. This induces progressive cartilage and bone destruction in the joint and subsequent disability. Since RA pathogenesis is likely to involve genetic elements, a number of groups have subjected samples from healthy and affected individuals to DNA microarray analyses for a broad-scale comparison. These studies have provided

Communicated by Mitsuo Oshimura

* To whom correspondence should be addressed. Tel. +81-6-6875-3980, Fax. +81-6-6875-5192, E-mail: snj-0212@biken.osaka-u.ac.jp

† These authors contributed equally to this work.

significant insights into RA pathogenesis.[1,2] The first samples tested were synovial specimens,[3–8] and peripheral blood mononuclear cells (PBMC),[9] from RA and osteoarthritis (OA) patients, and cluster analysis of the resulting microarray gene-expression data revealed some candidate genes that may play a specific role in RA pathogenesis.

In other studies searching for key factors in RA pathogenesis, immunoscreening by using an antirheumatoid synovial cell antibody identified synoviolin/Hrd1 to be a highly expressed enzyme (E3 ubiquitin ligase) in the rheumatoid synovium.[10] Synoviolin appears to be a pathogenic factor for RA because mice overexpressing this enzyme developed spontaneous arthropathy, while heterozygous knockdown results in increased synovial cell apoptosis and resistance to collagen-induced arthritis.[11] It was proposed that the excess elimination of unfolded proteins due to synoviolin overexpression triggers synovial cell overgrowth.[12] Thus, synoviolin may play a pivotal role in the pathogenesis of arthropathy due to its functions in the quality control of proteins through the endoplasmic reticulum (ER)-associated degradation (ERAD) system; its elevated expression may therefore have an anti-apoptotic effect that causes synovial hyperplasia.

Bone marrow-derived mononuclear cells (BMMC) are another target for analyses aiming to identify the key genes that participate in RA pathogenesis because accumulating evidence suggests that BMMC cell abnormalities may contribute to the pathogenesis of RA and experimental arthritis models.[13–17] Moreover, RA patients suffer from defective central and peripheral B-cell tolerance checkpoints,[18] the first of which occurs in the bone marrow between the early immature and immature B-cell stages (the second counter selection step of autoantibody-expressing B cells takes place in the periphery, when the new emigrant becomes a mature naive B cell).[18,19] In addition, inflammatory changes similar to those found in RA synovium seem to occur in the subchondral bone marrow of the involved RA joint,[20] and synovial inflammatory tissue can reach the adjacent bone marrow by fully breaking the cortical barrier.[21] Thus, BMMC cells are an interesting subject for studies seeking to identify specific genes involved in RA pathogenesis.

To identify the genes whose expressions are dramatically induced or reduced in the pooled BMMC mRNAs of 50 RA patients as compared with 50 OA patients, we here subjected these pooled mRNAs to stepwise subtraction, which is a unique technique that we have developed previously.[22] This method permitted the comprehensive identification of those genes that are specifically up- or down-regulated during RA pathogenesis. In addition, we also used microarray analysis, since DNA microarray analyses on the BMMC of RA patients have not been described previously. As a control, we also subjected the BMMC RNA from OA patients to stepwise subtraction and microarray analysis to identify the genes that are specifically involved in OA pathogenesis. These analyses together resulted in the isolation of 103 RA-upregulated genes, of which amphiregulin (AREG) was revealed by quantitative real-time RT–PCR (QRT–PCR) to be the most conspicuously induced gene in RA patients. Interestingly, we also show here that AREG operates upstream of synoviolin in isolated synovial cells through an epidermal growth factor receptor (EFGR) signaling pathway. We discuss how AREG upregulation could contribute to RA pathogenesis.

2. Patients, Materials and Methods

2.1. Human subjects and ethical considerations

All RA patients satisfied the 1987 revised diagnostic criteria of the American College of Rheumatology (ACR: formerly the American Rheumatism Association).[23] All OA patients fulfilled the ACR criteria for hip or knee OA.[24] The RA and OA patient groups were largely matched in terms of their average age and sex (Supplementary Figure S1A and B). This study was reviewed and approved by the Internal Review Board of the Research Institute for Microbial Diseases, Osaka University. Accordingly, a written informed consent was obtained from each participant before obtaining human tissues.

2.2. Cell proliferation assay

The synovial cells from each patient were seeded onto uncoated 35 mm tissue culture plates at 1×10^5 cells/well and cultured in 5% FBS/DMEM. After 12 h, the cells were incubated in fresh 5% FBS/DMEM with (100 ng/ml) or without AREG (Sigma-Aldrich, A 7080). Four photos were taken from fixed areas in four quadrants near the central area of each plate at the 0, 1, 3 and 4 day time points. The cells at each time point were counted from these four photos and expressed as mean ± standard error (SE).

2.3. Statistical analysis

Significant differences were determined using the Spearman's rank correlation (Supplemenatry Figure S4) or the Mann–Whitney *U*-test (Figs 2, 4 and Supplementary Figure S3). The data are expressed as means ± SE. $P < 0.05$ or $P < 0.01$ was considered to be statistically significant.

3. RESULTS

3.1. Identification of RA- or OA-specific genes by stepwise subtraction and DNA microarray analysis

To isolate the putative RA-specific genes that are upregulated in BMMC of RA patients relative to those

