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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.

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-

ous periods of time, as indicated in the Results, cells were fixed and stained for TRAP using a TRAP staining kit (Wako, Osaka, Japan). Osteoclasts were identified as TRAP-positive MNCs (more than five nuclei). AlexaFluor546-conjugated phalloidin was used to stain for F-actin (Invitrogen).

Bone resorption assay

In the presence of 25 ng/ml M-CSF, NCD14$^+$ monocytes were cultured on cortical bone slices in α-minimum essential medium supplemented with 10% FCS and further stimulated with 40 ng/ml RANKL and/or 100 ng/ml LIGHT. After 21 days, the bone slices were stained with Mayer's haematoxylin solution to detect resorption pits. The concentration of the type I collagen C-telopeptide (CTX-I) in the culture supernatant was quantified using the CrossLaps for Culture kit (Nordic Biosciences Diagnostics, Herlev, Denmark), according to the manufacturer's instructions.

Quantitative polymerase chain reaction analysis

Total RNA was prepared using an RNeasy Micro kit (Qiagen, Tokyo, Japan) and complementary DNA (cDNA) was generated from the RNA using Omniscript Reverse Transcriptase (Qiagen) following the manufacturer's instructions. The cDNA was used as a template for real-time quantitative polymerase chain reaction (PCR) in a LightCycler (Roche Diagnostics, Tokyo, Japan). The PCR was performed using SYBR Premix Ex Taq (Takara, Kyoto, Japan). The PCR primers used in this study were as follows: for NFATc1, 5′-TACCAGGTGCACCGCATCA-3′ and 5′-TTTCAGGATTCCGGCACAGTC-3′; for TRAP, 5′-TGCA GATCCTGGGTGCAGAC-3′ and 5′-GAGTATGCAATC TGGGCAGAGACA-3′; for cathepsin K, 5′-AGCT GCAATAGCGATAATCTGAACC-3′ and 5′-CGTTGTTC TTATTTCGAGCCATGA-3′; for carbonic anhydrase II, 5′-GCGACCATGTCCCATCACTG-3′ and 5′-TGGCTGTAT GAGTGTCGATGTCAA-3′; for gyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-GCACCGTCAAGGCTGAG AAC-3′ and 5′-ATGGTGGTGAAGACGCCAGT-3′; for LIGHT, 5′-TCACGAGGTCAACCCAGCAG-3′ and 5′-CC CAGCTGCACCTTGGAGTAG-3′; for HVEM, 5′-TTTG CTCCACAGTTGGCCTAATC-3′ and 5′-CAATGACTGT GGCCTCACCTTC-3′; for LTβR, 5′-ATGCTGATGCTG-GCCGTTC-3′ and 5′-AGGCTCCCAGCTTCCAGCTA-3′; for RANK, 5′-TTGTGCCGCCTAAGTGGA-3′ and 5′-ACC ACCTTGATCTGGGTAGCACATA-3′; for MMP-9, 5′-AC CTCGAACTTTGACAGCGACA-3′ and 5′-GATGCCATTC ACGTCGTCCTTA-3′; for MMP-12, 5′-TTGATGGCAAA GGTGGAATCCTA-3′ and 5′-AGGAATGGCCAATCTCGT GAAC-3′. The PCR was performed under the following conditions: initial denaturation at 95° for 10 seconds, then 40 cycles of 95° for 5 seconds and 60° for 20 seconds. SYBR green dye was used to detect amplified products and melting curves were routinely recorded to verify the singularity of the PCR product. In each sample, the level of cDNA was normalized based on the expression level of GAPDH.

Immunohistochemical and TRAP staining of tissue samples

Affected knee joints were resected during joint replacement surgery from five RA and three OA patients who had given written informed consent. Serial sections of the decalcified and paraffin-embedded subchondral bone were dewaxed and reacted with anti-human MMP-12 monoclonal antibody (clone 4D2, R&D Systems). Sections were then reacted with anti-mouse immunoglobulin G–horseradish peroxidase conjugate, chromogenic substrate and hydrogen peroxide. The neighbouring sections of those stained with anti-MMP-12 were subjected to staining with second antibody alone as a negative control or with TRAP staining as described above.

