TCCGTCACCTGCTCAGAATCA-3' and 5'-AGCACTGGCTGCTCGTCTCA-3'; for VCAM1, 5'-CGTGATCCTTGGAGCCTCAAATA-3' and 5'-GACGGAGTCACCAATCTGAGCA-3'; and for ICAM1, 5'-CCTGATGGGCAGTCAACAGCTA-3' and 5'-ACAGCTGGCTCCCGTTTCA-3'. PCR was performed under the following conditions: initial denaturation at 95°C for 10 seconds, then 40 cycles of 95°C for 5 seconds and 60°C for 20 seconds. When SYBR green dye was used to monitor PCRs, melting curves were routinely recorded to verify the singularity of the PCR product. In each sample, the level of cDNA was normalized to the expression of GAPDH.

Proliferation assay. RA FLS were seeded into a 96-well plate at a density of 5.0×10^3 cells/well. After 24 hours of preculture, the cells were stimulated for 48 hours by the addition of LIGHT or EGF. Bromodeoxyuridine (BrdU) was added for the last 24 hours of culture, after which the incorporation of BrdU was measured using a cell proliferation enzyme-linked immunosorbent assay (Cell Proliferation ELISA, BrdU; Roche Diagnostic) according to the manufacturer's instructions.

Measurement of cytokines and chemokines in culture supernatants. TNFα, IL-1β, IL-6, IL-8, and GM-CSF levels were measured in the supernatants of RA FLS cultures, using a Human Inflammatory Five-Plex Antibody Beads Kit (Biosource, CA) according

to the manufacturer's instructions, on a Luminex 100 instrument (Luminex, Austin, TX). The levels of MIP-1α, MIP-1β, MCP-1, eotaxin and RANTES in the supernatant were measured using a Human Chemokine Five-Plex Antibody Beads Kit (Biosource)

Transfection of RA FLS with siRNAs. All siRNAs were purchased from Qiagen. The sense strand sequences of the RNA duplexes were as follows: HVEM, 5'-GGCACUGCCUCACAGCCAAdTdT-3'; LTβR, 5'-CAUCUACAAUGGACCAGUAdTdT-3'; and control siRNA 5'-UUCUCCGAACGUGUCACGUdTdT-3'. The day before transfection, RA FLS were seeded into 6-well culture plates at a density of 4×10^4 cells/well, or 96-well plates at the 5×10^3 cells/well, in complete medium without antibiotics. The next day, siRNAs (at final concentration of 50 nM) were introduced into cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Twenty-four hours after transfection, media were replaced with regular culture media. The cells were then cultured for 96 hours before analyzing the gene-silencing effects. The mRNA levels were measured by quantitative real-time PCR analysis.

Immnofluorescence assay for NF-κB localization. To examine the nuclear translocation of NF-κB, RA FLS were seeded at a density of 5×10^3 cells/well in 8-well Lab-Tek chamber slides (Nalgen Nunc International, Naperville, IL). The cells were

stimulated with 10 ng/ml LIGHT for 40 minutes, washed with cold PBS, and then fixed in PBS with 4% paraformaldehyde for 10 minutes. The cells were permeabilized with PBS and 0.1% Triton-X100 for 10 minutes. Nonspecific binding was prevented with blocking buffer containing 2% goat serum diluted in PBS. The cells were incubated with mouse monoclonal anti-NF-κB p65 antibody or an isotype control, for 1 hour at room temperature, then with AlexaFluor 488-conjugated goat anti-mouse antibody for 30 minutes at room temperature. Slides were coverslipped and examined using a fluorescence microscope (Olympus, Tokyo, Japan).

Western blotting analysis. For measurement of IκBα by Western blotting, RA FLS at a density of 1.5×10^6/well were seeded into 6-well plates in culture medium for 24 hours. Following incubation with 10 ng/ml LIGHT for 40 minutes, cells were washed twice in ice-cold PBS and lysed in 100 μl of sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris HCl, pH, 10% glycerol, 2% SDS, 5% mercaptoethanol and 0.001% bromophenol blue). Cell lysates were separated by SDS-polyacrylamide gel electrophoresis in 12% polyacrylamide gels, and transferred onto nitrocellulose membranes (Invitrogen, CA). After blocking, membranes were incubated with either anti-β-actin or anti-IκBα antibody, overnight at 4°C, and then with secondary antibody conjugated to horseradish peroxidase (DAKO), at room temperature for 1 hour. The

signals were visualized using chemiluminescence reagent (ECL; Amersham Biosciences, Little Chalfont, UK).

