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*

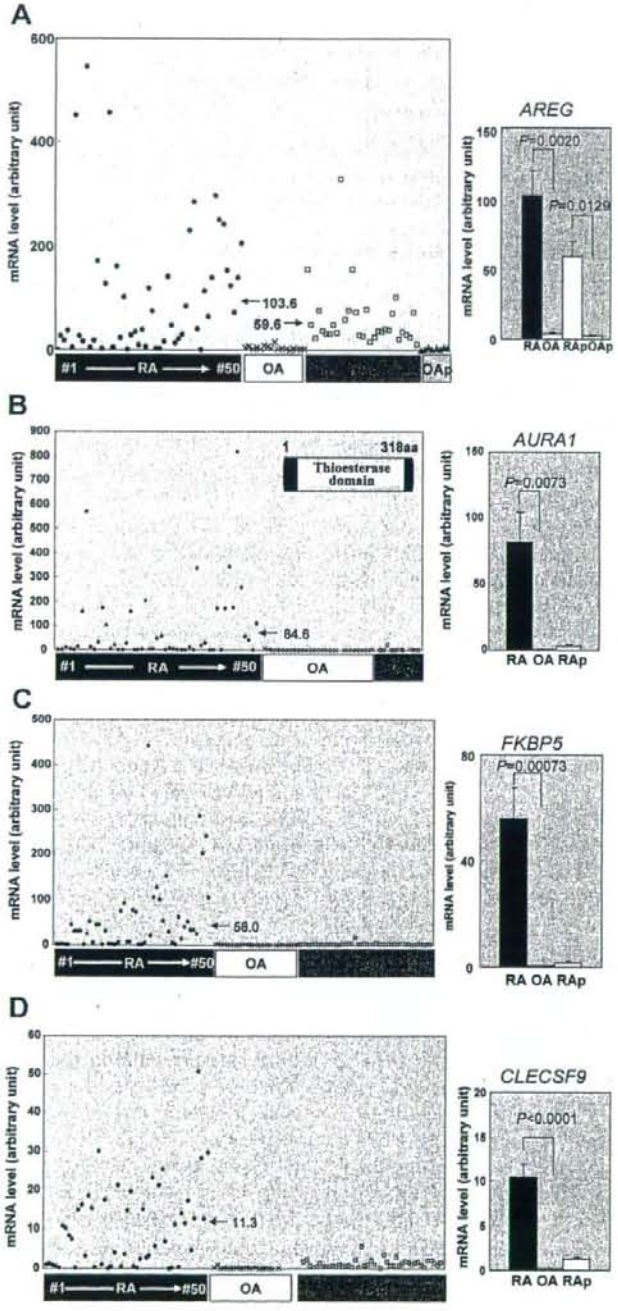
To determine whether the *AURA* genes are expressed in particular human blood cells, we performed RT–PCR on multiple tissue cDNA panels (MTC) from Clontech (Palo Alto, CA). As shown in Fig. 3, RT–PCR detected *AREG* mRNA in both monocytes (lane 4) and T and B cells (lanes 2–4), in particular in activated $CD4^+$ 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,



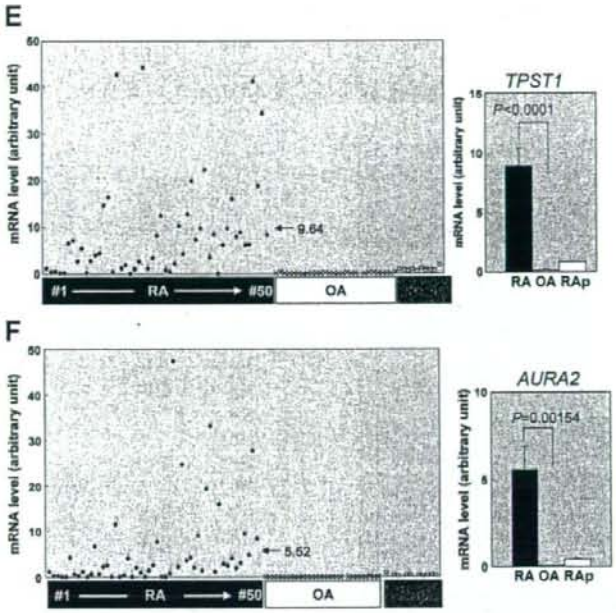


Figure 2. Expression levels of *AURA* genes in individual RA and OA patients. QRT–PCR analyses show that the mRNA levels of (A) *AREG*, (B) *AURA1*, (C) *FKBP5*, (D) *CLECSF9*, (E) *TPST1* and (F) *AURA2* are conspicuously upregulated in RA patient BMMC (and sometimes PBMC), while the BMMC and PBMC of 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 Aura1 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

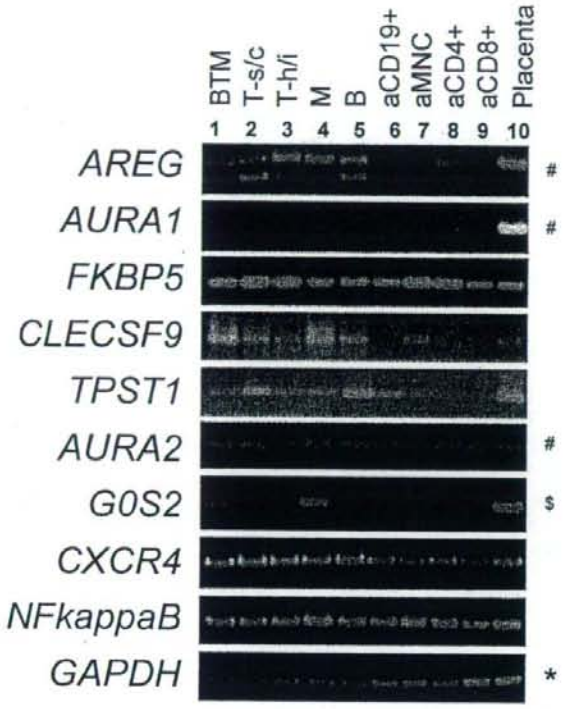


Figure 3. Determination by RT–PCR of the human blood cells that express *AREG, AURA1, FKBP5, CLECSF9, TPST1, AURA2, G0S2, CXCR4* and *NFκB*. RT–PCR was performed using the multiple tissue cDNA panel for human blood fractions (MTC, Clontech). *GAPDH* was also amplified as a loading control. PCR amplifications 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.