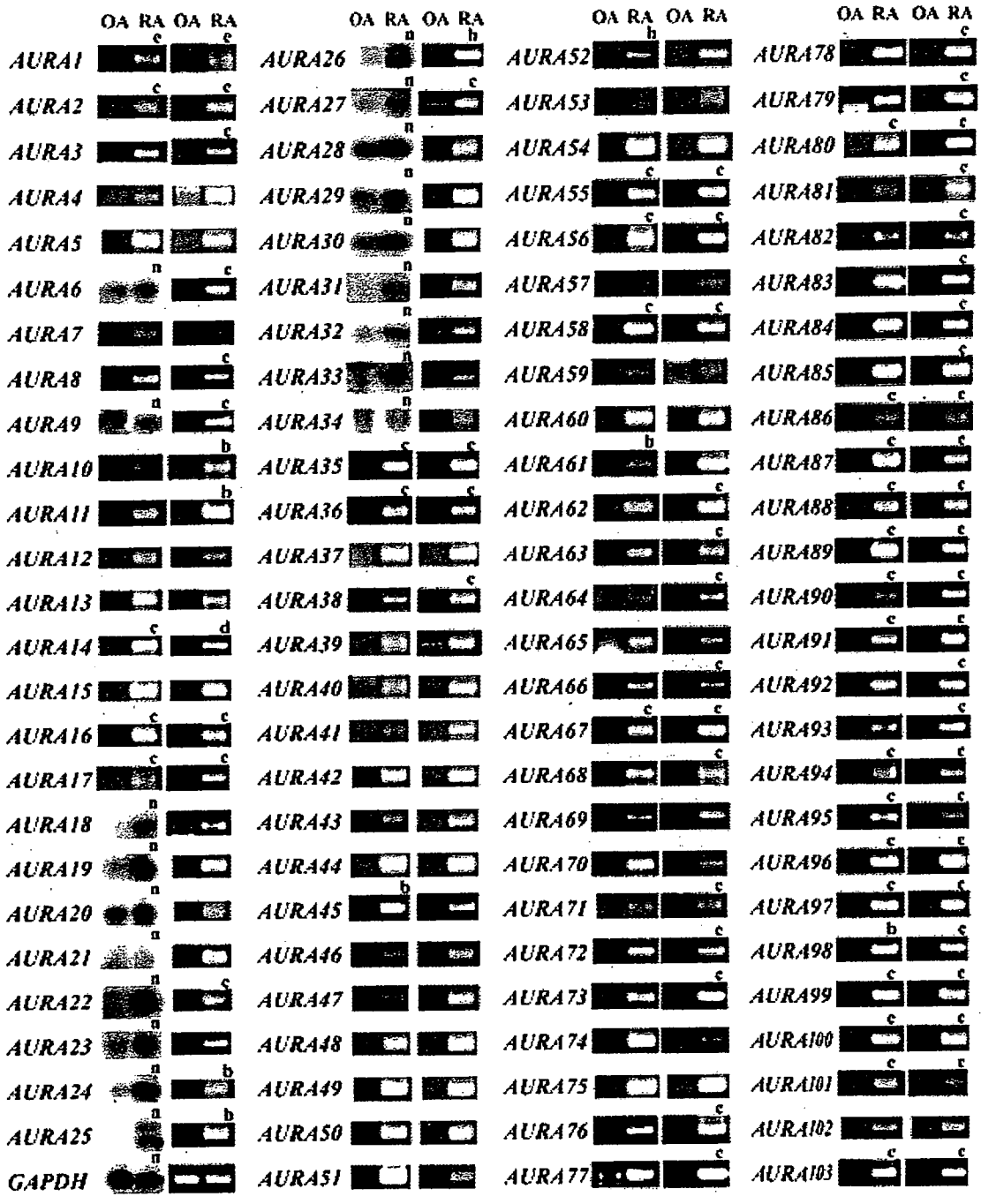


Figure 1. Northern blot or RT–PCR analysis of individual *AURA* cDNA clones to compare the expression levels of the genes in the BMMC of 50 RA patients and 50 OA patients (see Table 1 for their gene names). A northern blot or RT–PCR for *GAPDH* is also shown as a loading control. Left row: expression levels as detected by RT–PCR or northern blot analysis (denoted as n above each picture). Right row: confirmation of the expression level of each gene as determined by RT–PCR. The annealing temperature and amplification cycles for RT–PCR were always 50°C and 40 cycles, respectively, with the exception of the reactions denoted by a (50°C and 35 cycles, respectively), b (50°C and 30 cycles, respectively), c (55°C and 35 cycles, respectively), d (55°C and 40 cycles, respectively), and e (60°C and 40 cycles, respectively).

that are upregulated in OA patients, we first used our stepwise subtractive hybridization method. Briefly, we prepared a cDNA library from the pooled mRNA from the BMMC of 50 RA patients (Supplementary Figure S1A) by the linker-primer method using a pAP3neo vector.[25] Stepwise subtractive hybridization was then performed with the biotinylated pooled mRNA from the BMMC of 50 OA patients (Supplementary Figure S1A) to select candidate genes that may show upregulation in RA BMMC only as described previously.[22] To examine if the candidate genes are actually upregulated in RA but not OA BMMC, we performed northern blot analysis and/or RT–PCR using the pooled mRNA from the BMMC of 50 RA and 50 OA patients (Fig. 1). To reduce

the possibility of missing important RA-specific pathogenic genes by this method, we also performed a genome-wide complementary DNA microarray analysis using the Agilent Hu44K array with the same pooled RNA samples obtained from the BMMC of RA and OA patients that were described above. When we tested top 70 genes from the microarray list of RA-upregulated genes by northern blot analysis and/or RT–PCR as described above, we found that only 20 genes really displayed RA-upregulated expressions. Thus, we identified 103 RA-upregulated genes (Fig. 1) and named them *AURA* (*aug*mented in ***RA***). As shown in Table 1, 15 *AURA* genes (*AURA1*~*AURA7* and *AURA10*~*AURA17*) are uncharacterized novel genes.

We also performed similar experiments to obtain candidate OA-upregulated genes by generating a cDNA library from the pooled mRNA from the BMMC of 50 OA patients (Supplementary Figure S1A) and then using biotinylated pooled mRNA from the BMMC of 50 RA patients for subtraction (Supplementary Figure S1A). DNA microarray analysis also yielded a number of candidate OA-specific genes, as described above. However, when we checked whether these candidate genes are truly specifically up-regulated in OA BMMCs by northern blot analysis and/or RT–PCR, we could confirm this for only two genes (Supplementary Figure S2). These two OA-upregulated genes encode nuclear receptor coactivator 1 and a hypothetical protein (FLJ20581). This result suggests that the gain of function due to the enhanced expression of the RA-upregulated candidate genes is important in the pathogenesis of RA. Thus, we subsequently concentrated our study on the RA-upregulated genes.

3.2. *Expression profiles of RA-upregulated genes in individual RA or OA patients*

To determine whether the upregulation of the 103 RA-specific candidate genes is widespread in many RA patients or occurs in only a few patients, we performed QRT–PCR using individually prepared RNA samples from the BMMC or PBMC of RA patients. Of the 103 candidate genes, 5 genes whose functions are unknown and 12 genes that may be related to growth regulation or immune response were analyzed by QRT–PCR. OA patients were also examined as negative controls. In every QRT–PCR, a standard RNA from the PBMC of a healthy volunteer (male, age 52) was used (denoted as normal with a relative intensity of 1.0). This allowed us to compare the expression profiles of the genes tested in this study. In addition, since we used this control, we could also compare the expression profiles of the genes in this study with those of other genes tested in our previous reports on other autoimmune diseases.[26]

Of the 17 tested *AURA* genes (denoted x in Table 1), AREG (*AURA9*) was the most conspicuously upregulated in the BMMC of many of the RA patients, while in contrast OA BMMCs invariably expressed this gene at very low levels (Fig. 2A). Similarly, the PBMC of many RA patients strongly expressed AREG, while only very low expression was detected in the PBMC of the OA patients (Fig. 2A). AREG is one of the EGF-like growth factors that stimulate cell growth by activating the EGF receptor (EGFR) signaling of the target cells in an autocrine/juxtacrine fashion.[27]

AURA1 was the next most conspicuously upregulated gene in the BMMC of many RA patients, while the BMMC of all OA patients showed only very low expression of this gene (Fig. 2B). However, unlike *AREG*, the PBMC of RA patients showed negligible enhancement in the expression of *AURA1*. *AURA1* encodes an uncharacterized protein containing a thioesterase domain (Fig. 2B inset) that may cleave thioester bonds of an unknown target.

The gene encoding FK506 (tacrolimus)-binding protein 5 (FKBP5 = *AURA45*) also showed enhanced expression in nearly half of the RA patient BMMC samples, while no such increase was observed in the OA patient BMMC samples or in the PBMC of the RA patients (Fig. 2C). FKBP5 is a cellular receptor for FK506 and has an immunosuppressive effect on activated T cells because it inhibits the protein phosphatase calcineurin.[28]

Nearly half of the RA patient BMMC samples showed 5- to 50-fold greater expression of *CLECSF9* (=*AURA24*), *TPST1* (=*AURA52*) and *AURA2* than the normal control PBMC sample (Fig. 2D–F). No such increase was observed in the BMMC of OA patients or in the PBMC of the RA patients. *CLECSF9* encodes a macrophage-inducible C-type lectin (Mincle) that harbors a calcium-dependent carbohydrate-recognition domain. *TPST1* is one of the two Golgi tyrosylprotein sulfotransferases (*TPST1* and *TPST2*) that mediate the post-translational modification tyrosine O-sulfation.