Statistical analysis. Comparisons of ≥3 populations were made using the Kruskal-Wallis test. Comparisons of 2 independent data sets were made using the Mann-Whitney U-test. P values less than 0.05 were considered statistically significant.

Results

Increased expression of LIGHT in the synovial fluid of RA patients. To examine whether or not LIGHT is involved in the pathogenesis of RA, we analyzed the level of LIGHT in synovial fluids from 23 RA patients and 10 OA patients by ELISA. The synovial fluids from OA patients were used as controls, because synovial fluids were not available from healthy individuals. The concentration of LIGHT in synovial fluids from RA patients was significantly higher than in those from OA patients (Figure 1). Median levels of LIGHT in synovial fluids from RA and OA patients were 108.5 pg/ml and 7.8 pg/ml, respectively.

Expression of LIGHT and its receptors in RA synovial tissue and RA FLS. Because RA patients had more LIGHT in their synovial fluid than OA patients, we determined whether LIGHT and its membrane-bound receptors, HVEM and LTβR, were

expressed in the synovial tissues from RA patients and OA patients. Although quantitative real-time PCR analysis revealed that the mRNA expression of LIGHT in synovial tissue was significantly higher in RA patients than in OA patients (Figure 2A), HVEM and LTβR levels were not different between RA and OA patients.

Furthermore, we investigated the mRNA expression of LIGHT, HVEM and LTβR in RA FLS by RT-PCR. RA FLS from all seven patients expressed HVEM and LTβR mRNAs, but no LIGHT expression was detected (Figure 2B).

Induction of RA FLS proliferation by LIGHT. Previous studies reported that LIGHT induced cell proliferation in T lymphocytes (7,12) and vascular smooth muscle cells (13). Since the expression of HVEM and LTβR in RA FLS had been confirmed, we next evaluated the effect of LIGHT on the proliferation of RA FLS using a BrdU assay. As shown in Figure 3A, treatment with LIGHT significantly enhanced de novo DNA synthesis in RA FLS in a dose-dependent manner. LIGHT showed growth-promoting activity equivalent to that of epidermal growth factor (EGF), which was used as a positive control.

Next, to investigate the contributions of HVEM and LTβR to the LIGHT-induced proliferation of RA FLS, we transfected RA FLS with HVEM siRNA or LTβR siRNA. Quantitative real-time PCR analysis revealed that the HVEM mRNA level

in HVEM siRNA-transfected RA FLS was reduced by 75% compared with control siRNA-transfected RA FLS (Figure 3B). Similarly, treatment of RA FLS with LTβR siRNA led to a 75% reduction in the LTβR mRNA level compared with that in control siRNA-transfected RA FLS (Figure 3B). Under these conditions, LIGHT-induced growth of RA FLS was significantly decreased by LTβR siRNA, but not by HVEM siRNA, when compared with control siRNA (Figure 3C). Both LTβR siRNA and HVEM siRNA had little effect on the EGF-induced proliferation of RA FLS (data not shown).

LIGHT induces the expression of proinflammatory cytokines, chemokines and adhesion molecules in FLS via LTβR. Previous studies reported that LIGHT induces the secretion of various cytokines and augments the expression of adhesion molecules (13,15-17,19). We examined the effects of LIGHT on inflammatory cytokine production by RA FLS. Treatment with LIGHT enhanced both mRNA and protein expression of IL-8 and MCP-1 in RA FLS, in a dose-dependent manner (Figure 4A and 4B). LIGHT induced IL-1β, IL-6, GM-CSF, RANTES and MIP-1α, but not TNFα, eotaxin or MIP-1β (data not shown). Next, to assess whether LIGHT can induce cell surface adhesion molecules on RA FLS, we examined ICAM-1 and VCAM-1 expression on LIGHT-stimulated RA FLS. LIGHT treatment significantly increased the expression of ICAM-1 mRNA in a dose-dependent manner (Figure 4A and 4B). FACS analysis revealed the augmented