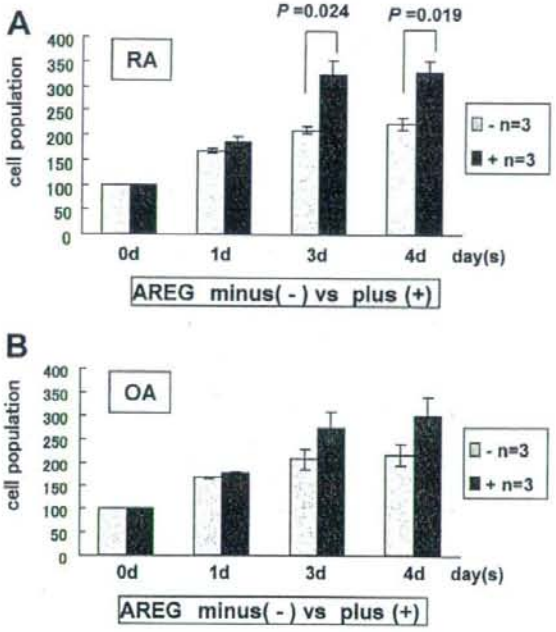


Figure 4. The effect of AREG on the proliferation of synoviocytes from RA and OA patients. The synovial cells from three RA patients (RA1, RA2 and RA3) (A) and three individual OA patients (OA1, OA2 and OA3) were counted on days 0, 1, 3 and 4 after incubation with or without AREG. The cell counts on 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

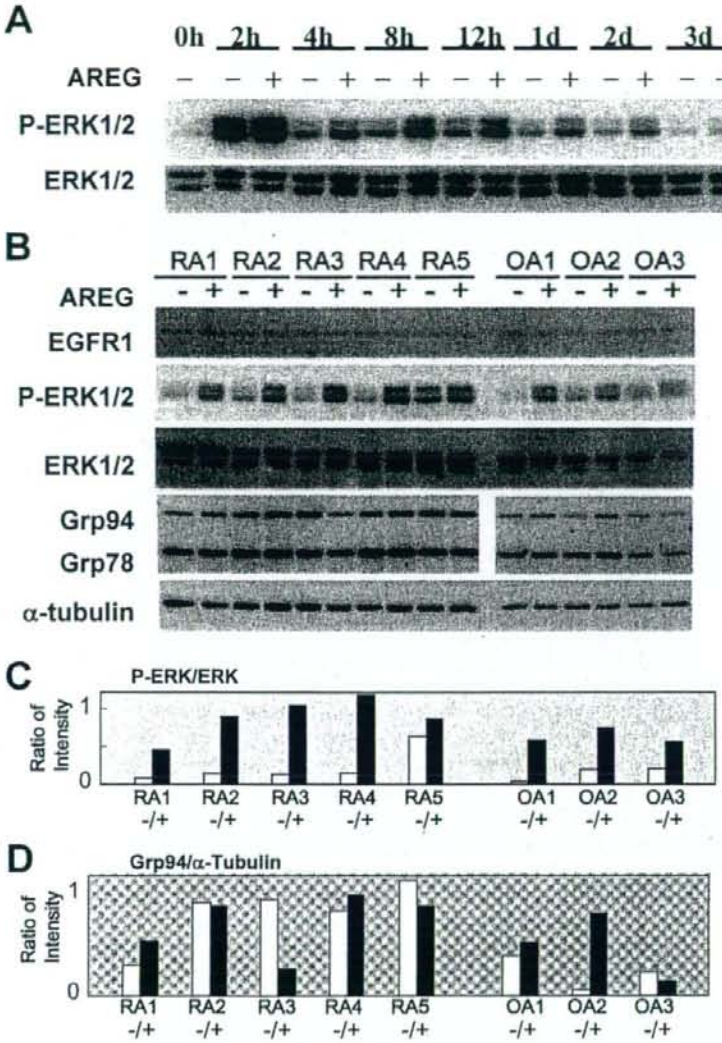


Figure 5. Western blot analysis of RA and OA synovial cells incubated in the presence or absence of AREG. (A) Expression levels of ERK1/2 and its phospho-form (P-ERK1/2) that is phosphorylated at Thr202 and Tyr204. Pooled synovial cells from five RA patients were incubated with (100 ng/ml) or without 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.

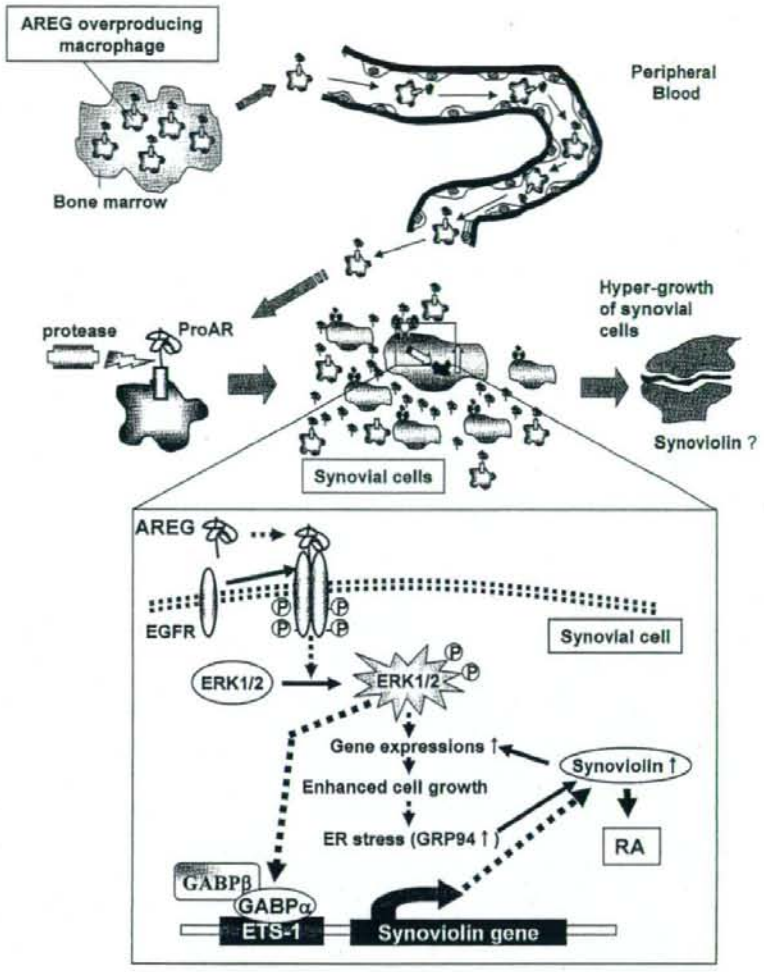


Figure 6. A working hypothesis to explain how augmented level of AREG in BMMC of RA patients may lead to hyperproliferation of synovial cells. Putative macrophages with enhanced expression of AREG precursor (ProAR) may approach to the synovial cells of the joint through blood flow, where they release 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

together, we propose that enhanced expression of AREG in BMMC and PMBC may play a pivotal role in the pathogenesis of RA.

