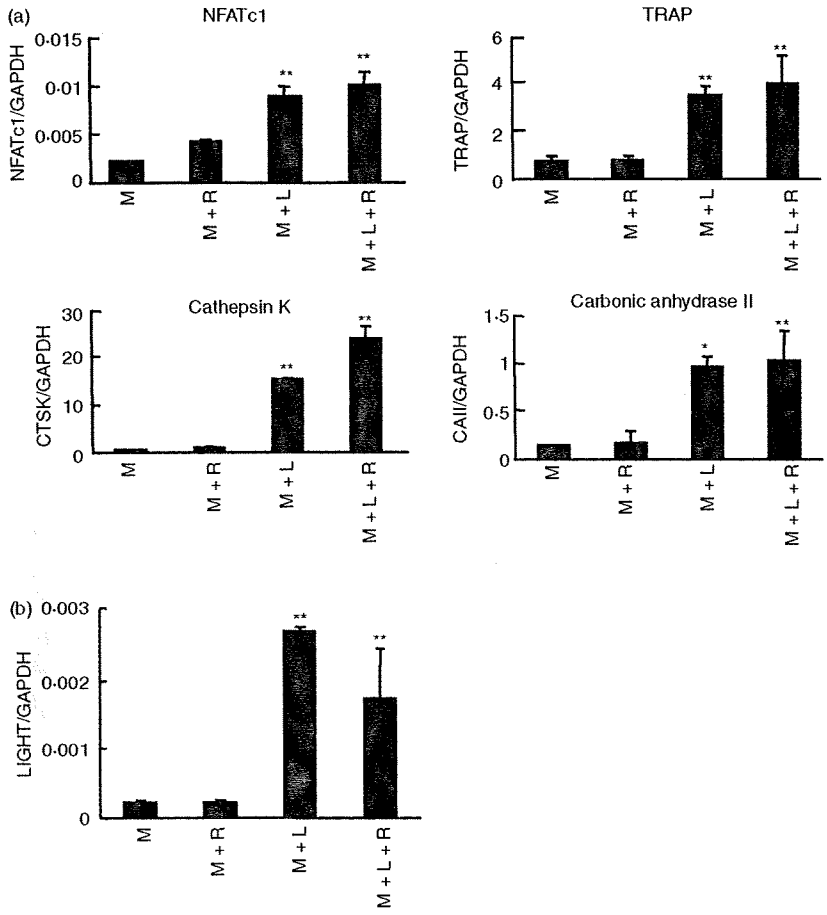

Figure 3. The molecular phenotype of LIGHT-induced tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) derived from NCD14$^+$ monocytes. In the presence of 25 ng/ml macrophage colony-stimulating factor (M-CSF; M), NCD14$^+$ monocytes were cultured for 6 days with 40 ng/ml 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