Statistical analysis

All data are expressed as means ± SD. A non-paired Student's *t*-test was used for comparison, using the STATVIEW program (Abacus Concepts, Berkeley, CA). $P < 0.05$ was considered to be statistically significant.

Results

LIGHT induces the differentiation of NCD14$^+$ monocytes into TRAP-positive MNCs

To investigate whether or not LIGHT is involved in local bone destruction, we examined the effects of LIGHT on osteoclastogenesis using established osteoclast precursors (NCD14$^+$ monocytes) in addition to freshly prepared CD14$^+$ monocytes, as described in the *Materials and methods* section.

In the presence of M-CSF, CD14$^+$ or NCD14$^+$ monocytes were cultured for 6 days with RANKL and/or LIGHT. As shown in Fig. 1(a,b), M-CSF alone did not induce TRAP-positive MNCs from either type of monocytes. CD14$^+$ monocytes were differentiated into TRAP-positive MNCs by RANKL, but not by LIGHT. The combination of RANKL and LIGHT had little effect on MNC formation. Conversely, NCD14$^+$ monocytes were strongly differentiated into TRAP-positive MNCs when treated with LIGHT. Although RANKL had only a slight effect on NCD14$^+$ monocytes, the combination with LIGHT enhanced the formation of TRAP-positive MNCs more than LIGHT alone (Fig. 1b). The LIGHT-induced formation of MNCs was time dependent (4–8 days, Fig. 1c), and dose dependent (1–100 ng/ml, data not shown). Freshly isolated

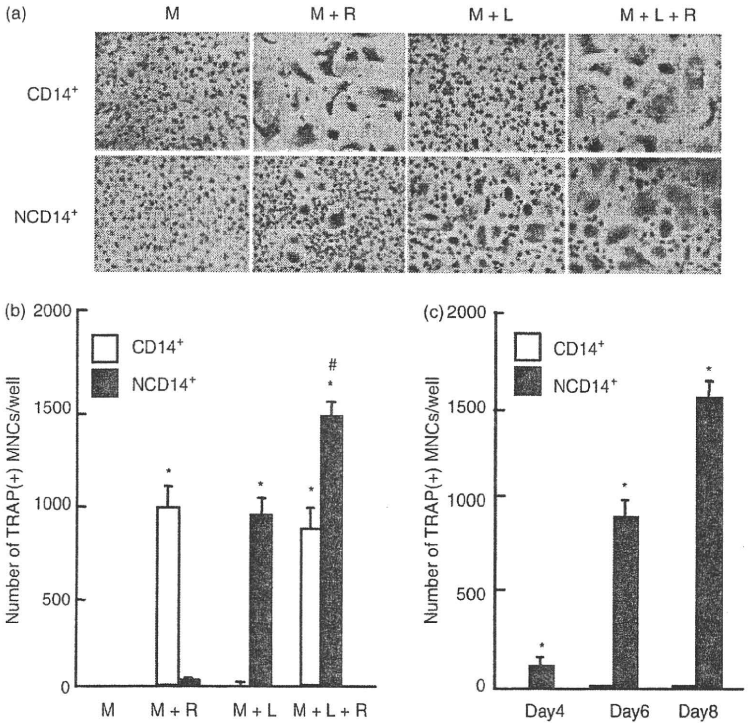

Figure 1. LIGHT induces the differentiation of NCD14$^+$ monocytes into tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs). (a, b) In the presence of 25 ng/ml macrophage colony-stimulating factor (M-CSF; M), CD14$^+$ or NCD14$^+$ monocytes were cultured for 6 days with 40 ng/ml receptor activator of nuclear factor-κB ligand (RANKL; M + R), 100 ng/ml LIGHT (M + L), or 40 ng/ml RANKL plus 100 ng/ml LIGHT (M + L + R). *P < 0·01 versus M-CSF alone. #P < 0·01 versus NCD14$^+$ monocytes stimulated with M-CSF plus LIGHT. (c) CD14$^+$ or NCD14$^+$ monocytes were cultured for the indicated periods in the presence of 25 ng/ml M-CSF plus 100 ng/ml LIGHT. Cultured cells were fixed and stained for TRAP. The number of TRAP-positive MNCs was counted. *P < 0·01 versus CD14$^+$ monocytes. Representative results of at least three independent sets of similar experiments are shown as means ± SD of triplicate experiments.