Acknowledgements: We thank the patients and healthy volunteers who participated in this study. We also thank Ms Tomoko Motoyama, Ms Kumiko Ikeue, Ms Maki Masuda, and Ms Yuki Hamada for technical assistance and Dr Patrick Hughes for critically reading the manuscript. This work was primarily supported by a grant-in-aid from the Health Science Research grant from the Ministry of Health and Welfare of Japan. This work was also supported in part by Innovation Plaza Osaka of the Japan Science and Technology Agency (JST), and by grants-in-aid for Scientific Research on Priority Areas Applied Genomics, Scientific Research (S), Exploratory Research, and the Science and Technology Incubation Program in Advanced Regions, from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Supplementary Data: Supplementary data are available online at www.dnaresearch.oxfordjournals.org.

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Proinflammatory role of amphiregulin, an epidermal growth factor family member whose expression is augmented in rheumatoid arthritis patients

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Published: 27 April 2008

Journal of Inflammation 2008, **5**:5 doi:10.1186/1476-9255-5-5

Received: 11 October 2007
Accepted: 27 April 2008

This article is available from: http://www.journal-inflammation.com/content/5/1/5

Abstract

Background: The epidermal growth factor (EGF) and EGF receptor (EGFR) families play important roles in the hyperplastic growth of several tissues as well as tumor growth. Since synovial hyperplasia in rheumatoid arthritis (RA) resembles a tumor, involvement of the EGF/EGFR families in RA pathology has been implied. Although several reports have suggested that ErbB2 is the most important member of the EGFR family for the synovitis in RA, it remains unclear which members of the EGF family are involved. To clarify the EGF-like growth factors involved in the pathology of RA, we investigated the expression levels of seven major EGF-like growth factors in RA patients compared with those in osteoarthritis (OA) patients and healthy control subjects.

Methods: The expression levels of seven EGF-like growth factors and four EGFR-like receptors were measured in mononuclear cells isolated from bone marrow and venous blood, as well as in synovial tissues, using quantitative RT-PCR. Further evidence of gene expression was obtained by ELISAs. The proinflammatory roles were assessed by the growth-promoting and cytokine-inducing effects of the corresponding recombinant proteins on cultured fibroblast-like synoviocytes (FLS).

Results: Among the seven EGF-like ligands examined, only amphiregulin (AREG) was expressed at higher levels in all three RA tissues tested compared with the levels in OA tissues. The AREG protein concentration in RA synovial fluid was also higher than that in OA synovial fluid. Furthermore, recombinant human AREG stimulated FLS to proliferate and produce several proinflammatory cytokines, including angiogenic cytokines such as interleukin-8 and vascular

endothelial growth factor (VEGF), in a dose-dependent manner. The VEGF mRNA levels in RA synovia and VEGF protein concentrations in RA synovial fluid were significantly higher than those in the corresponding OA samples and highly correlated with the levels of AREG.

Conclusion: The present findings suggest that AREG functions to stimulate synovial cells and that elevated levels of AREG may be involved in the pathogenesis of RA.

Background

Rheumatoid arthritis (RA) is a chronic inflammatory disease that is mainly characterized by synovial hyperplasia and progressive destruction of the affected joints. Activated synoviocytes in the hypertrophic synovia induce angiogenesis, and play pivotal roles in the recruitment and differentiation of inflammatory cells. However, the driving force of the synovial hyperplasia remains obscure.

The granulomatous tissues of RA synovia, referred to as pannuses, resemble tumors. Cultured fibroblast-like synoviocytes (FLS) from these pannuses share some features with transformed cells, *i.e.* anchorage-independent growth [1,2] and downregulation of tumor suppressors [3-5]. Similar to transformed cells, tyrosine-phosphorylated proteins are augmented in RA-FLS, and several growth factors whose receptors possess tyrosine kinase activities have been reported to promote the tumor-like behavior of RA synovial membranes [6-9]. Since platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) stimulate DNA synthesis and proliferation of FLS cultured in medium containing low concentrations of serum [10] and histochemical studies have revealed upregulated expression levels of PDGF and FGF and their receptors in RA synovial tissues [11-13], these molecules are considered to be the major contributors to synovial hyperplasia [2,14].

On the other hand, the proto-oncogene c-erb-B, referred to as epidermal growth factor (EGF) receptor (EGFR), is a well-known tyrosine kinase growth factor receptor. Four members of the EGFR family have been identified to date, namely c-erb-B/EGFR and its related products ErbB2, ErbB3 and ErbB4. The family members form homodimers or heterodimers in various combinations, and exhibit different ligand specificities for the 13 members of the EGF family [15]. Although expression of ErbB2, but not the other ErbB-related receptors, has been reported to be augmented in RA synovial tissues [7,16,17], it remains unknown which members of the EGF family are expressed in the affected joints and involved in the pathology of RA.

In previous studies, we investigated the involvement of bone marrow in the pathology of RA. An increase in myeloid cells expressing abnormal surface antigens in bone marrow was associated with the severity of RA [18-23]. Pathogenic synovial fibroblasts may be derived from bone marrow $CD34^+$ cells in RA [24]. Recently, we identified RA-associated genes in bone marrow cells using a cDNA subtraction technique [25]. In that report, we demonstrated that two EGF-like growth factors, amphiregulin (AREG) and epiregulin (EREG), were upregulated in RA bone marrow.

In the present study, we examined the extents of involvement of EGF family members in RA pathology by investigating the expression of seven major EGF-like growth factors, namely EGF, AREG, EREG, transforming growth factor α(TGFα), heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC) and neuregulin-1 (NRG1), in synovial tissues and mononuclear cells isolated from bone marrow and venous blood. The results revealed that AREG expression was augmented in all RA tissues and cells examined. Moreover, the AREG protein concentration in RA synovial fluid was significantly higher than that in osteoarthritis (OA) synovial fluid. Recombinant human AREG stimulated RA-FLS to proliferate and express several proinflammatory cytokines. These findings suggest that AREG may play a role in the pathogenesis of RA.