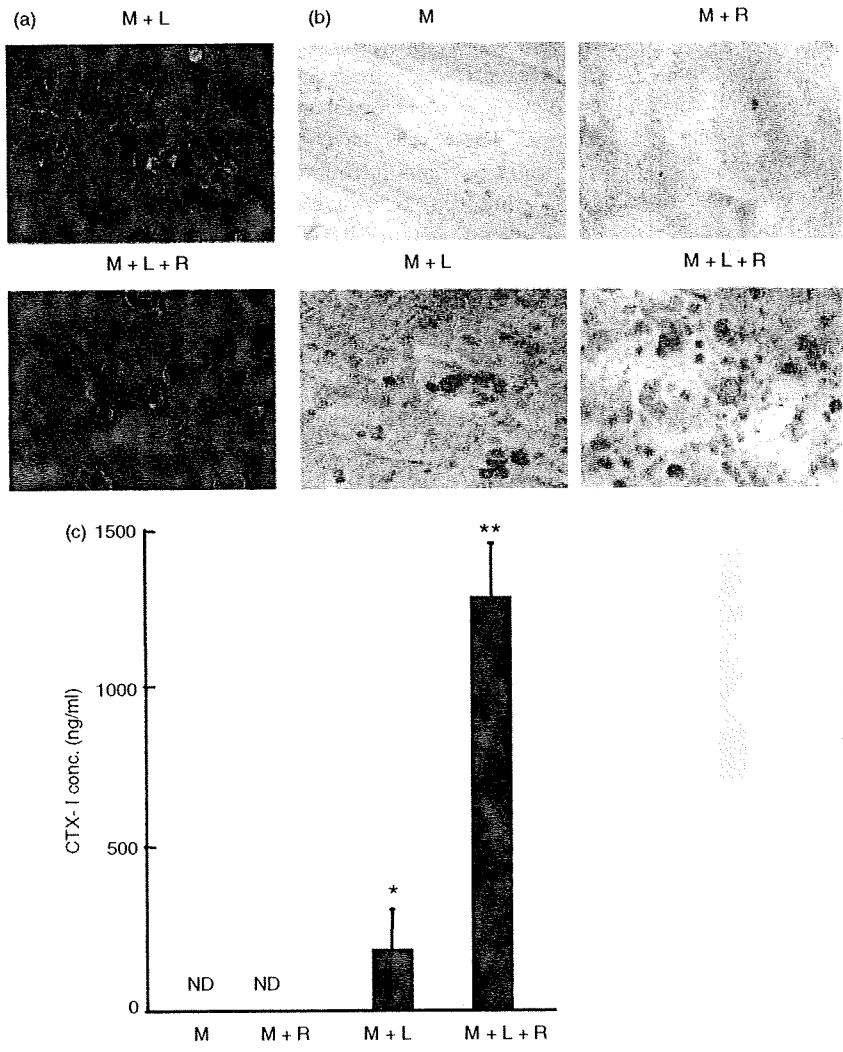

Figure 4. Osteoclastic functions of LIGHT-induced tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) from NCD14$^+$ monocytes. (a) For actin ring formation, NCD14$^+$ monocytes were stimulated, in the presence of 25 ng/ml macrophage colony-stimulating factor (M-CSF), with 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.

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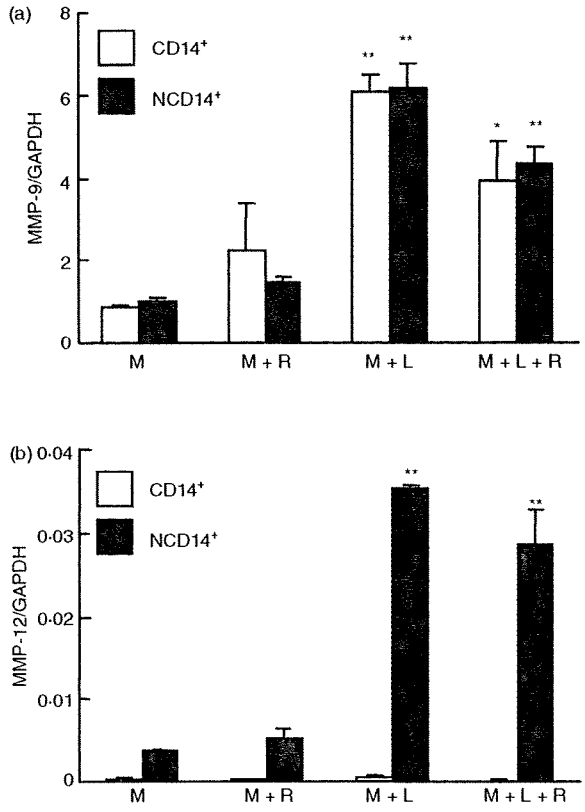

Figure 5. Comparison of matrix metalloproteinase-9 (MMP-9) and MMP-12 messenger RNA (mRNA) expression in $CD14^+$ or $NCD14^+$ monocyte derived tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs). In the presence of 25 ng/ml macrophage-colony stimulating factor (M-CSF; M), $CD14^+$ 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. $^*P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$ 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.