G0S2 (=*AURA8*), chemokine receptor 4 (CXCR4 = *AURA86*), nuclear factor-kappa B (NF-κB = *AURA25*) and *AURA17* showed augmented expression in both the BMMC and PBMC of some of the RA patients when compared to the expression in the BMMC and PBMC of the OA patients, although the differences between the RA and OA samples are not as significant as for the previously discussed genes (Supplementary Figure S3A–D). G0S2 is one of the G0/G1 switch (G0S) genes that are differentially expressed in lymphocytes during their lectin-induced switch from the G0 to the G1 phases of the cell cycle.[29] CXCR4, the receptor for a chemokine called stromal cell-derived factor-1 (SDF-1/CXCL12), is important in the migration, homing and survival of hematopoietic stem cells. SDF-1, which is secreted by ischemic myocardium, is involved in the homeostatic and inflammatory traffic of leukocytes, and is highly expressed in the synovial tissues of RA patients.[30] NF-κB

Table 1. List of *AURA* genes

AURA no.	Accession no.	Sequence description	SS/DM	QRT-PCR
AURA1	AK001968	Unknown cDNA (FLJ11106)	b	r
AURA2	BC022398	Unknown cDNA	b	r
AURA3	BC031341	Unknown cDNA (hypothetical protein MGC45871)		
AURA4	NM_052862.2	Unknown cDNA (hypothetical protein MGC21854)		
AURA5	AK097275.1	Unknown cDNA (FLJ39956) L-PLASTIN-like		
AURA6	BC019355	Unknown cDNA (ring finger protein 149: IMAGE:3956746)		
AURA7	AF078845.1	Unknown cDNA (16.7Kd protein)		
AURA8	M69199	Putative lymphocyte G0/G1 switch gene (G0S2)=Aile1	b	r
AURA9	AH002608	Amphiregulin	b	r
AURA10	AK026118	Unknown cDNA (Ch20-ORF43)		r
AURA11	AK094006	Unknown cDNA		
AURA12	AK095896.1	Unknown cDNA (FLJ38577)		
AURA13	BC014435	Unknown cDNA (IMAGE:4855747)		r
AURA14	ZF161365	Unknown cDNA (HSPC102)	m	
AURA15	FLJ23431	Unknown cDNA (FLJ23431) MHC class I -like		
AURA16	BC066334	Unknown cDNA (FLJ37760)		
AURA17	XM_058513	Unknown cDNA (DKFZp434H2111)	m	r
AURA18	BC016660	Heat shock 70 kDa protein 8		
AURA19	BC022347	Lactotransferrin		
AURA20	NM_001800.2	Cyclin-dependent kinase inhibitor 2D (p19) (CDKN2D)		
AURA21	X55668.1	Proteinase 3		
AURA22	BC013946	Kruppel-like factor 13		
AURA23	BC022463	Dual specificity phosphatase 1 (DUSP1)		r
AURA24	AY358499	C-type lectin, superfamily member 9 (CLECSF9)	b	r
AURA25	AY033600	NF-kB alpha	b	r
AURA26	AF194172	Androgen-regulated protein 6 (AIG6)	m	
AURA27	NM_021810	Cadherin-like 26 (CDH26)		
AURA28	X52053.1	HP-1 (corticostatin/defensin family)		r
AURA29	BC018857.2	Translation elongation factor 1 gamma		
AURA30	BC053585.1	Colony stimulating factor 3 receptor (granulocyte)		
AURA31	AY124010	Interleukin 1 receptor, type II (IL1R2)	m	
AURA32	BC020635	Ficolin 1 (FCN1: collagen/fibrinogen domain-containing)		
AURA33	BC106068	Microtubule-associated protein, RP/EB family, member 1		
AURA34	AF443591	Death effector domain-containing DNA binding protein2		
AURA35	BC032491	Ubiquitin-conjugating enzyme E2L 6 (UBE2L6)		
AURA36	BC004967	Ubiquitin associated domain containing 1 (UBADC1)		
AURA37	NM_006313.1	Ubiquitin specific protease 15 (USP15)		
AURA38	BC011358	ADP-ribosylation factor 1		
AURA39	AY366510.1	Pre-mRNA 3'end processing factor FIP1		
AURA40	NM_175039.1	Sialyltransferase 7D (SIAT7D). transcript variant 2		
AURA41	BC030230.2	Aminolevulinate, delta- synthase 2		
AURA42	NM_014390.1	Staphylococcal nuclease domain containing 1 (SND1)		
AURA43	NM_015999.2	Adiponectin receptor 1 (ADIPOR1)		
AURA44	BC033877.1	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV)		r
AURA45	NM_004117	FK506 binding protein 5 (FKBP5)	b	r
AURA46	NM_000211.1	Integrin beta 2 (antigen CD18 (p95)		
AURA47	BC015641.2	Enolase 1 (alpha)		

Table 1. continued.

AURA no.	Accession no.	Sequence description	SS/DM	QRT–PCR
AURA48	BC028299.1	Non-POU domain containing. octamer-binding.		
AURA49	BC000734.2	Eukaryotic translation initiation factor 3. subunit 648 kDa		
AURA50	NM_012198.2	Grancalcin. EF-hand calcium binding protein (GCA)		
AURA51	BC026690.2	CD97 antigen. transcript variant 2.		
AURA52	CR542060	Tyrosylprotein sulfotransferase 1 (TPST1)	m	r
AURA53	NM_005875.1	Translation factor sui1 homolog (GC20)		
AURA54	NM_004048.2	Beta-2-microglobulin (B2M)		
AURA55	BC017934	NudC domain containing 2 (NUDCD2)		
AURA56	NM_000569	Fc fragment of IgG, low affinity IIIa, receptor for (CD16)	b	
AURA57	BC018649.2	Polymerase (RNA) II (DNA directed)		
AURA58	BC013293	Synuclein, alpha (a molecular chaperone)		
AURA59	NM_033405.2	PRIC285		
AURA60	J02694.1	Myeloperoxidase		
AURA61	BC020219	Zinc finger protein 143 (clone pHZ-1)	m	
AURA62	BC071590	Nijmegen breakage syndrome 1 (nibrin)		
AURA63	BC003186	DNA replication complex GINS protein PSF2		r
AURA64	NM_006060	Zinc finger protein, subfamily 1A, 1 (ZNFN1A1)		
AURA65	BC015859	T-cell activation GTPase activating protein		
AURA66	Z50749	Sds22 (protein phosphatase regulatory subunit)-like		r
AURA67	AF411850	C-type lectin-like receptor CLEC-6	m	
AURA68	BC064831	HMT1 hnRNP methyltransferase-like 3		
AURA69	BC022797	Mof4 family associated protein 1		
AURA70	BC032437	Heterogeneous nuclear ribonucleoprotein A3		
AURA71	M87790	Anti-hepatitis A immunoglobulin lambda chain variable region		
AURA72	K01763	Haptoglobin alpha(1S)-beta precursor		
AURA73	BC016800	Aldolase A, fructose-bisphosphate, transcript variant		
AURA74	BC001391	Actin-like 6A, transcript variant 1		
AURA75	NM_003512.3	H2 histone, family 2AC (H2AC)		
AURA76	BC017558	H3 histone, family 3B (H3.3B)		
AURA77	BC032748	Myosin regulatory light chain MRCL3		
AURA78	S60099	APPH = amyloid precursor protein homolog		
AURA79	BC067100	Fas (TNFRSF6) associated factor 1		
AURA80	NM_000896	Cytochrome P450, family 4, subfamily F (CYP4F3)	b	
AURA81	BC010577	Granulin (an association partner of cyclin T1)		
AURA82	AF054186	p18		
AURA83	BC028626	Trinucleotide repeat containing 6B		
AURA84	L43631	Scaffold attachment factor B (SAF-B)		
AURA85	M11124	MHC HLA DQ alpha-chain mRNA from DRw9 cell line		
AURA86	AF025375	Chemokine (C-X-C motif) receptor 4 (CXCR4)	b	r
AURA87	BC000163	Vimentin (VIM)		
AURA88	BC071860	Lactate dehydrogenase B (LDHB)		
AURA89	BC100032	Ribosomal protein S13 (RPS13)		
AURA90	BC011852	Glutamine synthetase (GLUL)		
AURA91	NM_000045	Arginase, liver (ARG1)		
AURA92	BC006510	Cyclin B1		
AURA93	BC007063	Peroxiredoxin 1		
AURA94	NM_005746	Pre-B-cell colony enhancing factor 1 (PBEF1)	m	