Methods

Patients and samples

Bone marrow fluid, venous blood and/or synovial tissues were intraoperatively obtained from 15 RA patients (all women; mean age ± SD: 59.3 ± 8.7 years) and 12 OA patients (all women; mean age ± SD: 64.5 ± 11.8 years) undergoing joint arthroplasty. None of the patients had taken any medication for at least 1 week before the operation. The RA and OA patients fulfilled the 1987 revised criteria of the American College of Rheumatology for the classification of RA [26] and the diagnostic criteria for OA [27], respectively. Bone marrow fluid and venous blood were mixed with heparin and separated by centrifugation at 1700 *g* for 15 min. After removal of the plasma, the blood cells and bone marrow cell fractions were adjusted to their original volumes with Hank's balanced salt solution (HBSS) and fractionated by density-gradient centrifugation at 3000 *g* for 30 min on Ficoll-Hypaque (GE Healthcare Bioscience, Tokyo, JPN). Mononuclear cells were collected from both the bone marrow and peripheral blood and used for the experiments described below. For further separation, the collected mononuclear cells were fractionated by magnetic beads coated with immobilized

CD14, CD3 or CD19 antibodies (Miltenyi Biotec, Tokyo, JPN), since CD14, CD3 and CD19 are lineage-specific markers for monocytes, T lymphocytes and B lymphocytes, respectively. The cell populations fractionated by these antibodies were measured using flow cytometry, and confirmed to be > 95% pure. Synovial fluid was obtained from 24 RA patients and 10 OA patients and venous blood was obtained from 57 RA patients and 12 OA patients attending the outpatient clinic of our hospital. Synovial fluid was separated from cells and debris by centrifugation, and the clear supernatant was collected. Plasma was collected by centrifuging heparinized blood as described above. The synovial fluid and plasma samples were analyzed by ELISAs. All patients and healthy volunteers provided informed consent for participation in the study, which was approved by the Ethical Committee of the National Hospital Organization, Sagamihara National Hospital.

Isolation of FLS and establishment of cell lines

Synovial membranes were minced aseptically and then digested enzymatically with 1 mg/ml collagenase (Wako, Osaka, JPN) in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY, USA) for 2 h at 37°C. Single cell suspensions were filtered through a nylon mesh, seeded in culture dishes containing DMEM supplemented with 100 units/ml penicillin, 0.1 mg/ml streptomycin (GIBCO) and 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), and cultured at 37°C in humidified air containing 7.5% CO_2. Since freshly isolated FLS contain many lymphocytes, monocytes and granulocytes, we used homogeneous fibroblastic cell populations after more than 4 passages. In proliferation assays, FLS cultures were stimulated by various concentrations of recombinant human AREG (R&D Systems, Tokyo, JPN) for 2 days. Prior to cell harvesting onto glass fiber disks, FLS were cultured with ^{3}H-thymidine for 18 h. The radioactivities on the disks were measured using a liquid scintillation counter.

RNA extraction and cDNA synthesis

Total cellular RNAs were extracted using the TRIZOL™ reagent (Invitrogen, Tokyo, JPN) according to the manufacturer's instructions. For RNA extraction from synovia, minced tissues were homogenized in TRIZOL using a Polytron homogenizer and the extracted RNAs were further purified using an RNeasy micro kit (QIAGEN, Tokyo, JPN). In cytokine induction assays, FLS cultures were stimulated by various concentrations of recombinant human AREG and/or genistein (SIGMA, Tokyo, JPN) for 3 h and subjected to RNA extraction using the RNeasy micro kit. First-strand cDNAs were synthesized from 2 μg of total RNAs by priming with oligo dT and Omniscript™ reverse transcriptase (QIAGEN) according to the manufacturer's instructions.

Quantitative RT-PCR

Using real-time PCR, we estimated the mRNA expression levels of four EGFR family members and seven EGF family members. In subsequent investigations, the mRNA expression levels of five proinflammatory cytokines, namely interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor-α (TNF-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF) were measured. The mRNA expression levels of vascular endothelium growth factor (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), a disintegrin and metalloproteinase (ADAM) 10 and ADAM17 were also measured. The primer sequences used were: 5'-GTGATTCCATCATGTATCCCAGGAG-3', 5'-AGATGCACTGTCCATGCAAACAA-3' (EREG); 5'-CTTCACTGTGTGGTGGCAGATG-3', 5'-ATGCAGTAATGCTTGTATTGCTTGG-3' (BTC); 5'-CAACCAGTGGCTGGTGAGGA-3', 5'-GAGCCCTTATCACTGGATACTGGAA-3' (EGF); 5'-GTGGTGCTGTCGCTCTTGATACTC-3', 5'-TCAAATCCATCAGCACTGTGGTC-3' (AREG); 5'-GGGCATGACTAATTCCCACTGA-3', 5'-GCCCAATCCTAGACGGCAAC-3' (HB-EGF); 5'-AGATAGACAGCAGCCAACCCTGA-3', 5'-CTAGGGCCATTCTGCCCATC-3' (TGFα); 5'-AGAATGTGCCCATGAAAGTCCAA-3', 5'-GCAGATGCCGGTTATGGTCAG-3' (NRG1); 5'-GGTGCGAATGACAGTAGCATTATGA-3', 5'-AAAGGTGGGCTCCTAACTAGCTGAA-3' (EGFR); 5'-CAGGCACCGCAGCTCATCTA-3', 5'-TCCCAGGTCACCATCAAATACATC-3' (ErbB2); 5'-CCCAGCATCTGAGCAAGGGTA-3', 5'-TTTAGGCGGGCATAATGGACA-3' (ErbB3); 5'-TGATAGGCCGTTGGTTGTCTGA-3', 5'-CCAGGTAGACATACCCAATCCAGTG-3' (ErbB4); 5'-CCCACTGAGGAGTCCAACAT-3', 5'-AAATGCTTTCTCCGCTCTGA-3' (VEGF); 5'-CTCTGATCATGCTAATGGCTGGA-3', 5'-GCTGCAGTTAGCGTCTCATGTGT-3' (ADAM10); 5'-GTGACATGAATGGCAAATGTGAG-3', 5'-AGACCCAACGATGTTGTCTGCTA-3' (ADAM17); 5'-CCCCTGCCCATTCGGAGGAAGAG-3', 5'-TTGGCCACCTTGACGCTGCGGTG-3' (PDGF); 5'-GTTGTGACAACCACAAGCAC-3', 5'-CTCTCACACTATCCACTGGT-3' (bFGF); 5'-ACACTGCGCCAACACAGAAATTA-3', 5'-TTTGCTTGAAGTTTCACTGGCATC-3' (IL-8); 5'-AAGCCAGAGCTGTGCAGATGAGTA-3', 5'-TGTCCTGCAGCCACTGGTTC-3' (IL-6); 5'-CCAGGGACAGGATATGGAGCA-3', 5'-TTCAACACGCAGGACAGGTACAG-3' (IL-1β); 5'-CATGATGGCCAGCCACTACAA-3', 5'-ACTGGCTCCCAGCAGTCAAAG-3' (GM-CSF); 5'-GACAAGCCTGTAGCCCATGTTGTA-3', 5'-CAGCCTTGGCCCTTGAAGA-3' (TNF-α). Real-time PCR was performed using a LightCycler 2.0 (Roche Diagnostics, Tokyo, JPN) and SYBR Premix Ex Taq (Takara, Kyoto, JPN) following the manufacturers' protocols. The amounts of PCR products were