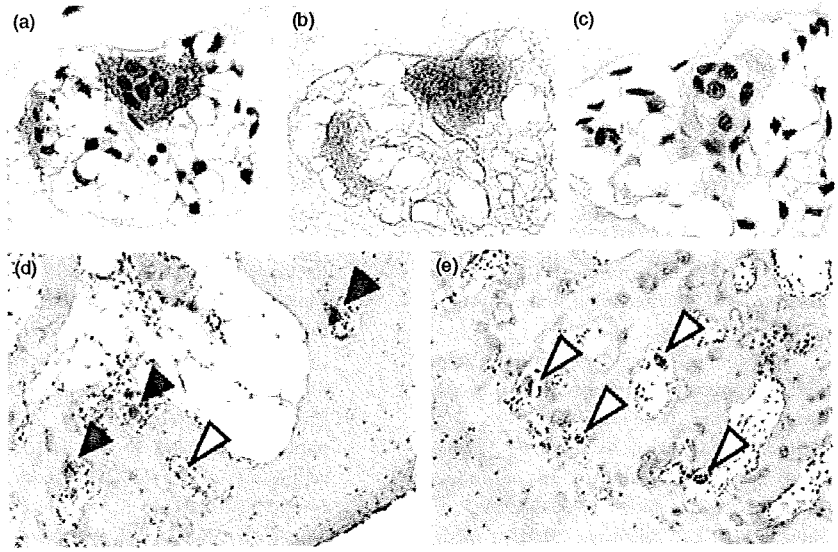

Figure 6. Detection of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) expressing matrix metalloproteinase-12 (MMP-12) in the erosive area of the rheumatoid arthritis (RA) joint. Affected subchondral bone samples were obtained from the resected knee joints of patients 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.

Acknowledgements

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Involvement of a disintegrin and metalloproteinase 10 and 17 in shedding of tumor necrosis factor-α

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Abstract: Tumor necrosis factor-α (TNF-α) is initially synthesized as a membrane-bound protein and converted into a soluble form by proteolytic cleavage. Although a disintegrin and metalloproteinase 17 (ADAM17) is considered to be the primary sheddase for TNF-α, it is not known whether ADAM17 is solely responsible for that process in any type of cells. To identify the TNF-α sheddase(s) in varieties of cells, we performed experiments using a unique screening system and observed that ADAM9, ADAM10, ADAM17, and ADAM19 were capable of cleaving TNF-α. We then performed RNA interference experiments and confirmed that ADAM10 and ADAM17 were in fact involved in TNF-α shedding in 293A cells. In mouse macrophages, ADAM17 was confirmed to be the primary sheddase, but the involvement of ADAM10 was also demonstrated. In NIH3T3 cells, ADAM10 could be more important in the shedding than ADAM17. In mouse vascular endothelial cell line UV♯2, ADAM10 and ADAM17 were equally involved in TNF-α shedding, whereas ADAM17 was a major sheddase in human osteoarthritic chondrocytes. From these observations and others, we concluded that both ADAM10 and ADAM17 can be a TNF-α sheddase and that their significance could be determined by their expression levels and the abundance of tissue inhibitor of metalloproteinases.

Key words: TNF-α, ADAM10, ADAM17, TIMP, ectodomain shedding.

Résumé : Le TNF-α est d'abord synthétisé sous forme de protéine liée à la membrane et sa forme soluble est produite par un clivage protéolytique. Même si la désintégrine métalloprotéase ADAM17 est considérée comme la principale sheddase agissant sur le TNF-α, on ignore si ADAM17 est la seule responsable de ce processus pour tous les types de cellules. Afin d'identifier les sheddases de TNF-α dans une variété de cellules, nous avons réalisé des expériences à l'aide d'un système de criblage unique et nous avons observé que ADAM9, ADAM10, ADAM17 et ADAM19 sont capables de cliver le TNF-α. Nous avons ensuite réalisé des expériences d'interférence par ARN et nous avons confirmé que ADAM10 et ADAM17 sont effectivement impliquées dans la libération du TNF-α chez les cellules 293A. Chez les macrophages de souris, ADAM17 s'est avérée comme sheddase principale mais l'implication de ADAM10 a aussi été démontrée. Chez les cellules NIH3T3, ADAM10 pourrait être plus importante pour la libération que ADAM17. Dans la lignée cellulaire vasculaire endothéliale UV♯2, ADAM10 et ADAM17 sont également impliquées dans la libération de TNF-α, alors que ADAM17 est la principale sheddase chez les chondrocytes ostéoarthritiques humains. À partir de ces observations et d'autres, nous avons conclu que ADAM10 et ADAM17 peuvent toutes deux être des sheddases du TNF-α et que leur importance pourrait être déterminée par leur niveau d'expression et par l'abondance des TIMP (« tissue inhibitor of metalloprotéinases »).