CD14$^+$ monocytes, however, did not differentiate into TRAP-positive MNCs, even after stimulation with 100 ng/ml LIGHT for 14 days (data not shown). When cultured with M-CSF for 4 weeks, CD14$^+$ monocytes could not differentiate into TRAP-positive MNCs in the presence of RANKL or LIGHT (data not shown).

Increased HVEM messenger RNA expression in NCD14$^+$ monocytes

Next, to clarify the reason for the difference in the efficiency of LIGHT-induced TRAP-positive MNC formation between NCD14$^+$ and CD14$^+$ monocytes, we analysed the messenger RNA (mRNA) expression of the LIGHT receptors, HVEM and LTβR, in both groups of monocytes. Quantitative real-time PCR analysis revealed that while the mRNA expression level of LTβR was not different between groups of monocytes, the level of HVEM mRNA was significantly higher in NCD14$^+$ monocytes than in CD14$^+$ monocytes (Fig. 2). Unexpectedly, the level of RANK mRNA in NCD14$^+$ monocytes was higher than that in CD14$^+$ monocytes (Fig. 2).

Analysis of the molecular phenotype of LIGHT-induced TRAP-positive MNCs derived from NCD14$^+$ monocytes

Furthermore, we investigated the mRNA expression of major osteoclast markers, such as nuclear factor of activated T cells (NFATc1), TRAP, cathepsin K (CTSK) and

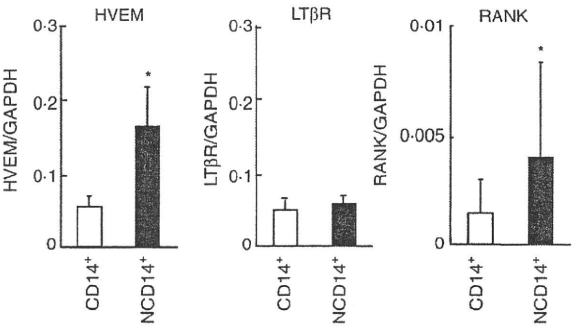

Figure 2. Expression of herpes virus entry mediator (HVEM), lymphotoxin β receptor (LTβR) and receptor activator of nuclear factor-κB (RANK) messenger RNA (mRNA) on CD14$^+$ and NCD14$^+$ monocytes. Total RNA was extracted from CD14$^+$ and NCD14$^+$ monocytes and the mRNA expression levels of HVEM, LTβR and RANK were analysed by quantitative real-time polymerase chain reaction. Representative results of at least three independent sets of similar experiments are shown as means ± SD of triplicate experiments. *P < 0·01 versus CD14$^+$ monocytes.

carbonic anhydrase II (CAII), in LIGHT-induced TRAP-positive MNCs derived from NCD14$^+$ monocytes, using quantitative real-time PCR analysis. In comparison with the control (M-CSF alone), the expression levels of all four genes were upregulated in TRAP-positive MNCs induced to differentiate by LIGHT for 6 days (Fig. 3a). The combination of LIGHT and RANKL stimulated their expression to a similar or slightly larger extent than LIGHT alone. When NCD14$^+$ monocytes are stimulated