assessed by the fluorescence of SYBR Green intercalated in the DNA fragments, and melting curves were routinely recorded to verify the singularity of the products. The amplified products using each primer pair were cloned into the pGEM-T vector (Promega, Tokyo, JPN) and plasmids linearized by enzymatic digestion were used as quantification standards. A reference cDNA was used in every assay to control the precision among assays. The cDNA levels among the samples were normalized by the expression level of the internal control gene GAPDH (5'-GCACCGTCAAGGCTGAGAAC-3', 5'-ATGGTGGTGAA-GACGCCAGT-3').

ELISAs

The AREG protein concentrations in plasma samples from 57 RA patients, 12 OA patients and 9 healthy volunteers and synovial fluid samples from 24 RA patients and 8 OA patients were determined using an AREG Duo-set ELISA kit (R&D Systems). The protein concentrations of IL-1β, IL-6, IL-8, TNF-α, GM-CSF and VEGF in culture supernatants of RA-FLS stimulated with recombinant human AREG (R&D Systems) and those of VEGF and IL-8 in synovial fluid samples from 9 RA patients and 7 OA patients were determined using a Quantikine ELISA kit (R&D Systems).

Statistical analysis

Statistical analysis was carried out using the StatView statistical analysis software (SAS, Cary, NC, USA). Differences between RA specimens and controls were determined to be significant when $P < 0.05$ by the Mann-Whitney U-test. The effects of AREG on RA-FLS were analysed by the Mann-Whitney U-test following the Kruskal-Wallis test. Correlation coefficients (ρ) were calculated by Spearman's rank correlation method and tested for statistical significance at the 0.05 (two-tailed) level.

Results

Expression profiles of EGF family members in bone marrow mononuclearcells (BMMCs)

First, we determined the mRNA expression levels of seven EGF family members in BMMCs obtained from 9 RA patients and 10 OA patients (Fig. 1A). EREG was the most abundantly expressed, and its mRNA level in RA-BMMCs was significantly higher than that in OA-BMMCs ($P = 0.0060$). The expression levels of AREG, TGFα and EGF were about 10-fold lower than that of EREG in OA-BMMCs, but significantly upregulated in RA-BMMCs ($P = 0.0258$, $P = 0.000045$ and $P = 0.0140$, respectively). Although the expression of HB-EGF was the next most abundant in RA-BMMCs, there was no significant difference between its expression levels in RA- and OA-BMMCs. The BTC and NRG1 mRNA expression levels were almost undetectable in both RA- and OA-BMMCs.

Expression profiles of EGF family members in peripheral blood mononuclear cells (PBMCs)

Next, we determined the mRNA expression levels of the seven EGF family members in PBMCs obtained from 8 RA patients and 7 OA patients (Fig. 1B). EREG, TGFα and EGF were highly abundantly expressed in PBMCs, and their mRNA levels in RA- and OA-PBMCs did not differ. The expression levels of AREG, HB-EGF and NRG1 were about 10-fold lower than the levels of the highly abundant members in OA-PBMCs. Although AREG and HB-EGF were markedly upregulated in RA-PBMCs ($P = 0.0017$ and $P = 0.0367$, respectively), NRG1 was not upregulated in RA-PBMCs. BTC was not detected in either type of PBMCs.

Expression profiles of EGF-like growth factors in synovial tissues

Next, we determined the mRNA expression levels of the seven EGF family members in synovial tissues obtained from 14 RA patients and 5 OA patients (Fig. 1C). Although HB-EGF was the most abundantly expressed and EREG was the next most abundantly expressed in synovial tissues from both RA and OA joints, their mRNA levels in RA synovia were somewhat lower than those in OA synovia. On the other hand, AREG expression, which was 1000-fold lower than HB-EGF expression in OA synovia, was markedly upregulated in RA synovial tissues ($P = 0.0110$). Expression of EGF, BTC or NRG1 was not detected in either OA or RA joints. Since only AREG expression was augmented in BMMCs, PBMCs and synovia of RA patients compared with the levels in control samples among the seven EGF-related growth factors examined, we narrowed the focus of the study to AREG.

Determination of plasma and synovial fluid concentrations of AREG

To confirm whether the protein concentration of AREG was augmented in RA patients, the AREG concentrations in plasma and synovial fluid samples were examined by ELISA. As shown in Fig. 2A, there were no significant differences among the AREG protein concentrations in the RA, OA and healthy control (HC) plasma samples, whereas the AREG concentration in RA synovial fluid samples was significantly higher than that in OA synovial fluid samples. Evaluation of BMMC, PBMC and plasma samples from 5 RA patients revealed that the AREG mRNA levels in RA-PBMCs were highly correlated with those in RA-BMMCs (data not shown), but not correlated with the plasma concentrations of this protein (Fig. 2B). Besides transformed cells, AREG-producing cells were previously reported to be activated monocytes [28] and activated T lymphocytes [29]. To clarify which lineage of blood cells expressed AREG in RA, PBMCs from RA patients were fractionated using magnetic beads coated with immobilized CD14, CD3 or CD19 antibodies. As shown in Fig. 2C,

