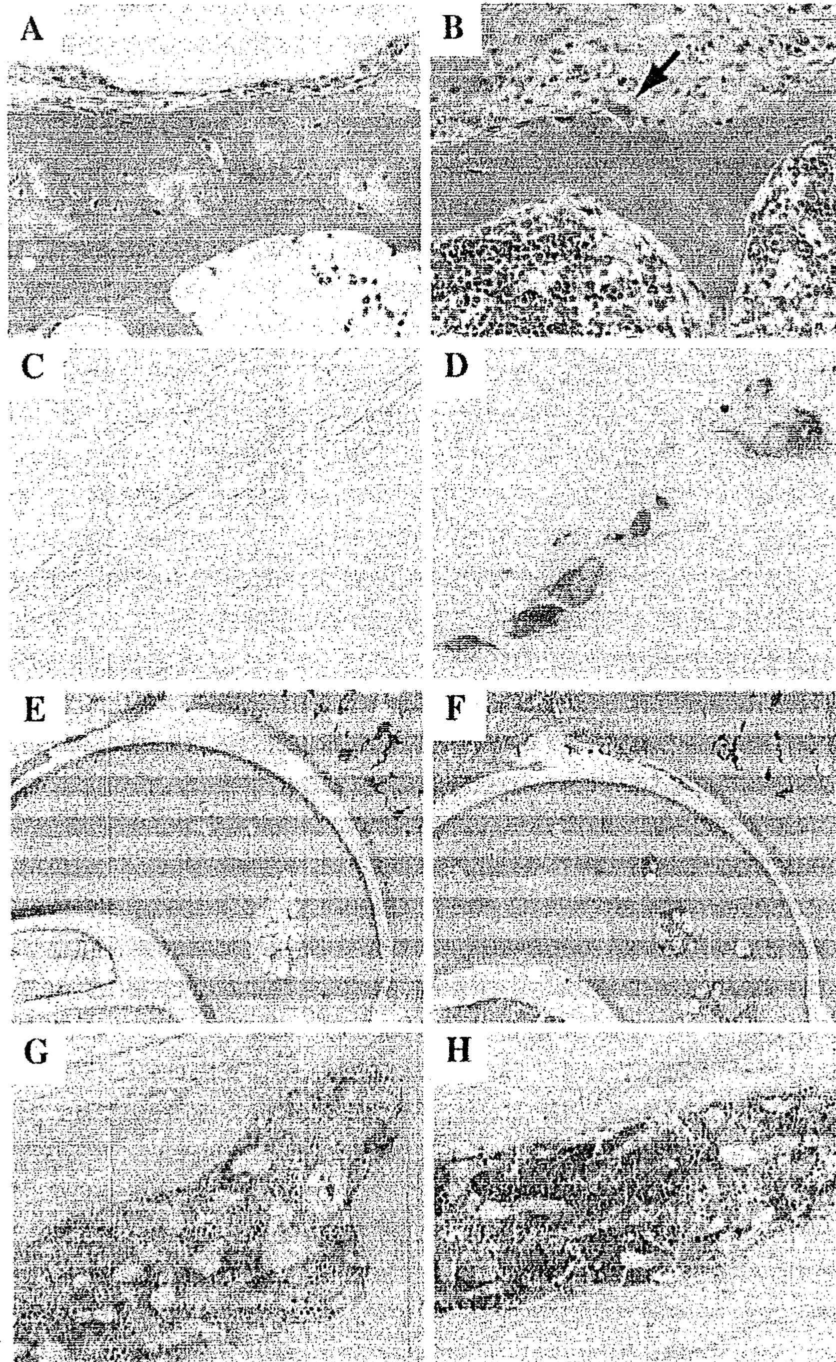

Fig. 6. Bone and cartilage destruction in athymic nude mice transferred with SKG T cell clones. High magnification of H&E-stained sections of a nude mouse recipient of dengue 2F7 (A) or 35S (B), showing bone erosion by pannus and BM activation (×400). Multinuclear cells (osteoclasts) (arrow) are also observed. Tartrate-resistant acid phosphatase-positive cells (osteoclasts) are detected in a 35S recipient (D) but not in a 2F7 recipient (C) (×400). By Safranin-O staining, proteoglycan stained red decreases in the articular cartilage matrix of a recipient of 35S (F) but not in a recipient of 2F7 (E) (×100). By immunohistochemistry, Gr-1-positive cells increase in the BM of a 35S recipient (H) but not in a 2F7 recipient (G) (×200). (A, C, E and G) 12 months after transfer; (B, D, F and H) 10 months after transfer.

Discussion

In this study, we have established two distinct CD8$^+$ T cell clones from arthritic lesions of SKG mice. Interestingly, both exhibited *in vitro* autoreactivity against not only synoviocytes but also a variety of MHC-matched cell lines and elicited both arthritis and interstitial pneumonitis when transferred to