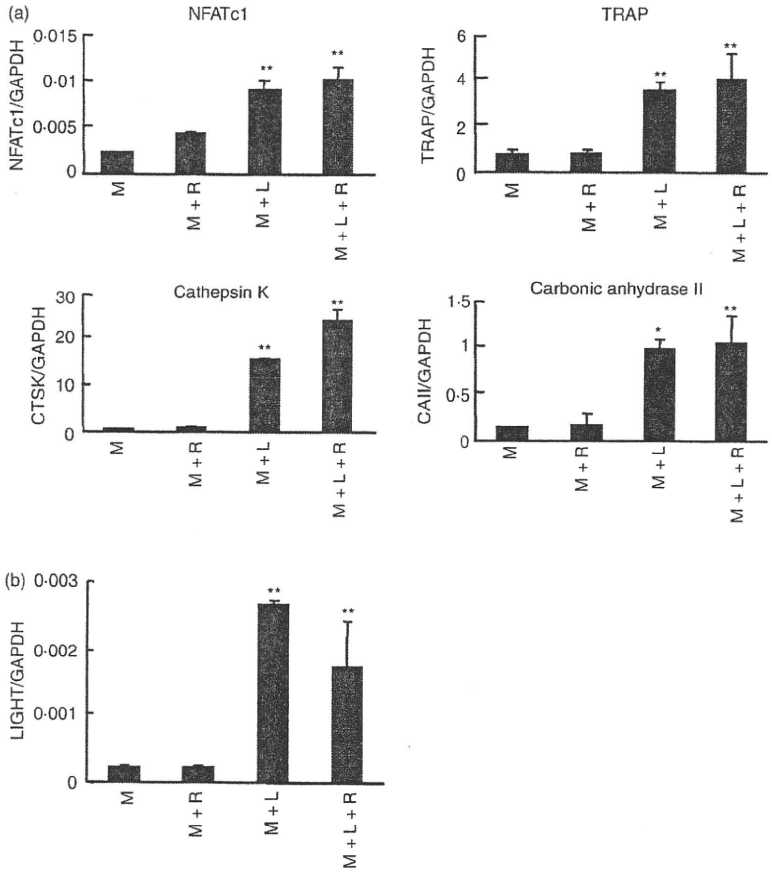

Figure 3. The molecular phenotype of LIGHT-induced tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) derived from NCD14$^+$ monocytes. In the presence of 25 ng/ml macrophage colony-stimulating factor (M-CSF; M), NCD14$^+$ monocytes were cultured for 6 days with 40 ng/ml receptor activator of nuclear factor-κB ligand (RANKL) (M + R), 100 ng/ml LIGHT (M + L), or both (M + L + R). The expression levels of (a) osteoclast markers and (b) LIGHT were determined by quantitative real-time polymerase chain reaction. Representative results of at least three independent sets of similar experiments are shown as means ± SD of triplicate experiments. $^*P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$ versus M-CSF alone.

by LIGHT or LIGHT plus RANKL, the CTSK and TRAP mRNA expression levels are higher in NCD14$^+$ monocytes stimulated for 12 days than in those stimulated for 6 days (data not shown). These inductions suggest that prolonged stimulation with LIGHT can induce more mature osteoclasts. Moreover, LIGHT induced an increase in its own expression level in NCD14$^+$ monocytes (Fig. 3b).

LIGHT-induced TRAP-positive MNCs from NCD14$^+$ monocytes have bone resorption activity

Since the expression levels of genes related to bone resorption by osteoclasts were upregulated in LIGHT-induced TRAP-positive MNCs from NCD14$^+$ monocytes, we assessed whether or not the MNCs could resorb bone. The formation of a ringed F-actin structure, called an actin ring, is closely related to osteoclast function.[35] Actin rings were recognized at the periphery of LIGHT-induced MNCs regardless of the presence of RANKL (Fig. 4a). Furthermore, resorption pits were observed on bone slices in the cultures treated with LIGHT, and the effect of LIGHT was enhanced by additional stimulation with RANKL (Fig. 4b). As expected, CTX-I release was induced in the presence of LIGHT and further increased by the combination with RANKL (Fig. 4c). By contrast, cells treated with M-CSF alone or with a combination of M-CSF and RANKL did not form resorption pits.

LIGHT-induced TRAP-positive MNCs from NCD14$^+$ monocytes express both MMP-9 mRNA and MMP-12 mRNA

In osteoclasts, MMP-9 is abundant and it is known to be a protease involved in bone resorption. We examined MMP-9 mRNA expression in both fresh CD14$^+$ and NCD14$^+$ monocytes stimulated by LIGHT or RANKL. Compared with RANKL, LIGHT or the combination of LIGHT and RANKL enhanced MMP-9 mRNA expression in both groups of monocytes (Fig. 5a). On the other hand, because we previously reported that MMP-12 was expressed in MNCs induced from NCD14$^+$ monocytes, but not in those from CD14$^+$ monocytes,[36] the influence of LIGHT upon the expression of MMP-12 mRNA in both monocytes was investigated. NCD14$^+$ monocytes strongly upregulated MMP-12 mRNA expression in response to LIGHT or the combination of LIGHT and RANKL, compared with control (M-CSF alone) or RANKL, whereas the MMP-12 mRNA levels