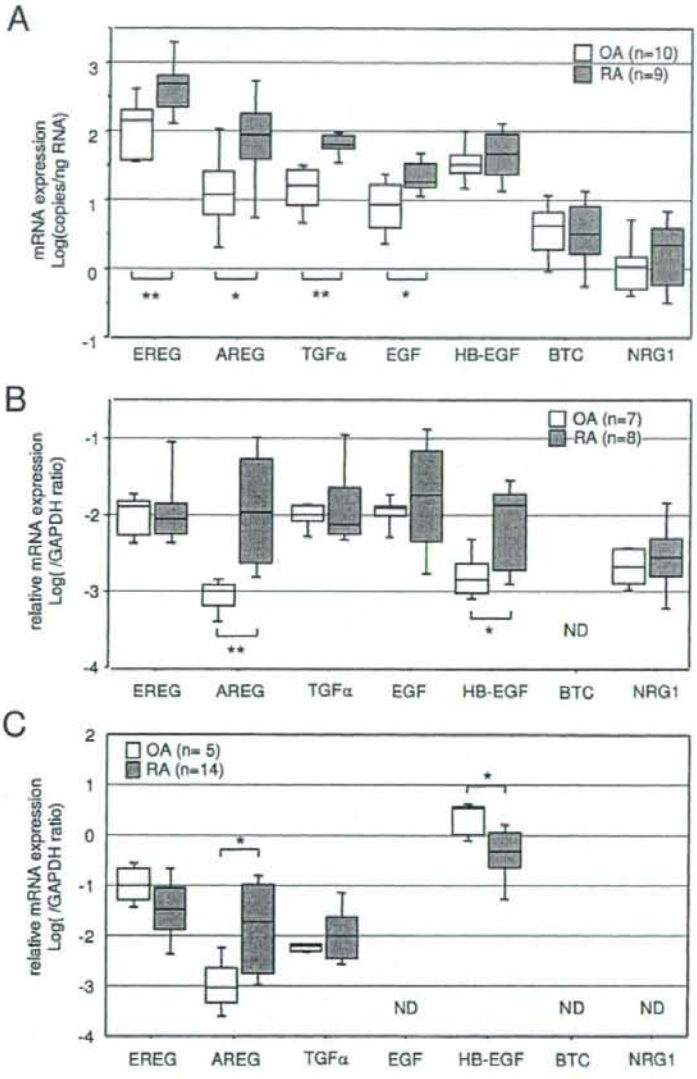

Figure 1

mRNA expression levels of EGF-related growth factors in BMMCs (A), PBMCs (B) and synovial tissues (C). The results of real-time PCR are shown as box-plots. The log ratio of the mRNA quantities relative to the total RNA amount (A) or GAPDH mRNA (B, C) is plotted on the y-axis of each graph. The upper and lower error bars indicate the 90th and 10th percentiles, respectively. The upper and lower edges of each box indicate the 75th and 25th percentiles, respectively, and the line inside the box shows the median. Genes not detected are shown as ND. The differences between the mRNA levels in the RA and control samples were analyzed by the Mann-Whitney U-test, and significant differences are shown by asterisks (*P < 0.05; **P < 0.01). RA: samples from RA patients; OA: samples from OA patients.

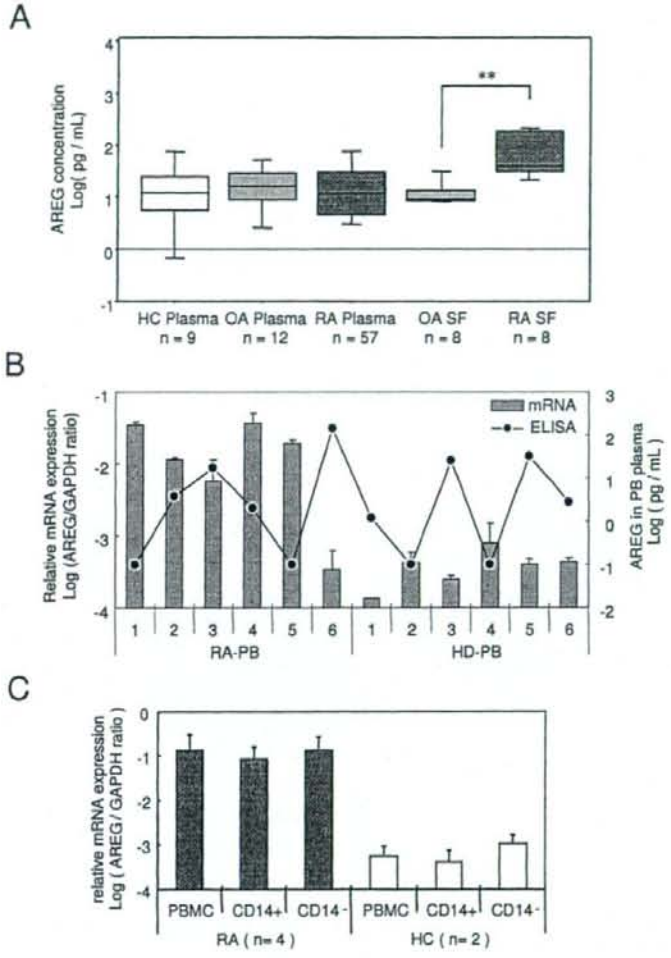

Figure 2

Amphiregulin expression in peripheral blood and synovial fluid samples. (A) The concentrations of AREG in plasma and synovial fluid samples are plotted as log-values on the y-axis of box-plots. Significant differences are shown by asterisks (**P < 0.01). RA: samples from RA patients; OA: samples from OA patients; HC: samples from healthy volunteers. (B) The AREG mRNA expression levels in PBMCs and AREG protein concentrations in plasma are shown. Venous blood samples from 6 RA patients and 6 healthy volunteers (HC) were separated into plasma and PBMCs. Total RNAs were extracted from PBMCs and subjected to cDNA synthesis. The AREG mRNA levels were measured by real-time PCR and normalized by the GAPDH mRNA levels. The relative AREG mRNA level relative to the GAPDH mRNA level is plotted as the log ratio on the primary y-axis (left), while the plasma concentration of AREG protein measured by ELISA is plotted as the log value on the secondary y-axis (right). The correlation coefficient (ρ) of the protein level in plasma to the mRNA level in PBMCs is -0.378 (P = 0.2104). (C) PBMCs from 4 RA patients and 2 HCs were separated by CD14 microbeads, and the AREG mRNA level in each fraction was measured by real-time PCR. The log ratio of the AREG mRNA level relative to the GAPDH mRNA level is plotted on the y-axis.

both CD14-positive and CD14-negative fractions of RA-PBMCs expressed equal amounts of AREG mRNA, and their levels were markedly higher than that in control PBMCs. The CD3 and CD19 separations yielded similar results (data not shown).