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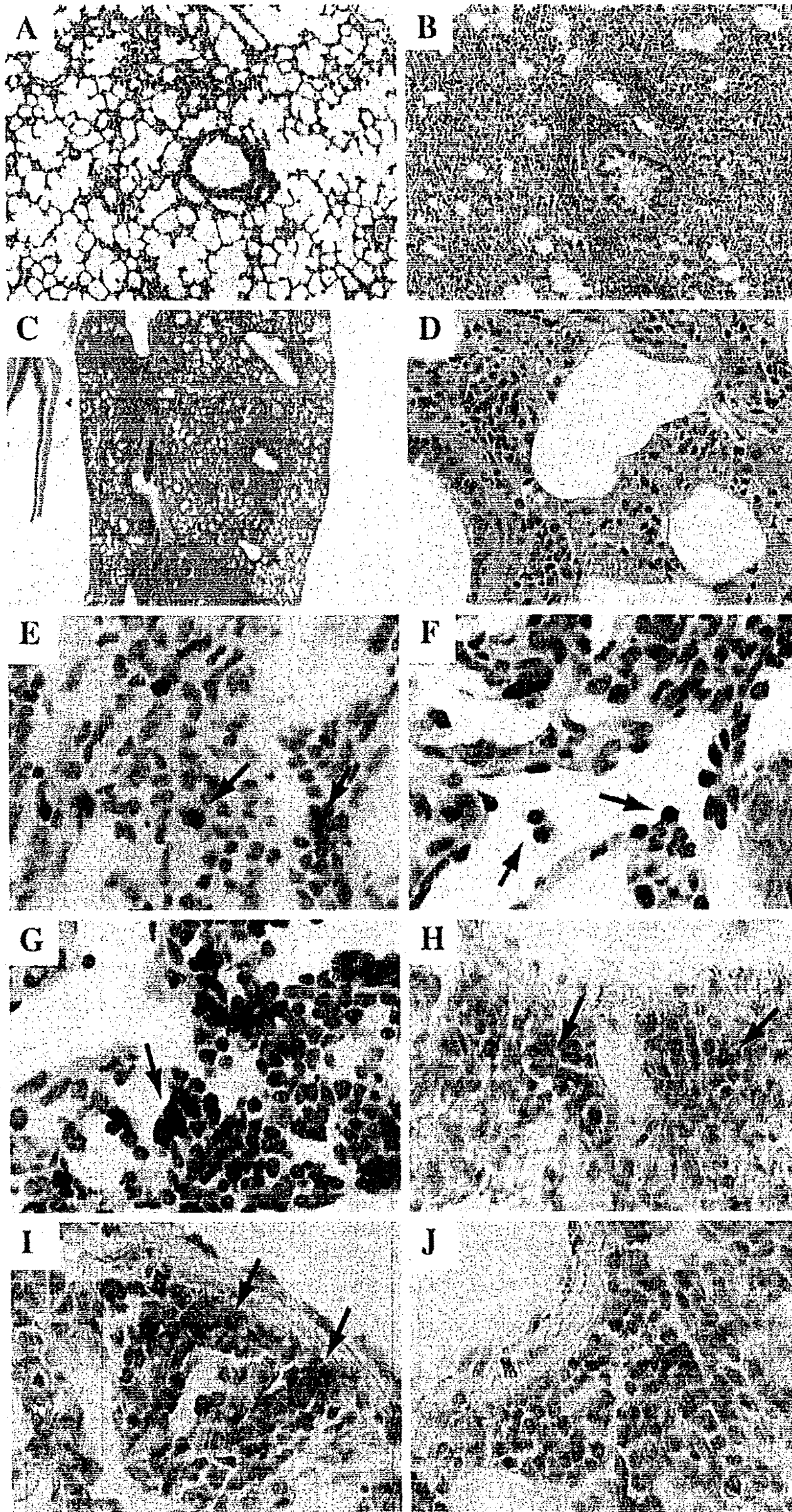

Fig. 7. Interstitial pneumonitis induced by the transfer of SKG T cell clones. (A–D) H&E-stained sections of the lungs of the recipients of control dengue 2F7 clone (A) or 73S clone (B–D) (A–B, ×100). Lower (C, ×10) and higher (D, ×400) magnification of the lung of 73S clone recipient show thickening of alveolar walls diffusely in the lung. (E–J) Serial sections of a lung of a 73S recipient mouse were stained for Ly-6G (Gr-1) (E), F4/80 (F), B220/CD45R (G), CD8a (H) or CD4 (I), with staining control (J) (×400). Typically positive cells in these stainings are arrowed. (A–J) 6 months after transfer.

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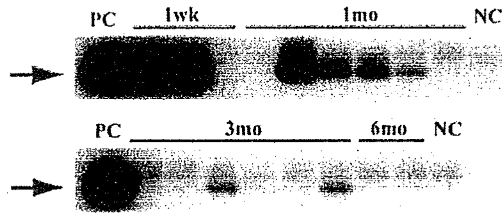

Fig. 8. Detection of a clone-specific TCR message of 35S clone in spleens by RT-PCR amplification and Southern blot analysis. After transfer of 1 × 10^7 clone cells to BALB/c nude mice, RNA was extracted from spleens at indicated days. PC, positive control (RNA from 35S clone diluted to 1%); NC, negative control (RNA from a 6-month-old non-treated BALB/c athymic nude mouse). The separate lanes represent individual mice.

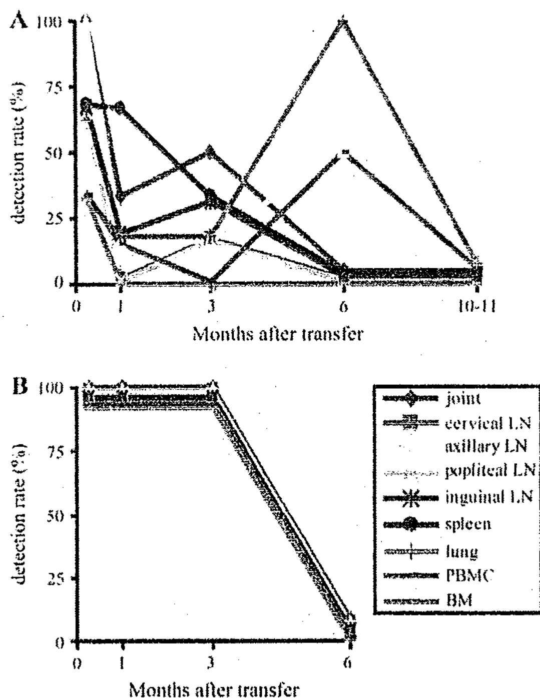

Fig. 9. Detection of TCR mRNA of the transferred clones in recipient BALB/c nude mice. 35S (A) or dengue 2F7 (B) in the recipients were detected by Southern blot analysis using primers and probes specific for TCR V and J region and CDR3 sequences of each clone. All mice with at least one positive signal out of four joints were considered to be positive. (A) $n = 3$ at 1 week; $n = 6$ at 1 month; $n = 6$ at 3 months; $n = 2$ at 6 months; $n = 2$ at 10–11 months. (B) $n = 2$ in every group. No signal was detected in control 6- or 11-month old BALB/c nude mice in each Southern blot analysis (data not shown).

histocompatible T cell-deficient mice. Furthermore, the arthritic and pulmonary lesions chronically progressed irrespective of the decline in the number of transferred T cell clones to hardly detectable levels in either lesion.