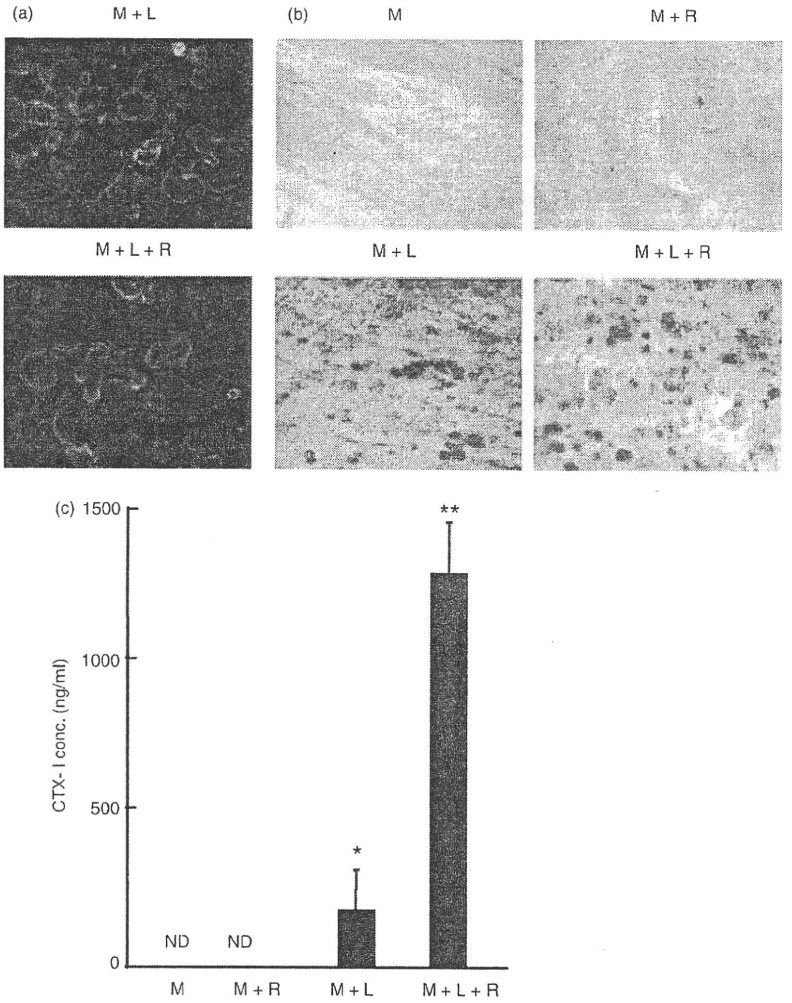

Figure 4. Osteoclastic functions of LIGHT-induced tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) from NCD14$^+$ monocytes. (a) For actin ring formation, NCD14$^+$ monocytes were stimulated, in the presence of 25 ng/ml macrophage colony-stimulating factor (M-CSF), with 100 ng/ml LIGHT (M + L), or 40 ng/ml receptor activator of nuclear factor-κB ligand (RANKL) plus 100 ng/ml LIGHT (M + L + R) for 6 days. Fixed cells were stained with Alexa-Fluor546-conjugated phalloidin. (b) In the presence of 25 ng/ml M-CSF (M), NCD14$^+$ monocytes were cultured for 21 days on bone slices with 40 ng/ml RANKL (M + R), 100 ng/ml LIGHT (M + L), or both (M + L + R). (c) The release of type I collagen C-telopeptide (CTX-I) was quantified in the culture supernatants using enzyme-linked immunosorbent assay. Representative results of at least three independent sets of similar experiments are shown as means ± SD of triplicate experiments. $^*P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$ versus M-CSF alone.

remained low in CD14$^+$ monocytes after any stimulation (Fig. 5b).