Mots-clés : TNF-α, ADAM10, ADAM17, TIMP, libération d'ectodomaine.

[Traduit par la Rédaction]

Introduction

Ectodomain shedding is a process in which transmembrane proteins are proteolytically released from the plasma membrane. Ectodomain shedding modifies the biologic and pathologic functions of the substrate protein. For example, amyloid precursor protein is cleaved by a disintegrin and metalloproteinase (ADAM) or the β-site of amyloid precursor protein cleaving enzyme and converted to a nonpathogenic and a pathogenic protein, respectively (Selkoe 1991; Buxbaum et al. 1998; Koike et al. 1999; Lammich et al. 1999; Vassar et al. 1999; Li et al. 2000). On the other hand, Fas ligand is cleaved by ADAM10 and largely loses its activity (Schneider et al. 1998; Schulte et al. 2007).

Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine that is involved in various pathologic conditions

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such as rheumatoid arthritis, osteoarthritis, Crohn's disease, and endotoxin shock (Tracey et al. 1987; Kriegler et al. 1988; Brennan et al. 1989; Reinecker et al. 1993; Fernandes et al. 2002). TNF-α is expressed mainly by hematopoietic cells such as macrophages and T lymphocytes but is also present in nonhematopoietic cells such as mast cells and vascular endothelial cells (Carswell et al. 1975; Kobayashi et al. 1986; Steffen et al. 1989; Nilsen et al. 1998). In those cells, TNF-α is initially synthesized as a transmembrane protein and then becomes a soluble form via ectodomain shedding (McGeehan et al. 1994; Mohler et al. 1994).

Like other proteins, the pathologic significance of TNF-α may differ between membrane-bound and soluble forms. Soluble TNF-α is responsible for endotoxin shock because the blockade of TNF-α release protected mice from death (Mohler et al. 1994). On the other hand, TNF-α-deficient mice reconstituted with mutated TNF-α (muTNF delta 1–12), which lost its activity when cleaved into a soluble form, were shown to develop experimental hepatitis (Küsters et al. 1997). The role of TNF-α shedding in arthritis is controversial. The muTNF delta 1–12 mice developed arthritis spontaneously, implying that membrane-bound TNF-α may be important in its etiology (Alexopoulou et al. 1997). On the contrary, tissue inhibitor of metalloproteinase (TIMP)-3-deficient mice were more susceptible to bovine serum albumin induced arthritis (Mahmoodi et al. 2005). Since TNF-α shedding by ADAM17 was likely to be enhanced in those mice, that result might indicate the importance of soluble TNF-α in the development of arthritis.