expression of ICAM-1 protein on the cell surface of RA FLS stimulated with LIGHT (Figure 4B). Similar increases in VCAM-1 mRNA and protein expression were also seen when stimulated with LIGHT (data not shown). Moreover, we investigated whether knockdown of HVEM or LTβR suppresses this series of LIGHT-induced gene expression in RA FLS. Compared with control siRNA, LTβR siRNA, but not HVEM siRNA, significantly decreased the expression of IL-8, MCP-1 and ICAM-1 mRNAs induced by LIGHT (Figure 5). Similarly, LTβR siRNA decreased the LIGHT-induced expression of IL-1β, IL-6, GM-CSF, RANTES and MIP-1α mRNAs in FLS (data not shown).

Activation of NF-κB in RA FLS via LTβR by LIGHT. It is known that activation of NF-κB has a key role in inflammatory disease (22). Several studies have shown that LIGHT activates the transcription factor NF-κB in different cell types (7,9,13,23-25). To demonstrate the involvement of NF-κB in LIGHT-induced gene expression, we examined the effect of the NF-κB inhibitor PDTC on the expression of IL-8, MCP-1 and ICAM-1 by real-time quantitative PCR. PDTC completely abolished the LIGHT-induced expression of IL-8, MCP-1 and ICAM-1 (Figure 6A). The LIGHT-induced expression of IL-1β, IL-6, GM-CSF, RANTES and MIP-1α mRNAs, in RA FLS, was also inhibited by PDTC treatment (data not shown). The concentration of PDTC used in these experiments had no cytotoxic effect, as demonstrated by cell viability studies using trypan blue

exclusion, which showed that >95% of cells remained viable over the entire period of the experiment (data not shown).

In the immunocytofluorescence analysis using anti-NF-κB p65 mAb, enhanced nuclear translocation of NF-κB p65 was observed in LIGHT-stimulated RA FLS (Figure 6B). Furthermore, Western blotting using anti-IκBα mAb showed that IκBα degradation was induced by LIGHT, and that IκBα degradation was inhibited by LTβR siRNA, but not by HVEM siRNA (Figure 6C).

Discussion

In the present study, we demonstrated that LIGHT, but not HEVM or LTβR, is overexpressed in the synovial tissues of RA patients compared with those of OA patients. The expression of LIGHT was not detected in RA FLS, which is one of the major components of the RA synovium. The RA synovium is histologically characterized by prominent infiltration of macrophages and lymphocytes (26). Although LIGHT has been supposed to be produced by activated T lymphocytes in vitro (4,5), a recent study reported that LIGHT was overexpressed in CD68-positive macrophages in RA synovial tissue compared with those in OA synovial tissue, and that expression levels of LIGHT

were low in areas rich in lymphocytes (19). Thus, macrophages rather than FLS and lymphocytes could be the major source of LIGHT in the RA synovium.

We further demonstrated that in vitro-cultured RA FLS express HVEM and LTβR, which implies that RA FLS are target cells of LIGHT. Indeed, in the present study, we first showed that LIGHT induced RA FLS proliferation to the same extent as EGF did. The proliferation of RA FLS is one of the most critical pathological changes in RA. Therefore, our findings suggested that increased expression of LIGHT might lead to the synovial hyperplasia of RA. Anti-cytokine therapies targeting TNFα, IL-1 and IL-6 have been used to treat patients with RA, and it has been demonstrated that such treatments may suppress the accompanying bone destruction as well as the synovitis (29,30). In addition, recent studies have indicated that LIGHT reduces Fas-mediated apoptosis in FLS (31), that LIGHT may function as a mediator of bone resorption through the induction of osteoclastogenesis (32), and that LTβR-Ig protein blocks the induction of experimental arthritis in mice (18). Thus, a neutralizing antibody against LIGHT could be a useful tool for the inhibition of synovial hyperplasia and bone destruction in RA.