Detection of TRAP-positive MNCs expressing MMP-12 in the subchondral bone of RA patients

To confirm the presence of MMP-12-expressing MNCs in the erosive area of the RA joint, serial sections of decalcified, paraffin-embedded subchondral bone of five RA patients and three OA patients were subjected to immunostaining with an anti-MMP-12 antibody. As shown in Fig. 6(a,b), both MMP-12-positive and MMP-12-negative MNCs expressing TRAP were present in the affected bone areas of all five RA patients examined in this study. The ratios of MMP-12-positive MNCs to TRAP-positive MNCs in RA patients were 46·7% (30/63) in patient 1, 52·5% (31/59) in patient 2, 41·7% (33/79) in patient 3, 2·2% (3/135) in patient 4, and 10·0% (9/90) in patient 5. By contrast, no MMP-12-positive MNCs were observed in the OA patients (Fig. 6b).

Discussion

Formation of osteoclasts and consequent joint destruction are hallmarks of RA. We previously reported that CD14$^+$ monocytes cocultured with NLCs (termed NCD14$^+$ monocytes in this article) were TRAP positive and that NCD14$^+$ monocytes differentiated into osteoclasts following treatment with RANKL or TNF-α. Consequently, NCD14$^+$ monocytes have been thought to be osteoclast precursors.[16,17]

In this study, to investigate whether LIGHT is involved in bone destruction in RA, we examined the effects of LIGHT on osteoclastogenesis using CD14$^+$ and NCD14$^+$ monocytes, and compared their abilities to differentiate into osteoclasts. When stimulated with RANKL for 6 days, a number of TRAP-positive MNCs, mature osteoclasts, were generated from CD14$^+$ monocytes, but only a few MNCs were generated from NCD14$^+$ monocytes. Conversely, LIGHT strongly induced MNCs from NCD14$^+$ monocytes, but not from CD14$^+$ monocytes.

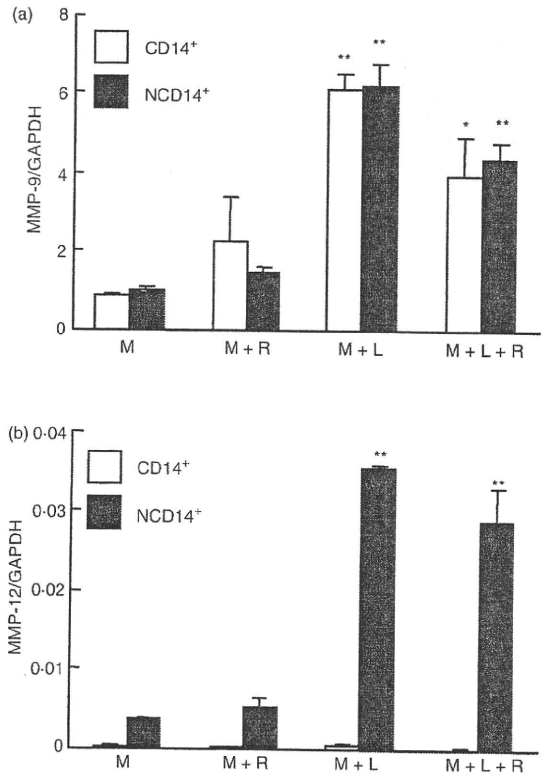

Figure 5. Comparison of matrix metalloproteinase-9 (MMP-9) and MMP-12 messenger RNA (mRNA) expression in $CD14^+$ or $NCD14^+$ monocyte-derived tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs). In the presence of 25 ng/ml macrophage-colony-stimulating factor (M-CSF; M), $CD14^+$ or $NCD14^+$ monocytes were cultured for 6 days with 40 ng/ml receptor activator of nuclear factor-κB ligand (RANKL; M + R), 100 ng/ml LIGHT (M + L), or both (M + L + R). The mRNA expression levels of (a) MMP-9 and (b) MMP-12 were determined by quantitative real-time polymerase chain reaction. Representative results of at least three independent sets of similar experiments are shown as means ± SD of triplicate experiments. $^*P < 0.05$ and $^{**}P < 0.01$ versus M-CSF alone.