Table 1. continued.

AURA no.	Accession no.	Sequence description	SS/DM	QRT-PCR
AURA95	BC018711	RNA-binding region (RNP1. RRM) containing 1		
AURA96	NM_001126	Adenylosuccinate synthase (ADSS)		
AURA97	BC008929	rab2 mRNA. YPT1-related and member of ras family		
AURA98	NM_004226	Serine/threonine kinase 17b (apoptosis-inducing) (STK17B)	m	
AURA99	BC096336	Insulin-degrading enzyme		
AURA100	AF501883	G protein Beta polypeptide 2 (GNB2)		
AURA101	BC007237	Myeloid/lymphoid or mixed-lineage leukemia		
AURA102	BC034149.1	Ribosomal protein S3		
AURA103	NM_020980	Aquaporin 9 (AQP9)	m	

Of 103 *AURA* genes, 83, 10 or 10 genes were identified by stepwise subtraction (SS) alone (no mark), by DNA microarray (DM) alone (denoted by m) or by both techniques (denoted by b), respectively. The *AURA* genes that were subjected to QRT–PCR analysis are denoted by r.
PRIC285: peroxisomal proliferator-activated receptor A interacting complex 285.

is a transcription factor that resides in the cytoplasm of every cell and translocates to the nucleus when activated by a wide variety of agents, including cytokines.[31] *AURA17* is an uncharacterized novel gene that encodes a large protein with 8 leucine rich repeats, Mitochondrial Rho (Miro) motif and protein tyrosine kinase domain (Supplementary Figure S3D inset).

We also tested seven other genes in RA and OA BMMC and PBMC samples by QRT–PCR, but none showed a widespread and conspicuous increase in expression in the RA BMMC samples (data not shown). Consequently, these genes appear to play a less significant role in RA pathogenesis. Since these experiments and those described above consumed almost all BMMC and PBMC samples from the RA and OA patients, the remaining *AURA* genes will have to be tested in the future with another RA patient set.

3.3. *Expression pattern of AURA genes in PBMC*

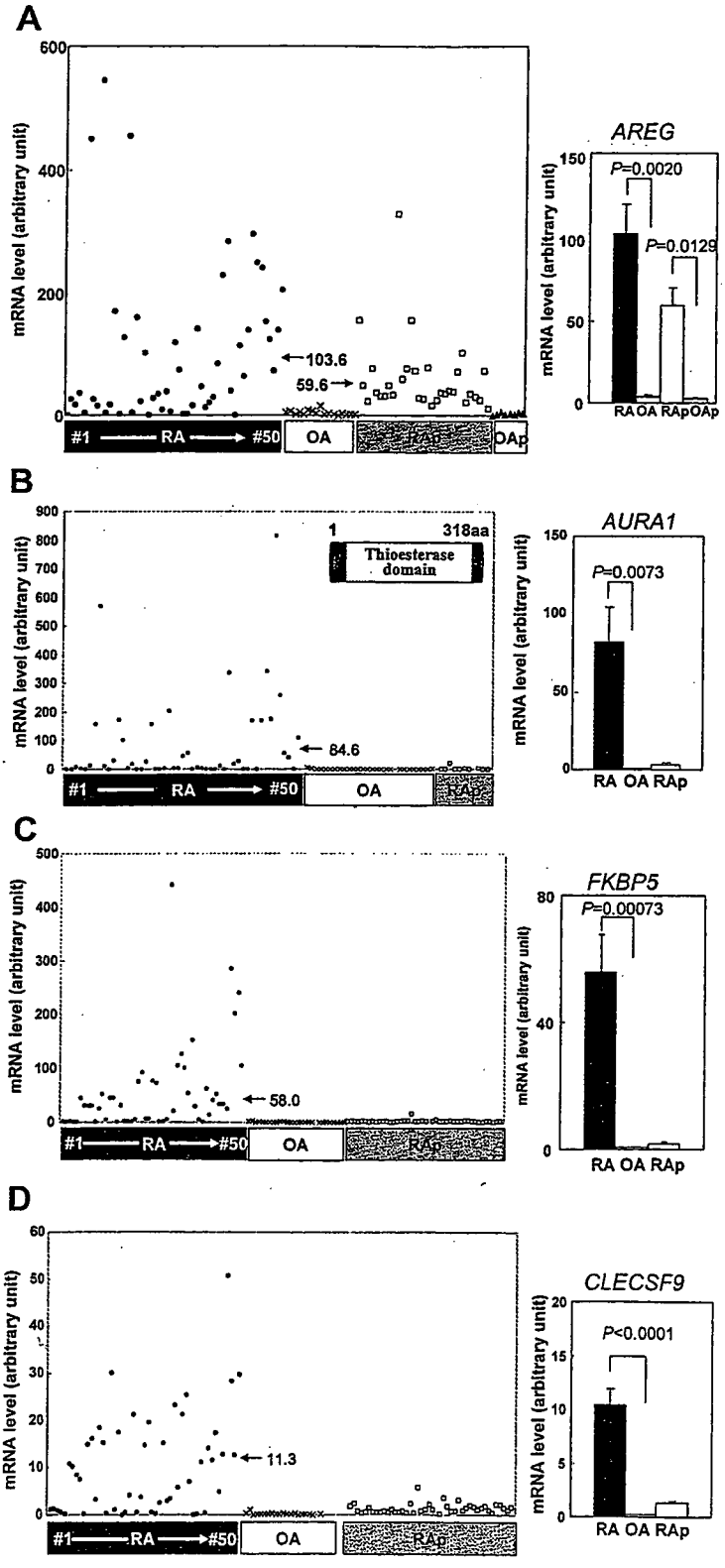
To determine whether the *AURA* genes are expressed in particular human blood cells, we performed RT–PCR on multiple tissue cDNA panels (MTC) from Clontech (Palo Alto, CA). As shown in Fig. 3, RT–PCR detected *AREG* mRNA in both monocytes (lane 4) and T and B cells (lanes 2–4), in particular in activated $CD4^+$ T cells (lane 8). *AURA1* is detected predominantly in resting $CD4^+$ (T helper/inducer; lane 3) and activated $CD4^+$ T (lane 8) cells. *CLECSF9* is expressed in most cell types except for activated $CD19^+$ T cells (lane 6), while *G0S2* is found primarily in monocytes (lanes a and 4). *FKBP5*, *TPST1*, *CXCR4*, *AURA2* and *NFκB* are ubiquitously expressed in most cell types. Thus, the analysis of the functions these *AURA* genes, apart from *AURA1* and *G0S2*, play in specific blood cells will not be easy because they are already expressed in normal blood. However, the function of *AURA1* can be studied by using $CD4^+$ T cells of RA and OA patients. In this study, however, we could not perform this analysis because of the low amounts of BMMC that we could obtain from the RA patients.