The enhanced effects of LIGHT on RA FLS proliferation were significantly inhibited by LTβR siRNA, but not by HVEM siRNA, suggesting that LTβR, rather than HVEM, is involved in the LIGHT-induced proliferation of RA FLS. The exact

mechanism by which LIGHT influences RA FLS proliferation through LTβR is unknown. A potential mechanism underlying RA FLS proliferation induced by LIGHT may involve cell cycle regulators, including cyclin-dependent kinase (CDK). The mammalian cell cycle is controlled by holoenzymes composed of a catalytic CDK and regulatory cyclin. The expression of p21 was reduced in RA synovial linings and FLS compared with the level in those of OA patients (33). Overexpression of p21 or p16 by adenovial-mediated delivery suppresses FLS growth in vitro (34,35). Furthermore, LIGHT induces cell proliferation, down-regulates the CDK inhibitors p21, p27 and p53, and inversely up-regulates cyclin D and Rb hyper-phosphorylation in vascular smooth muscle cells (13). Thus, it is possible that LIGHT promotes FLS proliferation by shortening the cell cycle of FLS in RA patients. Wang reported that LTβR null mice show reduced BrdU incorporation in dendritic cells (36). This supports our claim that LTβR signaling is involved in the proliferation of RA FLS.

In the current study, we have demonstrated that LIGHT also induces the production of inflammatory cytokines and chemokines and the expression of adhesion molecules on RA FLS. Inflammatory cytokines such as TNFα and IL-1β are involved in the pathogenesis of RA (1,2). The increased expression of ICAM-1 and VCAM-1 adhesion molecules on activated endothelial cells enhances the recruitment of monocytes,

lymphocytes and neutrophils, leading to inflammation. These findings indicate that LIGHT might play an important role in the inflammation in the synovial lining layer, as well as its hyperplasia. A recent study revealed that LIGHT upregulates the expression of ICAM-1, VCAM-1 and IL-6 in RA FLS via NF-κB activation (31,37). Although these reports are consistent with our present results, it has not been clear which of two receptors is involved in the induction of these genes in FLS. Our knockdown analysis using siRNA revealed that LIGHT induced the proliferation and the gene expression by signaling via LTβR, but not HVEM. Braun et al. has shown that LTβR is expressed on RA FLS, and that LTα1β2, which is a ligand for LTβR, induces the expression of inflammatory cytokines, chemokines and ICAM-1 (38). This supports our claim that LTβR signaling is involved in the activation of RA FLS. The NF-κB transcription factor is certainly involved in cytokine- and chemokine-driven responses and is a point of convergence for several upstream proinflammatory pathways (22). In fact, NF-κB activation appears to be an important factor in RA, as the expression of NF-κB is enhanced in lining cells (39,40) and in the cartilage-pannus junction in the RA synovium (41). In the present study, treatment with PDTC blocked LIGHT-induced IL-8, MCP-1 and ICAM-1 expression, suggesting that the effects of LIGHT are mediated through NF-κB. The involvement of NF-κB in LIGHT-induced proinflammatory responses was

further confirmed by the LIGHT-induced nuclear translocation of NF-κB p65. Moreover, LIGHT induced IκBα degradation in RA FLS, an effect that was inhibited by LTβR siRNA, but not by HVEM siRNA. These findings are consistent with previous studies showing that LTβR ligation can lead to activation of NF-κB (23,42-45). However, it is unknown why LIGHT prefers the LTbR signaling pathway in RA FLS, even though HVEM is also expressed on these cells.

In conclusion, we demonstrated that LIGHT is overexpressed in RA synovial tissues and synovial fluids. LIGHT induced increased production of inflammatory cytokines, chemokines and adhesion molecules through NF-κB activation, as well as proliferation of RA FLS. These findings indicate that LIGHT signaling via LTβR plays an important role in the pathogenesis of RA by affecting key process, such as the proliferation and activation of RA FLS. Therefore, regulation of LIGHT-LTβR signaling may represent a new therapeutic target for the treatment of RA.

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