Our previous study showed that bulk $CD4^+$ T cells alone from arthritic SKG mice were able to transfer the disease to athymic nude mice, whereas bulk $CD8^+$ T cells alone were not and that abundant $CD4^+$ T cells and only a small number of $CD8^+$ T cells were found by immunohistochemistry in the arthritic subsynovial tissue of arthritic SKG mice (14). These apparently opposing results with $CD8^+$ T cell clones versus bulk $CD8^+$ T cells indicate that potentially arthritogenic $CD8^+$ T cells are present in SKG mice and may usually need $CD4^+$ T cell help for induction of arthritis; yet, they are potentially able to mediate arthritis without $CD4^+$ T cell help if they are strongly activated, clonally expanded to a large number or possibly selected for stronger self-reactivity during *in vitro* culture. It remains to be determined how $CD8^+$ clones elicit proliferative synovitis rather than cytotoxic killing of certain cellular elements in the joint. One possibility is that these $CD8^+$ clones, which exert *in vitro* killing activity at a high T cell/target cell ratio, might also be able to stimulate synoviocytes through secreting cytokines. It is of interest in this regard that the joints and the lungs with severe pneumonitis in some recipients of the $CD8^+$ clones showed active transcription of IL-17 mRNA (Supplementary Figure 1, available at *International Immunology* Online). Although the $CD8^+$ clones did not produce detectable amounts of IL-17 by *in vitro* stimulation, they might produce the cytokine in the joints or interact with nude mouse-derived α/β or γ/δ T cells and stimulate them to secrete IL-17 (33, 34). It is of note that a large number of $Gr\text{-}1^+$ mature neutrophils exuded into the joint fluid and infiltrated into the subsynovial tissue of the recipient nude mice, as in the arthritic lesions of SKG mice (14). BM of the clone recipients also showed an increase in the number of $Gr\text{-}1^+$ mature neutrophils. It remains to be determined how $CD8^+$ T cells mediate arthritis and pneumonitis in SKG mice by recruiting other cellular elements including neutrophils, how they increase neutrophils in the BM and whether IL-17, which is capable of recruiting neutrophils, is involved in these processes (35, 36).

It also needs further investigation whether IFN-γ secreted by the transferred $CD8^+$ clones or their killing activity could contribute to the development of synovitis. IFN-γ may activate synoviocytes directly or indirectly through activating macrophages, facilitating synoviocyte proliferation. It might up-regulate the expression of MHC class I in synovial cells, rendering them susceptible to cytotoxic activity of $CD8^+$ T cells. With these apparently opposing activities of arthritogenic $CD8^+$ T cells (i.e. killing versus proliferation of synoviocytes), they mediate proliferative synovitis rather than synoviocyte destruction presumably because synoviocytes might be more sensitive *in vivo* to the stimulatory effect than the cytotoxicity (see Discussion below).

The $CD8^+$ clones exhibited *in vitro* cytotoxic activity against not only syngeneic synovial cells but also a variety of MHC-matched lymphoid and non-lymphoid cell lines. Although their precise antigen specificities need to be determined, this finding suggests that these clones may recognize a ubiquitous self-antigen (for example, ubiquitous cellular protein such as hsp complexed with MHC or the MHC molecule itself) expressed in the joint and lung and other tissues, rather than a common self-antigen exclusively expressed in the joint and lung. If this is the case, how are the joint and the lung selectively affected by these T cell clones? For the following reasons, one could attribute this to unique characteristics of the synoviocytes, and possibly the alveolar macrophage, as the target of this autoimmunity. Compared with other tissue cells, the synoviocytes are

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highly sensitive to pro-inflammatory cytokines, for example systemic overproduction of transgenic TNF-α or IL-1 almost exclusively produces chronic arthritis even in mice deficient of both T and B cells (37–39); similarly, systemic deficiency of the IL-1R antagonist, and resulting overproduction of IL-1, or systemic alteration of signal transduction via IL-6 receptor results in predominant development of arthritis with no inflammatory damage to other tissues (40, 41). These findings collectively indicate that synoviocytes are much more sensitive to the SKG self-reactive T cell clones (at least to those secreting pro-inflammatory cytokines) than other tissue cells, even if the common self-antigens recognized by the clones are ubiquitously expressed. In addition, synoviocytes are unique in that they are the target cells and also the mediators of autoimmunity, i.e. upon stimulation (e.g. by cytokines or via cell contact stimulation by self-reactive T cells), they proliferate and secrete pro-inflammatory cytokines (e.g. IL-1, IL-6 and TNF-α) and other inflammatory substances (matrix metalloproteinases and prostaglandins), mediating inflammation and tissue damage (42). It is likely that the cells composing the alveolar walls, in particular the alveolar macrophages, are sensitive and responsive to T cell self-reactivity in a similar manner as synoviocytes and that excessively and chronically activated macrophages might mediate alveolitis and interstitial inflammation. A similar mechanism might also be responsible for the development of colitis in SKG mice (Table 1).

We do not assert, however, that SKG arthritis and pneumonitis are solely mediated by T cells recognizing a ubiquitous common self-antigen. We have previously shown that SKG mice spontaneously produce IgG isotype auto-antibody specific for joint-rich type II collagen or IgG antibody cross-reactive with hsp-70 of Tuberculosis bacilli (14). This indicates that helper $CD4^+$ T cells that specifically react with these self-antigens may also be induced in SKG mice either primarily or secondarily to joint damage. Moreover, we have recently shown that some self-reactive T cells in SKG mice may not be arthritogenic but can polyclonally stimulate antigen-presenting cells in the spleen and lymph nodes to secrete IL-6 and other cytokines, which in turn facilitate differentiation of potentially arthritogenic self-reactive T cells to T_h17 effector T cells that mediate synovitis (19). In addition to our current approach to the characterization of antigen specificity of SKG autoimmune T cells by preparing T cell clones, efforts are being made to further characterize infiltrating T cells *in situ* at a single-cell level by amplifying their TCR message.

Tracing the fate of transferred T cell clones revealed that clone-specific TCR gene messages gradually diminished not only in the inflamed joints and the lungs but also in the regional lymph nodes and spleens of the recipients, becoming hardly detectable in 6–11 months; yet, inflammation in the joints and the lung continued to progress and severe arthritis and pneumonitis were apparent even 12 months after clone transfer. Thus, initial triggering of synovitis requires arthritogenic T cells; yet, synovitis apparently becomes less T cell dependent in a later phase, albeit it chronically progresses with the formation of pannus destroying adjacent cartilage and bone, as in human RA (2). This may correlate with the findings in humans that T cell-targeted mAb therapy is not much efficacious in the treatment of RA at a chronic stage (43). Further characterization of each stage of disease development in SKG mice will contribute to our understanding of the cellular and molecular basis of the T cell-dependent and -independent phases of disease progression in the joints and also in the lung in RA.

In conclusion, we have shown that $CD8^+$ T cell clones established from arthritogenic lesions of SKG mice are capable of mediating not only arthritis but also interstitial pneumonitis immunopathologically resembling ILD in RA. This provides a possible common pathogenetic basis between arthritis and ILD in RA. The etiology of RA is largely obscure at present (1, 2). Yet, there are recent findings that genetic polymorphism of the PTPN22-encoded lymphoid tyrosine phosphatase, which alters signal transduction at a TCR proximal step involving ZAP-70, contributes significantly (second only to MHC polymorphism) to the susceptibility to RA and other autoimmune diseases (22, 23, 44, 45). The polymorphism might be responsible for thymic generation of arthritogenic and other self-reactive T cells. Further elucidation of the mechanism by which such autoreactive T cells are generated and activated in SKG mice, and characterization of putative ubiquitous self-antigen recognized by self-reactive T cells capable of mediating arthritis and pneumonitis, would facilitate our understanding of the etiology and the pathogenetic mechanism of RA as a systemic autoimmune disease. This should help devising preventive or curative measures for the disease.