3.4. *AREG stimulates the growth of synovial cells*

Since AREG appears to be the most conspicuously unpregulated gene in many RA patients, we subjected it to further analysis. We first examined its ability to stimulate the growth of isolated synovial cells because AREG is one of the ligands of EGFR and is known to induce cell growth. Thus, we isolated synovial cells from synovial tissues that were obtained from five RA and three OA patients during joint reconstructive surgery. In the absence of AREG in the culture medium, the synovial cells from both the RA and OA patients grew at a similar rate (Fig. 4A and B). However, when AREG was present, the synovial cells from RA patients appeared to grow slightly faster than the synovial cells from OA patients, which is statistically significant ($P < 0.05$) (Fig. 4A).

To examine if this phenomenon is reflected in the signal transduction machinery of synovial cells, we investigated the activation of the EGFR signaling pathway in the AREG-treated and untreated RA synoviocytes. We first examined the phosphorylation of the extracellular signal-regulated kinases (ERK1/2) at Thr202 and Tyr204 by western blot analysis. ERK1/2 phosphorylation indicates the activation of the EGFR signaling pathway.[32] As shown in Fig. 5A, the phosphorylated ERK1/2 bands in the RA synoviocytes showed an increase in intensity when the cells had been treated with AREG; this effect peaked 8–12 h after AREG treatment but continued for 2–3 days. In contrast, the ERK1/2 protein levels remained largely unaffected by AREG treatment.

To compare the activation of EGFR signaling between RA and OA patients, we examined the activation of the EGFR signaling pathway in the synoviocytes from the five RA and three OA patients (Fig. 5B). We thus assessed the phosphorylated ERK1/2 expression levels by western blot analysis and expressed the results quantitatively by measuring the intensity of the lower phosphorylated band by densitometry and comparing it with the ERK1/2 band intensity (Fig. 5C). We found that the synoviocytes from the RA and OA patients expressed equivalent levels of EGFR and ERK1/2 proteins, regardless of AREG treatment. In contrast,



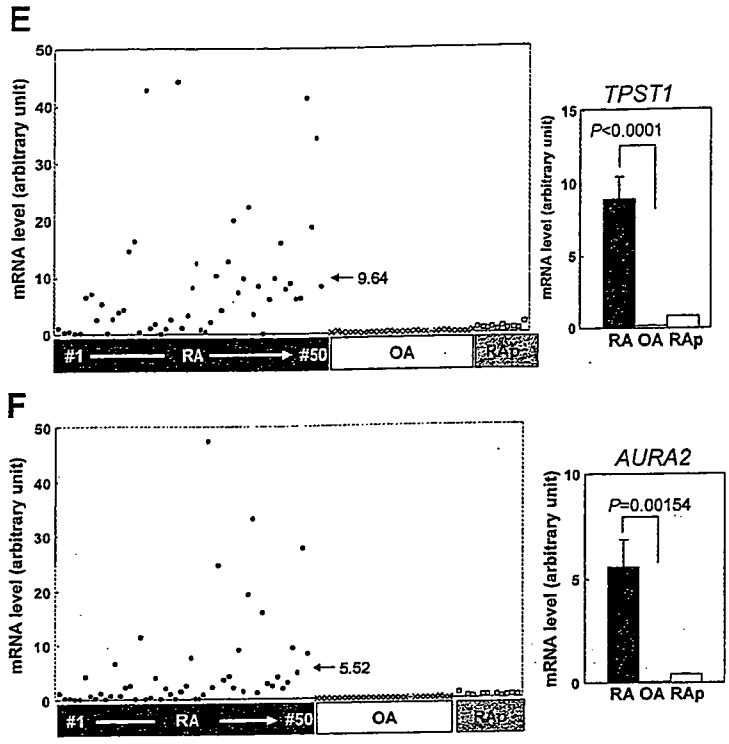


Figure 2. Expression levels of *AURA* genes in individual RA and OA patients. QRT–PCR analyses show that the mRNA levels of (A) *AREG*, (B) *AURA1*, (C) *FKBP5*, (D) *CLECSF9*, (E) *TPST1* and (F) *AURA2* are conspicuously upregulated in RA patient BMMC (and sometimes PBMC), while the BMMC and PBMC of OA patients show negligible upregulation. Expression levels in the BMMC for 50 RA patients (from #1 to 50) are arranged in the denoted order. The inset in (B) shows that the thioesterase domain occupies most of the Aural protein. The mean values of the samples analyzed in triplicate from each individual RA BMMC, RA PBMC, OA BMMC and OA PBMC are indicated by filled circles, open squares, x's, or filled triangles, respectively. The average values for the RA patient group are shown by the horizontal arrows. The bar graphs in the right panels show the average ± SE values of these measurements using the RA or OA BMMC or PBMC. All measurements are statistically significant when RA and OA are compared ($P < 0.01$).

AREG treatment upregulated the phosphorylated ERK1/2 expression levels much more strongly in the synoviocytes from RA2, RA3 and RA4 than in the synoviocytes of any of the OA patients. RA1 is an exception to this pattern as its limited phosphorylated ERK1/2 expression levels were similar to those in OA1–3. The AREG-induced upregulation of ERK1/2 phosphorylation was less apparent in the RA5 synovial cells because ERK1/2 was already activated in the absence of AREG.

Synoviolin plays a role in the synovial hyperplasia of RA by controlling the ERAD system.[10] To determine if the RA synovial cells have an abnormal ERAD system, we measured their levels of the ER stress proteins GRP78/BiP and GRP94, which protect cells from the stress-induced ER dysfunction that could lead to the accumulation of unfolded proteins.[33] We found that while the synovial cells of the RA and OA patients have similar levels of GRP78/BiP (Fig. 5B and D), the RA synoviocytes show enhanced levels of GRP94, irrespective of whether they have been stimulated with AREG. This suggests that at least part of the ER-stress responsive pathway, namely, that mediated by GRP94, is more activated in RA synoviocytes than in OA synoviocytes. Thus, the ERAD pathway does appear to be abnormally upregulated in RA synoviocytes. We confirmed by QRT–PCR that the BMMC and PBMC cells of RA patients RA1–5 show enhanced AREG mRNA levels, unlike the BMMC and PBMC of OA patients OA1–3 (Supplementary Figure S5A). Thus, chronic activation of AREG/EGFR signaling appears to be augmented in RA patients. Since AREG is expressed as transmembrane precursors that are cleaved in the extracellular domain to release soluble growth factor,[34] we speculated that the sera (PB) and bone marrow fluid (BM) of RA1–5 may show enhanced levels of cleaved AREG compared to the equivalent fluids of OA1–3. We tested this by enzyme-linked immunosorbent assay but found only one patient, RA2, showed levels of cleaved AREG that exceeded the detection level of the assay (Supplementary Figure S5B). Thus, it is not clear whether RA patients indeed secrete higher AREG levels than OA patients.