Effects of AREG on the proliferation of RA-FLS

To investigate the biological activity of AREG in joints affected by RA, we assessed the effects of recombinant human AREG on RA-FLS. Since AREG is a member of the EGF-like growth factor family, its growth-promoting activity was measured first. As shown in Fig. 3A, recombinant human AREG enhanced *de novo* DNA synthesis by RA-FLS in a dose-dependent manner. Fig. 3B shows the expression levels of the four EGFR family members in the cell lines used in the proliferation assay. In all FLS cell lines, ErbB2 and EGFR were the predominantly expressed receptors and ErbB3 and ErbB4 were expressed at about 100-fold lower levels than the most abundant ErbB2 level. There were no differences among the three RA-FLS lines and the one OA-FLS line. Although the amounts of radioactivity incorporated into the RA-FLS lines were higher than that incorporated into the OA-FLS line, the issue of whether RA-FLS are more sensitive to AREG than OA-FLS requires further examination. EGF-like growth factors are expressed as transmembrane-type precursors, and ectodomain shedding by ADAMs is essential for their effects as well as the expression of their receptors [30,31]. Since ADAM10 and ADAM17 are known to be sheddases for EGF-like growth factors, we measured the expression levels of the four EGFR-like receptors and ADAM10 and ADAM17 in synovial tissues from 10 RA patients and 6 OA patients (Fig. 3C). Similar to the findings for FLS, EGFR and ErbB2 were the predominantly expressed receptors in synovial tissues and their expression levels were not augmented in RA samples. Although AREG is supposed to be mainly processed by ADAM17, ADAM17 was expressed at a lower level than ADAM10, and neither ADAM10 nor ADAM17 was upregulated in RA synovia.

Effects of AREG on cytokine production by RA-FLS

Next, we tested the expression levels of five proinflammatory cytokines (IL-1β, TNF-α, IL-8, GM-CSF and IL-6) in RA-FLS stimulated by recombinant AREG. To clarify whether the recombinant AREG stimulated RA-FLS to proliferate via the induction of other growth factors, we also tested the expression levels of PDGF, bFGF and VEGF, which are involved in synovial hyperplasia. Recombinant AREG upregulated the mRNA expression levels of VEGF, IL-8, GM-CSF and IL-6 (Fig. 4A), but not those of PDGF, bFGF, IL-1β or TNF-α (data not shown). Recombinant AREG stimulated RA-FLS to express these cytokines in a dose-dependent manner, and the EGFR-tyrosine kinase inhibitor genistein suppressed the AREG-dependent expression in a dose-dependent manner. ELISA analysis revealed elevated levels of VEGF, IL-8, GM-CSF and IL-6 proteins in culture supernatants of AREG-stimulated RA-FLS (Fig. 4B), consistent with the results of the real-time PCR. We also tested the expression levels of ADAM10 and ADAM17 in AREG-stimulated RA-FLS. Contrary to the effect on the cytokine induction, AREG downregulated the expression of ADAM17 in a dose-dependent manner, and the AREG-dependent suppression was abolished by genistein (Fig. 4C). Analyses of ADAM10 expression produced similar results to those for ADAM17 (data not shown).

Correlation between VEGF and AREG expression levels

Since higher inductions of IL-6, IL-8 and GM-CSF than those induced by AREG have been observed and induction of VEGF has not yet been observed in our previous studies [32,33], we hypothesized that AREG would be closely related to VEGF in RA joints. To examine the relationship of AREG with this angiogenic factor in affected joints, VEGF expression was assessed in synovial tissues from 10 RA patients and 6 OA patients. Fig. 5A shows the mRNA levels of VEGF measured by real-time PCR. Ikeda et al. reported that the $VEGF_{165}$ transcript may be augmented in RA synovial tissues, and that the products of this transcript may be associated with RA pathology [34]. The primers for the real-time PCR amplification of VEGF used in our study were also designed to detect $VEGF_{165}$. VEGF expression in RA synovia was significantly higher than that in OA synovia (Fig. 5A, left panel), and highly correlated with AREG expression (Fig. 5A, right panel). Fig. 5B shows the VEGF protein concentrations in synovial fluid samples measured by ELISA. The ELISA system is able to detect all isoforms of VEGF-A, although it was designed for $VEGF_{165}$. Consistent with the results of the mRNA expression analyses, the VEGF protein levels in RA synovial fluid samples were significantly higher than those in OA synovial fluid samples (Fig. 5B, left panel), and highly correlated with the AREG concentration (Fig. 5B, right panel).

Discussion

Several previous studies have reported the involvement of c-erb-B family members, especially ErbB2, in the pathology of RA. Hallbech et al. showed immunohistochemically that the expression levels of ErbB2 and TGFα were augmented in RA synovia [17]. Satoh et al. demonstrated that ErbB2 was predominant in RA synovia and primary RA-FLS, but not in OA synovia or primary OA-FLS, and that a neutralizing antibody against ErbB2 suppressed the proliferation of primary RA-FLS, but not primary OA-FLS [16]. In the present study, we investigated which members of the ErbB family are predominantly expressed in RA-FLS and RA synovial tissues. Among the four ErbB family members, the mRNA level of ErbB2 was the highest, followed by that of EGFR, while the others were expressed at