Supplementary data

Supplementary figure is available at *International Immunology* Online.

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Disclosures

The authors declare no conflicting interests.

Abbreviations

BM	bone marrow
CDR3	the third complementarity-determining region
H&E	haematoxylin & eosin
hsp	heat shock protein
ILD	interstitial lung disease
MHA	microplate hybridization assay
PMA	phorbol myristate acetate
RA	rheumatoid arthritis
RT	reverse transcription
SSC	standard saline citrate
TNF	tumor necrosis factor
ZAP-70	ζ-associated protein of 70 kDa

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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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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'-ATGGTGGTGAAGACGCCAGT-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,

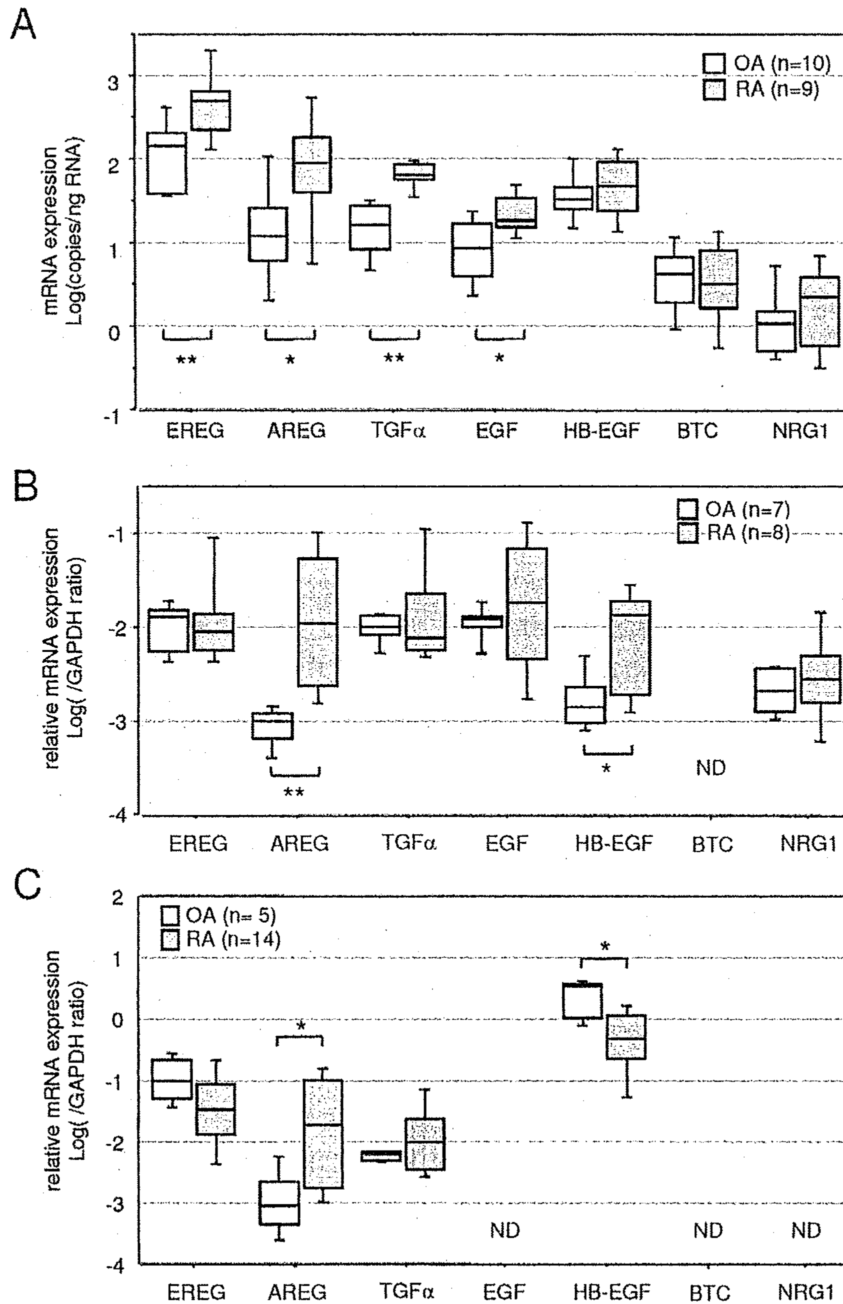

Figure 1
mRNA expression levels of EGF-related growth factors in BMMCs (A), PBMCs (B) and synovial tissues (C). The results of real-time PCR are shown as box-plots. The log ratio of the mRNA quantities relative to the total RNA amount (A) or GAPDH mRNA (B, C) is plotted on the y-axis of each graph. The 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.