We also examined whether RA synoviocytes expressed higher synoviolin mRNA levels than OA synoviocytes in the presence or absence of AREG. However, we could not detect any significant differences between the RA and OA patients in this regard (Supplementary

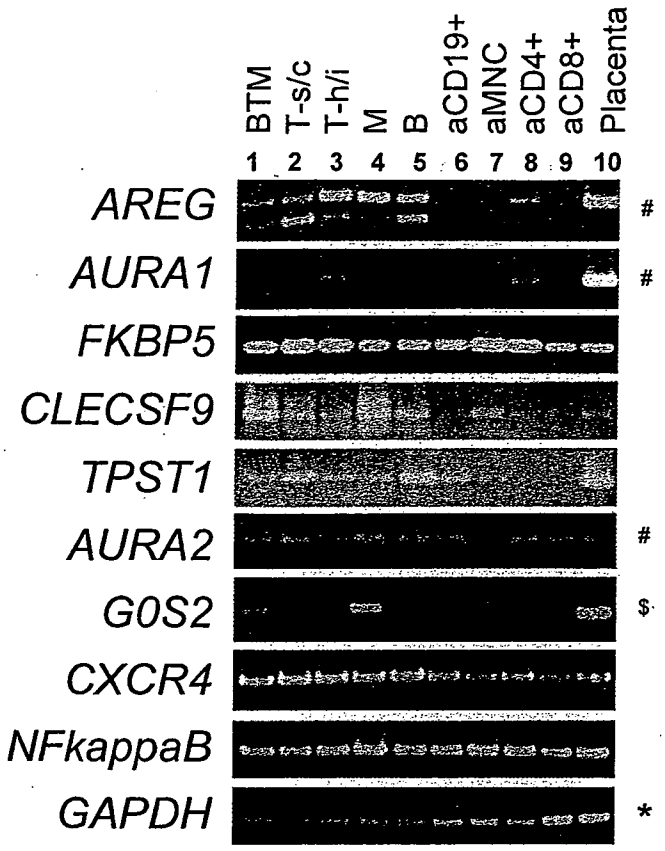


Figure 3. Determination by RT–PCR of the human blood cells that express *AREG*, *AURA1*, *FKBP5*, *CLECSF9*, *TPST1*, *AURA2*, *G0S2*, *CXCR4* and *NFκB*. RT–PCR was performed using the multiple tissue cDNA panel for human blood fractions (MTC, Clontech). *GAPDH* was also amplified as a loading control. PCR amplifications were conducted at 55°C and over 30 cycles except as indicated on the right of the panels: 55°C and 35 cycles (#), 55°C and 27 cycles (*) or 53°C and 25 cycles ($). Lane 1, mononuclear cells (B, T cells and monocytes). Lane 2, resting CD8+ cells (T-suppressor/cytotoxic cells). Lane 3, resting CD4+ cells (T-helper/inducer). Lane 4, resting CD14+ cells (monocytes). Lane 5, resting CD19+ cells (B cells). Lane 6, activated mononuclear cells. Lane 7, activated CD4+ cells. Lane 8, activated CD8+ cells. Lane 9, activated CD19+ cells. Lane 10, human placenta control cDNA served as a DNA size marker.

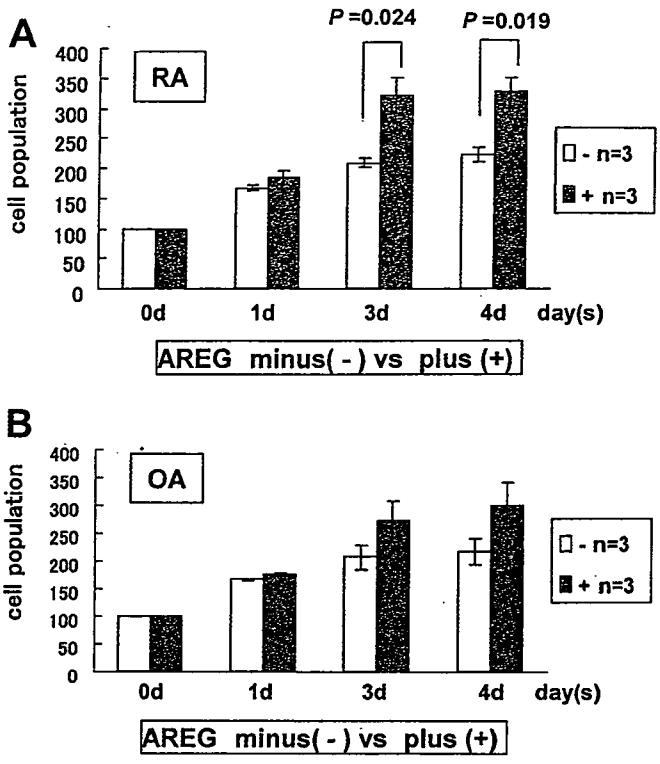


Figure 4. The effect of AREG on the proliferation of synoviocytes from RA and OA patients. The synovial cells from three RA patients (RA1, RA2 and RA3) (A) and three individual OA patients (OA1, OA2 and OA3) were counted on days 0, 1, 3 and 4 after incubation with or without AREG. The cell counts on days 1, 3 and 4 are expressed relative to 0 day. Statistically significant measurements are indicated ($P < 0.05$).

Figure S5C). It is not clear whether the synovial tissues of the patients would, like their cultured derivatives, show a similar lack of synoviolin upregulation.

4. Discussion

In this study, we report our comprehensive isolation of *AURA* genes that show augmented mRNA expression in the BMMC of RA patients as compared to their expression in OA patient BMMC (Fig. 1 and Table 1). Since RA patients suffer from defective central and peripheral B-cell tolerance checkpoints, and often display unusual immunoglobulin light chain repertoires that suggest impaired secondary recombination regulation,[18] we had expected that many immune response genes would be identified as *AURA* genes. Indeed, >10% of the *AURA* genes are directly related to immune responses; moreover, while the other *AURA* genes may seem at first glance to be unrelated to immune responses, many of these can also be linked to immune responses (Table 1). QRT–PCR analysis on individual patient samples revealed that the *AURA* genes discussed below are significantly increased in the BMMC of many of the 50 RA patients we tested (Fig. 2). Thus, the identification of these genes may help us to understand the pathogenesis of RA.

FKBP5, one of the cellular receptors for the immunosuppressant FK506, was expressed at higher mRNA levels in many RA patients than in the OA patients; this was true for the BMMC of the RA patients but not for their PBMC (Fig. 2C). FK506 has been suggested to be an effective drug for reducing the pain associated with RA.[35] This is because it can suppress inflammation by inhibiting the production by synovial cells of prostaglandin E2; it does so by suppressing the IL-1β production by leukocytes.[36] The enhanced FKBP5 expression in RA BMMC is not due to FK506 treatment since at the time of this study, treatment with FK506