Furthermore, LIGHT-induced MNCs derived from $NCD14^+$ monocytes showed several characteristics of osteoclasts, including the expression of genes encoding NFATc1, TRAP, CTSK and CAII, actin-ring formation, and the ability to resorb bone. These results indicate that LIGHT might be involved in bone destruction by forming osteoclasts from precursors through the interaction with NLCs.

RANKL enhanced LIGHT-induced osteoclast formation and bone resorption. Recent reports have shown that synoviocytes from patients with RA express a higher level of RANKL and could induce osteoclastogenesis from synovial macrophages.[7–9] Hence, the synergistic effects of RANKL and LIGHT on osteoclastogenesis may play an important role in the bone destruction in RA. LIGHT has been reported to be augmented in RA synovial fluids compared with those in OA patients.[34] Reports on the LIGHT-expressing cells in RA synovial tissue remain confused because of inconsistent immunohistochemical evaluations. These cells have been separately identified as macrophages[32] and T lymphocytes.[34] In the present study, LIGHT induced its own expression in $NCD14^+$ monocytes, suggesting that LIGHT might drive a positive feedback loop of osteoclastogenesis. LIGHT induced the expression of intercellular adhesion molecule-1 (ICAM-1) in RA synovial fibroblasts,[33,34] and the expression of IL-8 and monocyte chemoattractant protein-1 in RA synovial macrophages.[32] Since ICAM-1 and these chemokines could play a crucial role in the recruitment of monocytes into the synovial sublining,[37] LIGHT might not only induce osteoclastogenesis, but also increase the number of osteoclast precursors in the synovium of RA patients by recruiting monocytes. The exact mechanism by which $NCD14^+$ monocytes gain the ability to differentiate into osteoclasts in response to LIGHT is unclear. In this study, we confirmed the upregulation of HVEM only at the mRNA level. If HVEM mRNA expression correlates with protein expression, augmented HVEM can explain how the cells gain the ability to differentiate into osteoclasts in response to LIGHT. On the other hand, the decreasing responsiveness of $NCD14^+$ monocytes to RANKL alone contradicts the increasing expression of RANK mRNA. Because RANKL drastically enhanced LIGHT-induced osteoclastogenesis from $NCD14^+$ monocytes and the bone-resorbing activity, $NCD14^+$ cells could be responsive to RANKL. These findings indicate that the decreasing responsiveness to RANKL may be the result of alterations in the downstream pathways of RANK/RANKL signalling in $NCD14^+$ monocytes. Whereas LIGHT could not induce osteoclast differentiation from $CD14^+$ monocytes, it has been previously reported that LIGHT induces the expression of MMP-9 in monocytes.[31,32,38] These findings suggest that $CD14^+$ monocytes could somehow respond to LIGHT via HVEM or LTβR.

A recent study reported that LIGHT promotes osteoclastogenesis in RANKL-dependent and -independent manners;[39] however, in the present study, we could not observe LIGHT-induced osteoclastogenesis from $CD14^+$ monocytes. The discrepancy might be caused by differences in culture conditions or in the monocyte preparation methods (an adherence technique versus a magnetic bead method).

In addition to MMP-9 expression, LIGHT-induced MNCs derived from $NCD14^+$ monocytes expressed MMP-12 mRNA, which was not expressed in osteoclasts induced from $CD14^+$ monocytes by RANKL. The TRAP-positive MNCs expressing MMP-12 were present in the erosive areas of RA joints, but were not found in the affected bones of OA joints, implying that they may be unique to RA.