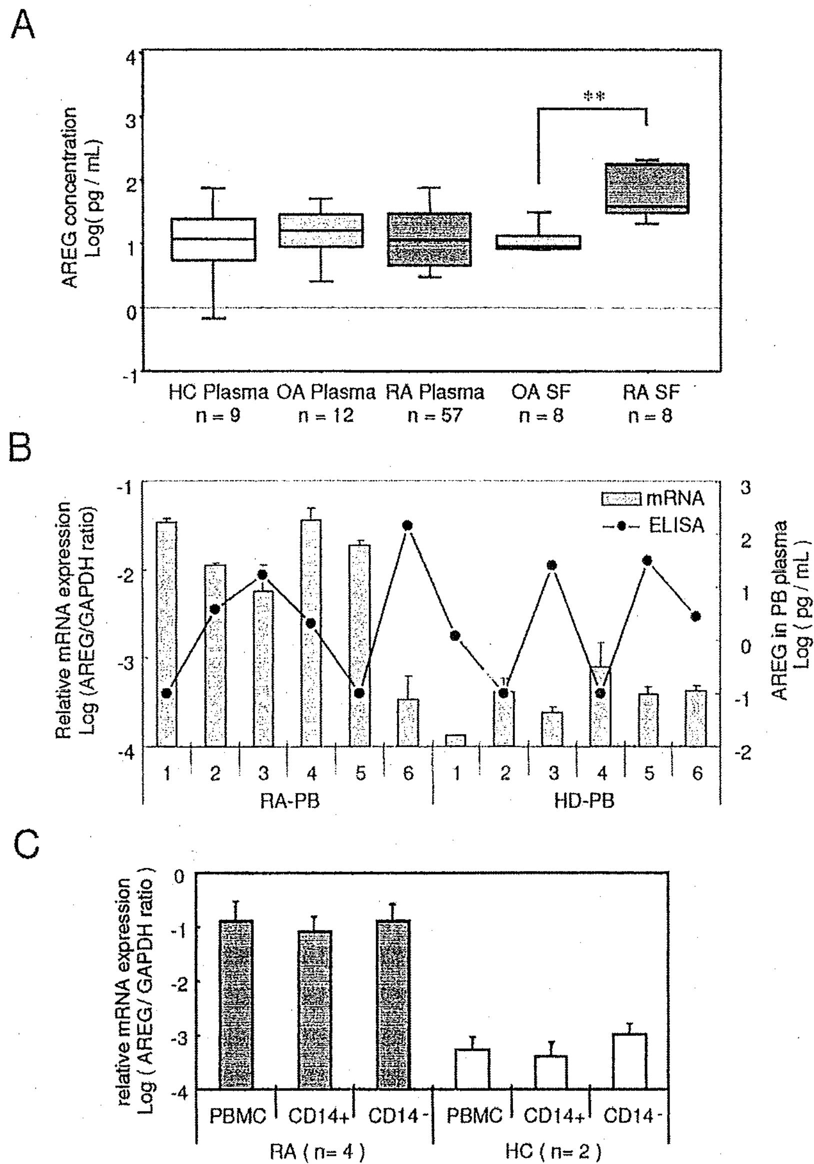

Figure 2

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

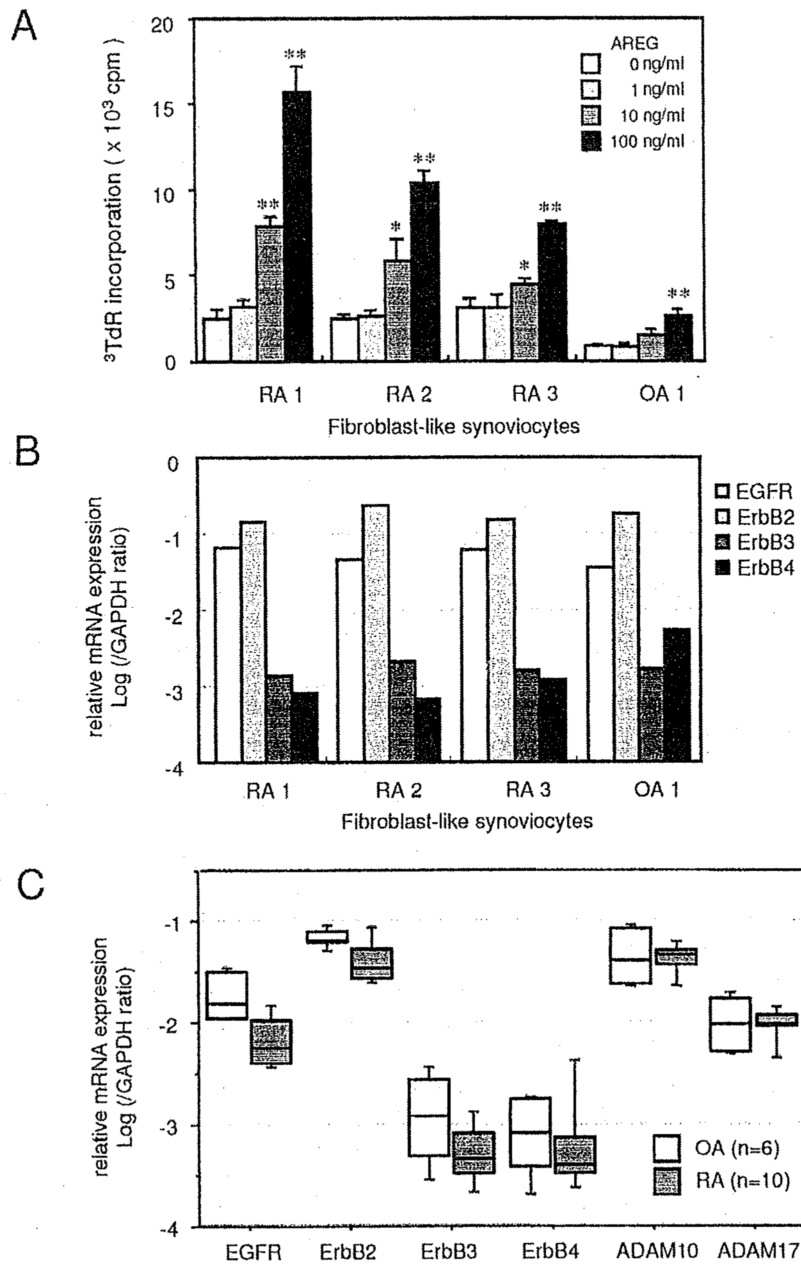

Figure 3
Stimulatory activity of AREG on the proliferation of RA-FLS. (A) Effect of AREG on the proliferation of RA-FLS. Three RA-FLS lines and one OA-FLS line were cultured with the indicated concentrations of AREG. After 24 h of stimulation, the cells were labeled with ^{3}H-thymidine for 18 h and 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.