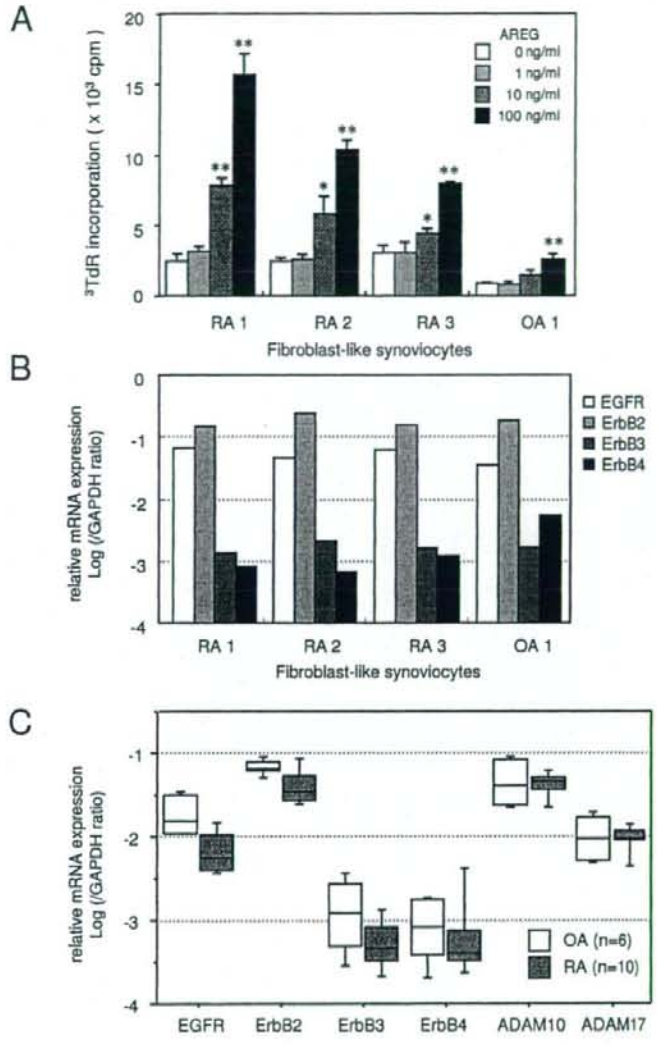

Figure 3

Stimulatory activity of AREG on the proliferation of RA-FLS. (A) Effect of AREG on the proliferation of RA-FLS. Three RA-FLS lines and one OA-FLS line were cultured with the indicated concentrations of AREG. After 24 h of stimulation, the cells were labeled with ^{3}H-thymidine for 18 h and then harvested on glass filters with a cell harvester. The incorporated radioactivity was measured by liquid scintillation counting. The values are shown as the means ± SD of three independent experiments. Significant differences are shown by asterisks (*P < 0.05; **P < 0.01). (B) Expression profiles of EGFR family members in FLS. cDNA samples of the four FLS lines used in the proliferation assay were subjected to real-time PCR analysis. (C) Expression profiles of the receptors and sheddases of the EGF family in synovia. cDNAs of synovial tissues from 10 RA patients and 6 OA patients were subjected to real-time PCR analysis.

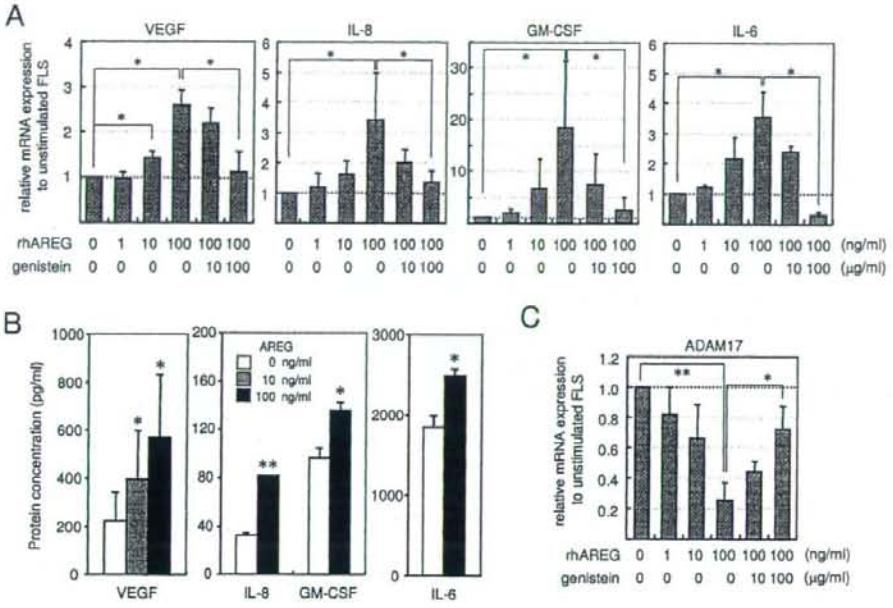

Figure 4

Stimulatory activity of AREG on cytokine production by RA-FLS. (A) Effects of AREG on cytokine expression in RA-FLS. Four RA-FLS lines were cultured with the indicated concentrations of recombinant human AREG and/or genistein. After 4 h of stimulation, total RNAs were extracted and the mRNA levels of PDGF, bFGF, VEGF, IL-1β, IL-6, IL-8, TNF-α and GM-CSF were measured by real-time PCR. The results for VEGF, IL-8, GM-CSF and IL-6 are shown. The results for the other molecules were omitted from the figure, since AREG had no effect on their expressions. (B) Effects of AREG on cytokine production by RA-FLS. Four RA-FLS lines were cultured with the indicated concentrations of AREG for 24 h. The GM-CSF, IL-6, IL-8 and VEGF concentrations in the supernatants were measured by ELISA, and are shown as means ± SD. (C) Effects of AREG on the expression of sheddases. The same cDNA samples used in panel A were subjected to real-time PCR analysis for ADAM10 and ADAM17. The results for ADAM10 were omitted from the figure, since they were similar to those for ADAM17. Each panel shows a representative result of three independent experiments. Significant differences from unstimulated cells are shown by asterisks (*P < 0.05; **P < 0.01).

almost undetectable levels in FLS and synovial membranes. While these results are consistent with the previous report [16], there were no differences in the expression levels between RA and OA samples. This discrepancy may reflect differences in the synovial specimens, although it will be necessary to confirm this hypothesis by assessing the ErbB2 protein concentrations in RA and OA samples. The OA synovial samples used in the present study were obtained from synovia with villous formation, rather than from the joint capsule, and thus our OA samples may be more activated than those used in the previous study. In any case, ErbB2 and EGFR were confirmed to be predominantly expressed in RA-FLS and RA synovia.

On the other hand, there have been very few reports of the expression profiles of EGF-like growth factors in RA synovia. In the present study, we found that AREG expression, which was 1000-fold lower than the most abundantly expressed HB-EGF in OA synovia, was markedly upregulated in RA synovia. Since it was correlated with the expression of AREG ($\rho = 0.532$, $P = 0.0241$), the expression of TGFα may tend to be augmented in RA synovia, as reported previously [17].

Since we recently reported augmented expression of AREG in BMMCs and PBMCs from RA patients [25], we examined the expression levels of other EGF family members in the present study. In RA-BMMCs, EGF and TGFα were also