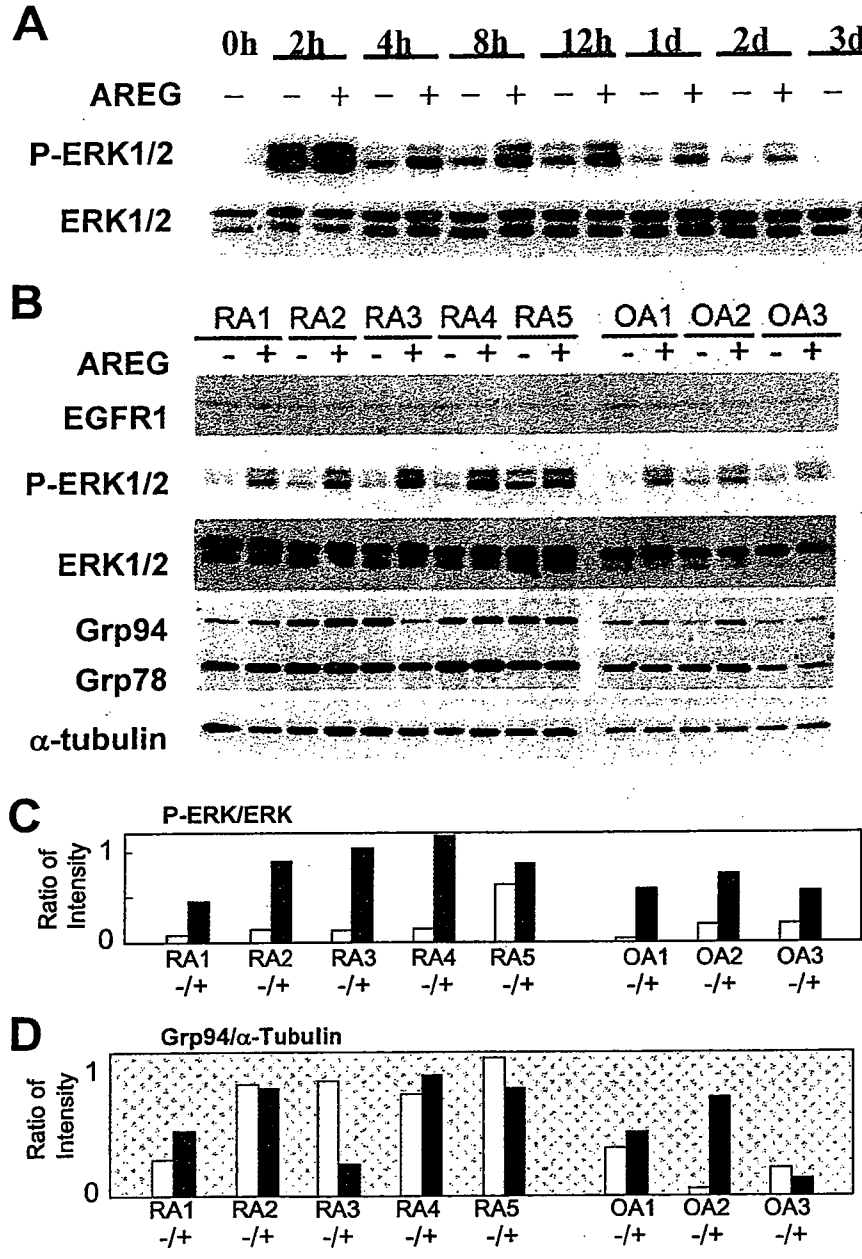


Figure 5. Western blot analysis of RA and OA synovial cells incubated in the presence or absence of AREG. (A) Expression levels of ERK1/2 and its phospho-form (P-ERK1/2) that is phosphorylated at Thr202 and Tyr204. Pooled synovial cells from five RA patients were incubated with (100 ng/ml) or without AREG for varying periods ranging from 0 h to 3 days. (B) Expression levels of EGFR1, ERK1/2, P-ERK1/2, Grp94, Grp78 and synoviolin in synovial cells from individual RA and OA patients that were incubated with or without AREG (100 ng/ml) for 8 h. Alpha-tubulin served as a loading control. (C) Relative optical densities of the western blot bands in (B) to determine P-ERK1/2 expression relative to ERK1/2 expression. (D) Relative optical densities of the western blot bands in (B) to determine Grp94 expression relative to alpha-tubulin expression.

was not permitted in Japan; consequently, none of the patients tested here have ever received FK506. In addition, the enhanced FKBP5 expression by RA BMMC does not correlate with therapeutic treatment using steroids. It remains possible, however, that the increased FKBP5 mRNA levels in the BMMC of RA patients may be due to treatment with other drugs. Alternatively, it may reflect genuine and spontaneous pathological events. Nevertheless, regardless of the cause of its elevated expression, the augmented FKBP expression may strongly inhibit the phosphatase activity of calcineurin, which could increase the dephosphorylation and thus inactivation of various substrates, including the NFAT family proteins and cytokines that are required for the expression of immunoregulatory molecules.

TPST1 mediates tyrosine sulfation within the trans-Golgi system, which affects 1% of all tyrosines in eukaryotic cells. It has been previously suggested that this post-translational modification may play an

important role in the pathogenesis of autoimmune diseases because it regulates mononuclear cell function at various stages of the immune response by enhancing interactions between ligands and receptors.[37] Notably, of the 62 identified target proteins of tyrosine sulfation, nine are cell adhesion molecules and chemokine receptors, which are both central players in leukocyte trafficking. Thus, the augmented expression of *TPST1* in RA patients may elevate the sulfation of crucial tyrosine residues in chemokine receptors that could constitutively increase their binding affinities with their ligands (e.g. the binding of CXCL12–CXCR4).

CLECSF9 belongs to the macrophage-inducible C-type lectin that serves multiple functions by recognizing carbohydrate chains; it plays important roles in macrophage function. Notably, a C-type lectin called DC-specific intercellular adhesion molecule 3-grabbing non-integrin is also highly expressed by macrophages in the synovium of RA patients.[38] However, the HH mRNA expression of macrophage-inducible C-type lectins is strongly induced in response to several inflammatory stimuli. Thus, the augmented expression of *CLECSF9* in the BMMC of RA patients may simply be due to the inflammation in the joint.

Unlike *FKBP5* and *TPST1* genes, the mRNA levels of G0S2, CXCR4 and NF-κB are increased in both the BMMC and PBMC of RA patients (Fig. 2 and Supplementary Figure S3). We previously showed that the PBMC of both systemic lupus erythematosus (SLE) patients and healthy young females express enhanced levels of *G0S2* mRNA.[26] Thus, G0S2 may not actually be involved in the pathogenesis of RA. With regard to the chemokine receptor CXCR4, it was also identified as a inflammation-related gene that is upregulated in synovial cells of patients with pigmented villonodular synovitis (PVNS), which is a joint problem that usually affects the hip or knee and involves the lining of the joint becoming swollen and growing.[8] The enhanced tyrosine sulfation of CXCR4 by augmented TPST1 activity, as described above, may also activate CXCR4, thereby elevating the ability of the CXCR4 ligand to induce the migration of bone marrow cells that could enhance the growth of synovial cells.[39] CXCR4 expression is also upregulated in the spinal cord of animals with experimental autoimmune encephalomyelitis, which is an animal model of autoimmune central nervous system inflammation.[40] With regard to NF-κB, this molecule along with the receptor activator of NF-κB (RANK) and its ligand RANKL have been found to play pivotal roles in the pathophysiological process of RA.[41] Thus, the increased mRNA levels of NF-κB in both the BMMC and PBMC of RA patients may contribute to the bone destruction mediated by activated NF-κB signaling pathway.[42]

AURA1 encodes a novel protein that is similar to thioesterase. Since the thioesterase homologs are widespread, functions of thioesterase vary in the human genome.[43] Thus, the physiological function of *AURA1* remains unknown. A possible role that it could play in RA pathogenesis is suggested by the following observations. First, the stable overexpression of acyl-CoA thioesterase III in human and murine T-cell lines increased both peroxisome numbers and lipid droplet formation, which suggests that it participates in the metabolic regulation of peroxisome proliferation in T cells.[44] Second, altered immune responsiveness is observed in mice deficient in palmitoyl protein thioesterase (PPT1) gene that is mutated in infantile neuronal ceroid lipofuscinosis.[45] Third, $CD4^+$ T cells are the prime mediators of RA in a mouse model SKG strain,[46] and *AURA1* expression is detected predominantly in resting and activated $CD4^+$ T cells (Fig. 3).