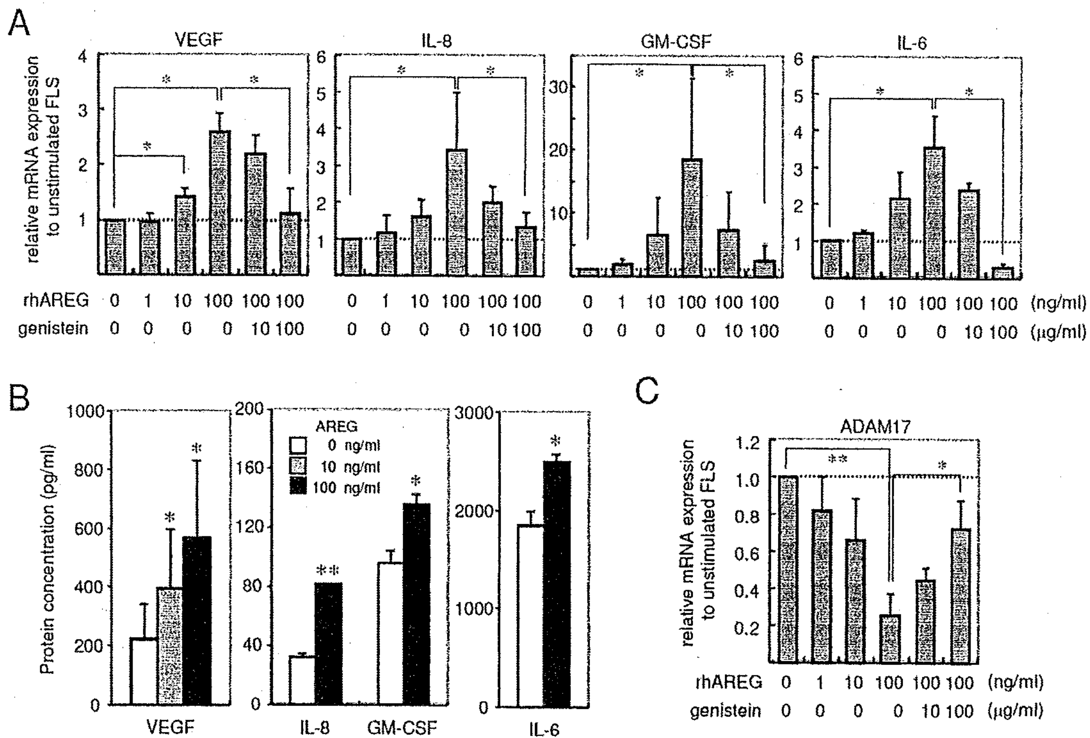

Figure 4
Stimulatory activity of AREG on cytokine production by RA-FLS. (A) Effects of AREG on cytokine expression in RA-FLS. Four RA-FLS lines were cultured with the indicated concentrations of recombinant human AREG and/or genistein. After 4 h of stimulation, total RNAs were extracted and the mRNA 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

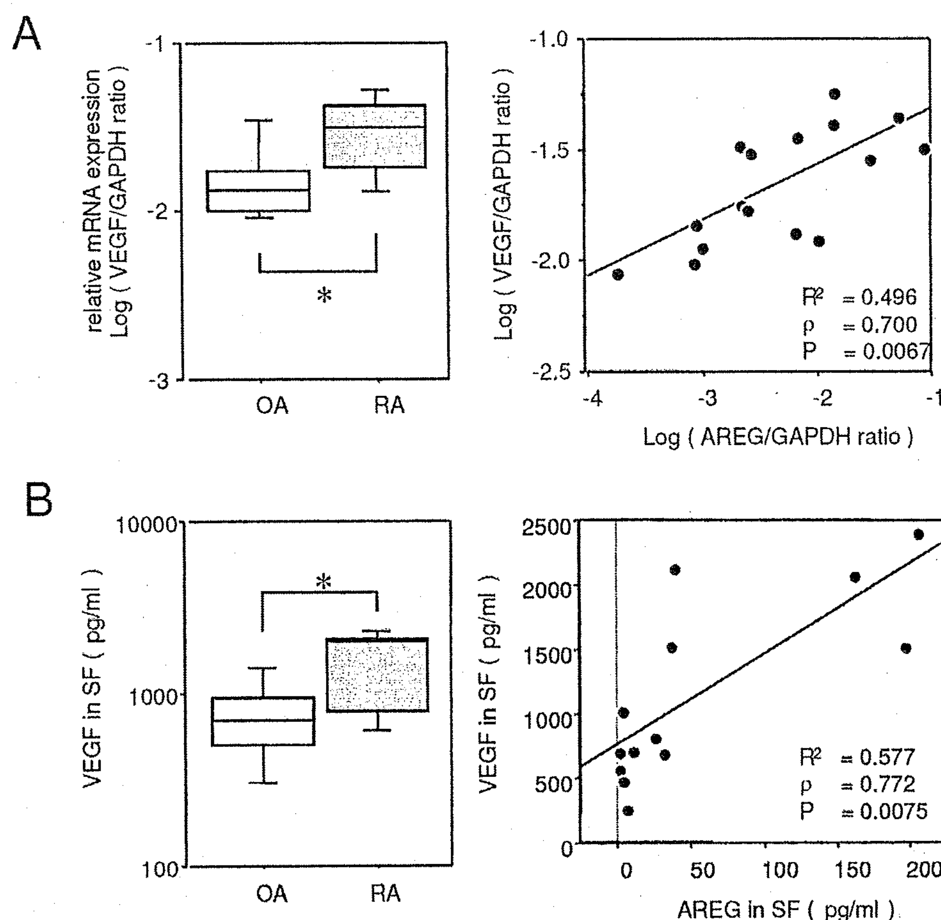

Figure 5
Correlation between AREG and VEGF expression levels. (A) The VEGF mRNA expression levels in synovia from 10 RA patients and 6 OA patients were measured by real-time PCR, and are shown by box-plots (left). Significant differences from unstimulated cells are shown by asterisks (*$P < 0.05$). The correlation between the mRNA levels of VEGF and AREG is shown by a distribution chart (right). The linear regression coefficient (R^2) is 0.496 and the correlation coefficient (ρ) is 0.700 ($P = 0.0067$). (B) The concentrations of VEGF in synovial fluids from 7 RA patients and 6 OA patients were measured by ELISA, and are shown by box-plots (left). Significant differences from unstimulated cells are shown by asterisks (*$P < 0.05$). The correlation between the protein concentrations of VEGF and AREG is shown by a distribution chart (right), in which R^2 is 0.577 and ρ is 0.772 ($P = 0.0075$).

upregulated, similar to AREG and EREG whose augmented expressions were reported in our previous study. Although there was no significant difference between the levels of HB-EGF in the RA and OA samples, its high correlations with AREG and EREG ($\rho = 0.788$, $P = 0.0008$ and $\rho = 0.823$, $P = 0.0005$, respectively) imply that HB-EGF may be upregulated in RA. Although EGF and TGFα were also upregulated in RA, their expression levels showed no correlations with those of other members. These results suggest that AREG, EREG and HB-EGF may be regulated by a common expression-controlling system.

In RA-PBMCs, AREG and HB-EGF were significantly upregulated, and their expression levels were correlated with each other ($\rho = 0.600$, $P = 0.0305$). Among the seven EGF family members examined, only AREG expression was augmented in all three tissues tested in the present study. To confirm which lineage of blood cells expresses AREG, PBMCs were further separated into monocyte-, T lymphocyte- and B lymphocyte-rich fractions. None of these fractions was enriched in AREG-expressing cells in healthy controls or RA patients. In our recent report, we speculated that bone marrow-derived abnormal monocytes expressing AREG may migrate via the blood circula-

tion, and bring about disease in synovia and/or other tissues they infiltrated [25]. Although our present findings strongly support that hypothesis, the abnormal cells expressing AREG among RA-PBMCs were not restricted to monocytes. We conclude that they are mononuclear leukocytes and not of a particular lineage.