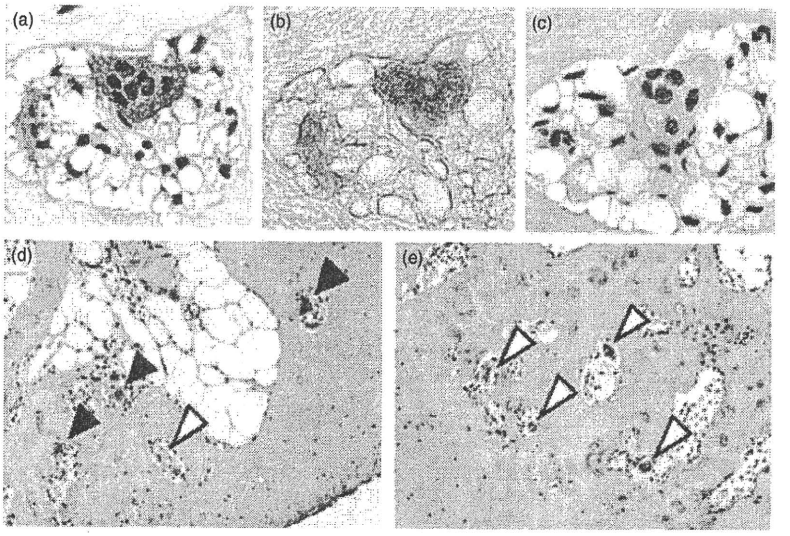

Figure 6. Detection of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) expressing matrix metalloproteinase-12 (MMP-12) in the erosive area of the rheumatoid arthritis (RA) joint. Affected subchondral bone samples were obtained from the resected knee joints of patients with RA and patients with osteoarthritis (OA). Serial sections were cut from decalcified and paraffin-embedded subchondral bone. (a) RA tissue samples were stained with anti-human MMP-12 monoclonal antibody. Neighbouring sections were stained for TRAP activity (b) or with second antibody alone as a negative control (c). All three sections were consecutive. (d) RA and (e) OA tissue samples were stained with anti-human MMP-12 monoclonal antibody. Solid arrowheads and open arrowheads show MMP-12-positive and -negative MNCs, respectively. Representative results from five RA patients and three OA patients are shown.

Although MMP-12 was expressed in rabbit and mouse osteoclasts,[40] MMP-12-expressing osteoclasts have not been identified in humans. This is the first report to demonstrate the presence of MMP-12-expressing osteoclasts in human RA joints. Increased expression of MMP-12 in RA synovial tissues and synovial fluids indicates the involvement of this enzyme in RA pathogenesis.[41] A study using MMP-12-deficient mice demonstrated that MMP-12 was not critical for bone resorption or osteoclast recruitment.[40] Although MMP-12 might not be directly involved in bone resorption in humans, several reports suggest that MMP-12 could be involved in cartilage destruction.[42,43] We previously reported that $NCD14^+$ and MMP-12-producing osteoclast-like cells differentiated from $NCD14^+$ monocytes could degrade the proteoglycan of bovine cartilage.[36] MMP-12 is critical for invasion and destruction in pathologies such as emphysema[44] and cutaneous granulomas.[45] Rheumatoid arthritis is also a granulomatous disease with tissue hyperplasia and destruction. Osteoclasts invading uncalcified cartilage beyond the tidemark are frequently found in the erosive areas of RA joints. Consequently, MMP-12-producing osteoclasts might play important roles in inflammation or cartilage destruction as well as in the bone destruction in RA.

Synovial fluid macrophages from RA patients could differentiate into osteoclasts via both RANKL and TNF-α signalling pathways, whereas OA macrophages differentiated into osteoclasts only through the RANKL pathway.[46] Moreover, osteoclasts derived from circulating precursors in RA patients have an increased bone-resorbing activity.[47] Osteoclast precursors responsive to inflammatory cytokines such as LIGHT would be present in RA patients, and the cytokine-induced osteoclastogenesis might be involved in enhanced bone destruction in RA. Although it should become clear from further investigations whether or not LIGHT-induced MNCs are identical to the MMP-12-expressing osteoclasts of RA joints, our *in vitro* differentiation system is robust and reproducible. All monocytes become LIGHT-responsive precursors of osteoclast-like cells via their interactions with RA-NLCs. We believe that our coculture system should be useful for elucidating the interactions between synovial cells and infiltrating monocytes in the pathogenic condition of RA, and the mechanism underlying inflammation-associated osteoclastogenesis.

The present study strongly suggested that LIGHT might be involved in the progression of bone destruction in RA, and implied that the blocking of LIGHT signalling may be a therapeutic target for the enhanced bone destruction in RA.

Disclosures

The authors have no financial conflict of interest.

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－関節マーカーを用いた早期診断と予後予測の確立に関する研究－

平成20年度～平成22年度　総合研究報告書

（3／4冊）

研究代表者　山田　治基

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