AREG is not directly related to immune responses but of all the genes examined, it showed the most conspicuously enhanced expression in both the BMMC and PBMC of many RA patients (Fig. 2A). We also found that the synovial cells of RA patients showed higher sensitivity to AREG, in terms of proliferation, than those of OA patients (Fig. 4). This is not due to augmented expression of EFGR (Fig. 5B, uppermost pane), but due to elevated activation of EGFR signaling pathway because the phosphorylation of ERK1/2 was more enhanced in AREG-treated RA patient synovial cells than that of AREG-treated OA patient synovial cells (Fig. 5). We here present a working hypothesis to explain how augmented AREG expression in BMMC and PBMC of RA patients and subsequent activation of EGFR signaling pathway lead to hyperproliferation of synovial cells in the joints of the RA patients (Fig. 6). Namely, this enhanced phosphorylation of ERK1/2 elevates the expression of many downstream target genes, which may also require the activation of the ERAD system. [12] Given that the Ets-binding site (EBS) of the proximal promoter of the synoviolin gene is responsible for its expression,[47] and that EBS-carrying genes are also activated by signaling events from the ERK pathway,[48] it is possible that the enhanced activation of EGFR signaling induced by AREG may directly activate the expression of synoviolin as well as that of other genes, thereby inducing the hyperproliferation of synovial cells. Thus, it is possible that the ERAD system in RA patients is hyperactivated by synoviolin because of augmented AREG expression in blood cells, possibly in the macrophages that occur in the vicinity of the synovial cells of RA patients, releasing augmented amount of AREG. This hypothesis should be tested more rigourously *in vivo* in the future because the experiments using the isolated synoviocyte cells in tissue culture medium may display distinct response to AREG. Likewise, examination of other EGF family proteins *in vivo* can also be interesting future subjects.

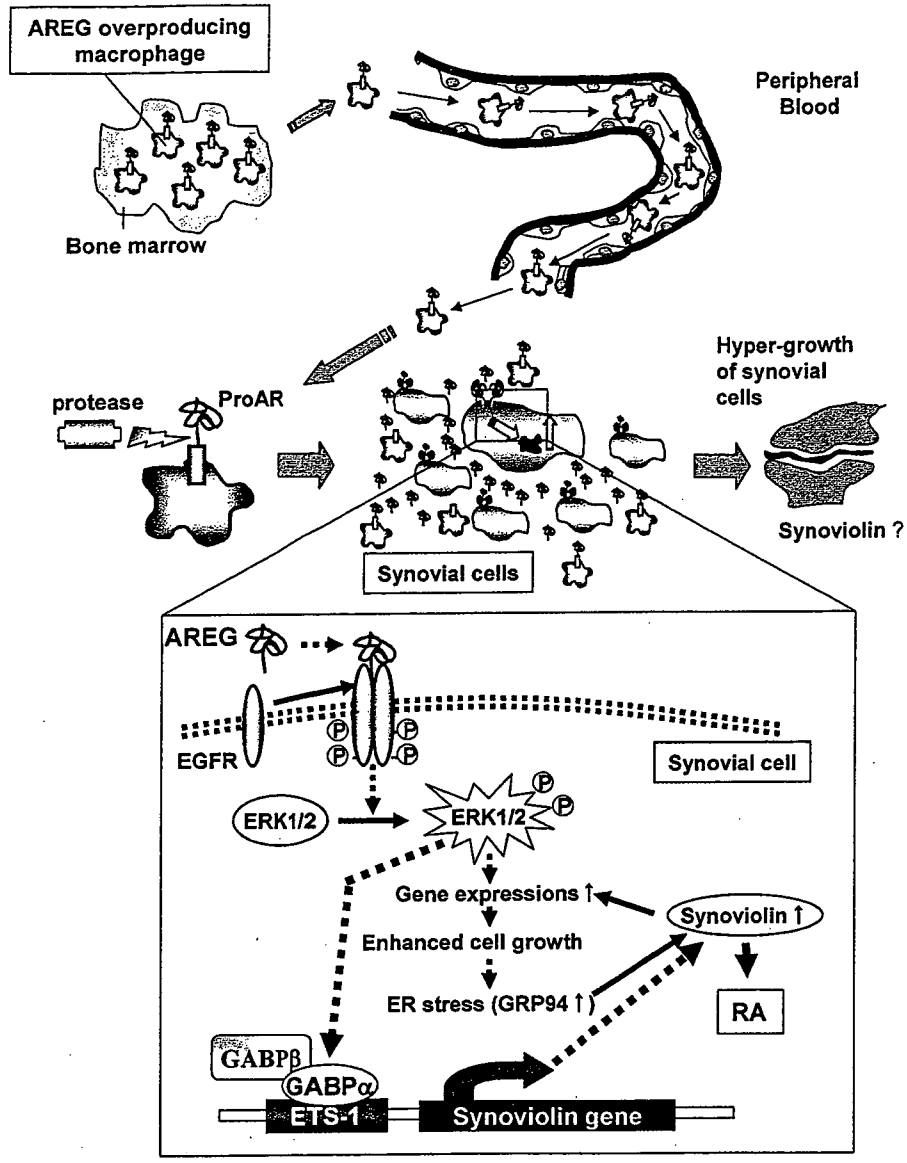


Figure 6. A working hypothesis to explain how augmented level of AREG in BMMC of RA patients may lead to hyperproliferation of synovial cells. Putative macrophages with enhanced expression of AREG precursor (ProAR) may approach to the synovial cells of the joint through blood flow, where they release AREG and activate the EGFR signaling pathway of synovial cells. Since Ets-binding site (ETS-1) of the proximal promoter of the synoviolin gene is one of the downstream targets of ERK pathway, the enhanced activation of EGFR signaling may directly activate the expression of synoviolin gene. The enhanced level of synoviolin activates the ERAD system, which may lead to hyperproliferation of synovial cells.

Overexpression of AREG has been linked to psoriasis in mice and humans.[49,50] Psoriasis is characterized by the hyperproliferation of keratinocytes and the loss of epidermal barrier function that leads to the infiltration of inflammatory cells into the epidermis and dermis.[51] AREG is also upregulated in a synoviocyte cell line derived from an RA patient in which the wild type and a dominant negative form of the orphan nuclear receptor Nurr1 were overexpressed.[52] Interestingly, AREG overexpression in the basal epidermis of transgenic mice induces a phenotype that is associated with synovial membrane inflammation.[49] Moreover, we showed previously that AREG expression is also enhanced in the PBMC of SLE and idiopathic thrombocytopenic purpura patients,[26] which suggests that AREG overexpression may also be associated with other autoimmune diseases. Notably, metalloprotease-mediated AREG shedding and the subsequent activation of EGFR appears to play a critical role in the secretion of IL-8 by the human airway epithelium-like NCI-H292 cells that is induced by tumor necrosis factor-α (TNF-α), a potent multifunctional cytokine that plays a central role in the pathogenesis of many inflammatory diseases like RA.[53] Since TNF-α-induced IL-8 secretion was completely inhibited by the neutralizing antibody against AREG,[53] this antibody could constitute a novel therapeutic tool for RA. Taken