Herceptin, a specific inhibitor of ErbB2, was reported to suppress the proliferation of RA-FLS, but not OA-FLS, and augmented expression of ErbB2 was considered to be a major contributor to the autonomous proliferation of RA-FLS [16]. In the present study, EGFR and ErbB2 were found to be predominantly expressed in synovial tissues and cultured FLS, with no differences between their expression levels in RA and OA. Furthermore, the expression levels of ADAM10 and ADAM17, which are also important for the functions of EGF-like growth factors, showed no differences between RA and OA. On the other hand, the mRNA and protein levels of AREG were upregulated in RA synovial tissues. Furthermore, recombinant human AREG enhanced the proliferation of FLS in a dose-dependent manner. In our study, differences were detected for EGF-like growth factors between RA and OA synovia, but not for their receptors or sheddases. AREG induces tyrosine phosphorylation of EGFR and transduces a stronger signal when bound to EGFR/ErbB2 heterodimers [15]. It has been reported that synovitis with granulomatous hyperplasia occurs in AREG transgenic mice [35]. These findings suggest that overexpression of AREG may promote the proliferation of synoviocytes in affected joints of RA patients. We investigated whether recombinant AREG induced the expression of PDGF and bFGF, which are well-known growth factors for hyperplastic proliferation of RA-FLS. We found that AREG had no effects on the expression of these factors, suggesting that AREG did not stimulate RA-FLS to proliferate via these growth factors. However, AREG stimulated RA-FLS to express VEGF, an angiogenic factor involved in synovial hyperplasia.

A large number of reports have shown that RA-FLS produce proinflammatory cytokines when stimulated by various stimuli [36,37]. In addition, we previously reported that RA-FLS produce proinflammatory cytokines, such as IL-6, IL-8, GM-CSF, IL-1β and/or TNFα, when co-cultured with monocytes or lymphocytes [32,33]. Our analyses of cytokine production in the present study revealed that AREG enhanced the production of several proinflammatory cytokines (IL-6, IL-8 and GM-CSF) and VEGF in RA-FLS. Since they were suppressed by an EGFR tyrosine kinase inhibitor, the AREG-dependent induction of these cytokines seemed to occur via activation of EGFR/ErbB2. Interestingly, AREG downregulated the expression of ADAM10 and ADAM17 in a dose-dependent manner. These results suggest the presence of negative feedback regulation of ADAMs via AREG/EGFR signaling. To assess the involvement of AREG in the elevated expression of VEGF in affected joints of RA patients, the correlations between their mRNA levels and protein levels in synovial fluid samples were analyzed, and good correlations were found for both the mRNA levels ($\rho = 0.700$, $P = 0.0067$) and protein levels ($\rho = 0.772$, $P = 0.0075$). There have been very few reports about cytokine induction by AREG to date. The present results suggest that the increased level of AREG may be involved in the upregulation of VEGF in RA joints, and demonstrate, for the first time, that AREG stimulates RA-FLS to produce proinflammatory cytokines, including angiogenic factors. IL-8 has been reported to be an angiogenic factor as well as a chemoattractant factor [38,39]. Although several studies have recently shown that AREG plays important roles in hyperplasia or angiogenesis of skin diseases or tumors [40-43], the role of AREG in RA pathology remains unknown. Ma et al. speculated on the involvement of AREG in the angiogenesis of tumors [40]. Although it is known that EGF and TGFα are potent angiogenic mediators [44,45], the proangiogenic activity of AREG has not been directly determined to date. Its induction of angiogenic factors, such as IL-8 and VEGF, strongly suggests that AREG may be involved in the angiogenesis of synovial hyperplasia in affected joints of RA patients.

Myeloid cells expressing abnormal cell surface markers have been observed in RA bone marrow [18,19] and reported to be correlated with the disease severity [20-23]. Consistent with these reports, the present study revealed that several EGF-like growth factors were upregulated in RA bone marrow cells, suggesting that the onset and/or progression of chronic synovitis may be influenced by alterations to the bone marrow in RA patients.

Conclusion

Among the seven EGF-like growth factors, AREG was upregulated in synovial tissues of RA patients. Recombinant human AREG stimulated RA-FLS to proliferate and produce several proinflammatory cytokines, including angiogenic factors. These results suggest that the elevated expression of AREG in synovial tissues may be involved in RA pathology containing synovial hyperplasia. AREG-expressing cells were observed in both the blood and bone marrow of RA patients as well as in RA synovial tissues. Abnormal leukocytes may lead to the upregulated expression of AREG in affected joints of RA patients.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SY designed the study, carried out the experiments, analyzed the data and drafted the manuscript. SI, KK and YH

carried out the RNA extractions and cDNA syntheses. RM and KT carried out the quantitative real-time PCR. NS performed the measurements of ^{3}H-TdR incorporation into fibroblast-like synoviocytes. TM, TJ and TO participated in the study design and collection of clinical samples. NY, NF, TI and RS participated in the study design and coordination as well as editing of the manuscript. All authors have read and approved the final manuscript.

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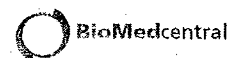

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IMMUNOLOGY ORIGINAL ARTICLE

The interaction of monocytes with rheumatoid synovial cells is a key step in LIGHT-mediated inflammatory bone destruction

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Summary

Formation of osteoclasts and consequent joint destruction are hallmarks of rheumatoid arthritis (RA). Here we show that LIGHT, a member of the tumour necrosis factor (TNF) superfamily, induced the differentiation into tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) of $CD14^+$ monocytes cocultured with nurse-like cells isolated from RA synovium, but not of freshly isolated $CD14^+$ monocytes. Receptor activator of nuclear factor-κB ligand (RANKL) enhanced this LIGHT-induced generation of TRAP-positive MNCs. The MNCs showed the phenotypical and functional characteristics of osteoclasts; they showed the expression of osteoclast markers such as cathepsin K, actin-ring formation, and the ability to resorb bone. Moreover, the MNCs expressed both matrix metalloproteinase 9 (MMP-9) and MMP-12, but the latter was not expressed in osteoclasts induced from $CD14^+$ monocytes by RANKL. Immunohistochemical analysis showed that the MMP-12-producing MNCs were present in the erosive areas of joints in RA, but not in the affected joints of osteoarthritic patients. These findings suggested that LIGHT might be involved in the progression of inflammatory bone destruction in RA, and that osteoclast progenitors might become competent for LIGHT-mediated osteoclastogenesis via interactions with synoviocyte-like nurse-like cells.

Keywords: differentiation; LIGHT/TNFSF14; monocyte; osteoclast; rheumatoid arthritis

Introduction

Osteoclasts are large, tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs). Receptor activator of nuclear factor-κB ligand (RANKL) is a key regulator of osteoclast differentiation from haematopoietic precursors of the monocyte/macrophage lineage.[1–3] Although osteoclasts have an essential role in physiological bone remodelling, increases in their number and activity, would lead to diseases accompanied by local bone destruction. Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by arthritis affecting multiple joints and the progressive destruction of cartilage and bone.[4] Osteoclasts are important contributors to the joint destruction in RA. Inflammatory cytokines, such as tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1), which are upregulated in RA synovial tissues, are known to induce the differentiation and activation of

Abbreviations: CTX-I, type I collagen C-telopeptide; HVEM, herpes virus entry mediator; LTβR, lymphotoxin β receptor; MMP, matrix metalloproteinase; MNCs, multinucleated cells; NLCs, nurse-like cells; OA, osteoarthritis; RA, rheumatoid arthritis; RANKL, receptor activator of nuclear factor-κB ligand; TRAP, tartrate-resistant acid phosphatase.

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osteoclasts.[5,6] Invasive synovial tissue at sites of bone destruction, also termed pannus, plays important roles in osteoclastic bone resorption.[7–9]

We previously established nurse-like cells (NLCs) from the synovial tissues of RA patients.[10] Although having the same appearance as fibroblast-like synoviocytes, NLCs have a number of distinct activities that could contribute to rheumatoid inflammation.[10–14] Among these are their ability to promote antibody production by B cells, the capacity to protect lymphocytes from apoptosis, and the ability to secrete large amounts of cytokines and chemokines such as IL-6 and IL-8 that could promote the accumulation and activation of lymphocytes and monocytes. However, fibroblast-like synoviocytes from patients with osteoarthritis (OA) hardly show any such activities.[10,15] Therefore, to distinguish them from general fibroblast-like synoviocytes, we have defined synovial NLCs as those that go through the active cell population from the RA synovium. The NLCs promote the survival of peripheral blood monocytes via macrophage colony-stimulating factor (M-CSF) production.[16,17] Monocytes cocultured with NLCs for 4 weeks possessed TRAP activity and differentiated into osteoclasts in response to some cytokines, including RANKL.[17] These reports have suggested that NLCs might be involved in RA-induced bone destruction by maintaining osteoclast precursors in areas of progressive synovial expansion.

LIGHT, which is homologous to lymphotoxin, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes, was recently identified as a type 2 transmembrane glycoprotein of the TNF ligand superfamily (TNFSF14).[18] LIGHT is expressed on activated T lymphocytes,[18,19] monocytes,[20] granulocytes[20] and immature dendritic cells.[21] LIGHT signalling is transduced via two members of the TNFR family, herpes virus entry mediator (HVEM, TNFRSF14) and lymphotoxin β receptor (LTβR, TNFRSF3). The HVEM is expressed prominently on monocytes, dendritic cells and lymphocytes,[19,22–24] whereas LTβR is expressed on many cell types with the exception of lymphocytes.[18,20,25] LIGHT has been shown to regulate cell proliferation[21,26,27] and apoptosis,[20,28] to induce the secretion of various cytokines, and to augment the expression of adhesion molecules.[26,29–31] Recently, Kim *et al.* reported that LIGHT was overexpressed in the synovial tissue of RA patients and that it induced the production of chemokines, cytokines and matrix metalloproteinase 9 (MMP-9) from macrophages in synovial fluid.[32] Moreover, LIGHT contributes to the survival and activation of synovial fibroblasts in RA.[33,34] These studies have suggested that LIGHT may be an important inflammatory cytokine in the development of RA. However, the roles of LIGHT in the bone destruction in RA have not yet been elucidated.

In this study, we compared the abilities to differentiate into osteoclasts in response to LIGHT, between fresh $CD14^+$ monocytes and $CD14^+$ monocytes cocultured with NLCs. We found that LIGHT induced osteoclast differentiation from $CD14^+$ monocytes cocultured with NLCs, but not from freshly isolated $CD14^+$ monocytes. Furthermore, LIGHT-induced osteoclasts express MMP-12, which was not expressed in osteoclasts induced by RANKL, and the MMP-12-expressing osteoclasts were observed at the erosive areas in the subchondral bones of RA patients, but not in those of OA patients. These findings suggest that $CD14^+$ monocytes gain the ability to differentiate into osteoclasts in response to LIGHT through their interactions with NLCs, and that LIGHT plays a critical role in the inflammatory joint destruction in RA.

Materials and methods

Cells and cultures

Nurse-like cells were established from synovial tissues obtained from RA patients, as described previously.[10] NLCs were cultured in Dulbecco's modified Eagle's minimum essential medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS). The NLCs from passages 4–9 were used for each experiment. Mononuclear cells were collected from the venous blood of healthy volunteers and $CD14^+$ monocytes were prepared by further separation using anti-CD14 antibody-coated beads, as described previously.[16] $CD14^+$ monocytes ($2{\cdot}0 \times 10^6$ cells/well) were cocultured with NLCs ($2{\cdot}0 \times 10^5$ cells/well) in six-well plates. Half of the medium was replaced every 3 days with fresh medium. After coculture for 4 weeks, floating or weakly adherent monocytes were harvested as NLC-supported $CD14^+$ monocytes ($NCD14^+$ monocytes) by gently washing the culture with fresh medium. Over 97% of $NCD14^+$ monocytes were TRAP positive, and their purity was confirmed cytochemically, as reported previously.[16] All human specimens were obtained with written informed consent according to the study protocol, which was approved by the review board of the Sagamihara National Hospital.

Osteoclast formation assay

In the presence of 25 ng/ml recombinant human M-CSF (R&D Systems, Minneapolis, MN), freshly isolated $CD14^+$ monocytes ($1{\cdot}0 \times 10^5$ cells/well) and $NCD14^+$ monocytes ($2{\cdot}0 \times 10^4$ cells/well) were cultured in 96-well plates in α-minimum essential medium (Invitrogen) supplemented with 10% FCS. As indicated, the cells were further stimulated with 40 ng/ml recombinant human RANKL (Peprotech, London, UK) and/or various concentrations of recombinant human LIGHT (R&D Systems). After vari-

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