Several proteinases are known to cleave TNF-α into a soluble form. At present, ADAM17 (also called TNF-α converting enzyme, or TACE) is thought to be the major sheddase for TNF-α (Black et al. 1997; Moss et al. 1997; Condon et al. 2001; Zheng et al. 2004; Bell et al. 2007; Horiuchi et al. 2007). In T lymphocytes, deficiency or suppression of ADAM17 blocked solubilization of membrane-bound TNF-α (Black et al. 1997; Condon et al. 2001). In mouse embryonic fibroblasts, deficiency of ADAM17 resulted in suppression of phorbol 12-myristate 13-acetate stimulated TNF-α shedding (Zheng et al. 2004). More recently, two other groups reported that ADAM17 is the primal sheddase for TNF-α in mouse macrophages (Bell et al. 2007; Horiuchi et al. 2007). However, these results do not necessarily mean that ADAM17 is the primal TNF-α sheddase in all types of cells because the endogenous sheddase for a substrate protein can differ from cell to cell. For example, the sheddase for receptor activator of NF-κb ligand (RANKL) in the mouse bone stromal cell line TM8B2 is mainly ADAM10, but a large part of soluble RANKL is produced by membrane type-1 matrix metalloproteinase (MT1-MMP) in osteoblasts (Hikita et al. 2006). Thus, the endogenous sheddase for TNF-α should be determined in the respective cell types.

In this study, we attempted to identify the endogenous TNF-α sheddase in several types of cells using a screening system for proteinases.

Materials and methods

Reagents

DNA polymerase, KOD plus, was purchased from TOYOBO (Osaka, Japan). Antibodies were obtained from the following companies: TNF-α, Cell Signaling Technology Inc. (Beverly, Massachusetts); ADAM10, Kamiya Biochemical (Seattle, Washington); ADAM17, Santa Cruz Biotechnology, Inc. (Santa Cruz, California); actin, Sigma-Aldrich Co. (St. Louis, Missouri); V5 tag, Invitrogen (Carlsbad, California). Lipopolysaccharide (LPS) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Recombinant mouse TIMP-1 and TIMP-2 were purchased from Acris Antibodies GmbH (Hiddenhausen, Germany) and recombinant mouse TIMP-3 from R&D Systems (Minneapolis, Minnesota). Recombinant human TIMP-1 and TIMP-3 were obtained from Daiichi Fine Chemical (Toyama, Japan) and R&D Systems, respectively.

Cell culture

The human kidney cell line 293A (Invitrogen), mouse fibroblastic cell line NIH3T3, and mouse vascular endothelial cell line UV♀2 (RCB1994) (Riken BioResource Center, Tsukuba, Japan) were cultured in Dulbecco's modified Eagle medium (D-MEM) (Wako Pure Chemical Industries, Ltd.) supplemented with 10% FBS (SAFC Biosciences, Lenexa, Kansas) and 1% penicillin–streptomycin solution (Sigma-Aldrich Co.). Macrophages were collected from spleens of 4- to 6-week-old male ddy mice and cultured in α-MEM (Invitrogen) supplemented with 10% FBS, 1% penicillin–streptomycin solution, and 200 ng/mL M-CSF from the culture supernatant of CMG14-12 (a generous gift from Dr. Sunao Takeshita, Department of Bone and Joint Disease, Research Institute, National Center for Geriatrics and Gerontology, Obu City, Japan). Methods for collection and culture of human chondrocytes were previously described (Fukui et al. 2006). In brief, human articular chondrocytes were obtained from osteoarthritic knee cartilage at prosthetic surgery. The chondrocytes were isolated from the surrounding matrix by serial enzymic digestion and cultured in monolayer at the density of 2×10^5 cells/cm^2 in D-MEM/F-12 containing 10% FBS, 25 μg/mL ascorbic acid, and penicillin–streptomycin. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Expression vectors were transfected to these cells using FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, Indiana) according to the manufacturer's instructions.

Constructs

cDNA of mouse TNF-α was cloned from cDNA of mouse macrophages by PCR and inserted in pCR-blunt II TOPO (Invitrogen) using protocols recommended by the manufacturer. The cDNA encoding TNF-α was subcloned into pSG5 vector (Stratagene, La Jolla, California) together with cDNA for myc or V5 and His tag subcloned from pCMV-Tag5A (Stratagene) or pcDNA3.1-V5HisA (Invitrogen), respectively. The (TNF-SEAP expression vector was constructed by inserting the cDNA fragment of the mouse TNF-α (corresponding to amino acids 1–90 including the cytoplasmic region, the transmembrane domain, and the stalk region) and the cDNA for SEAP subcloned from pSEAP2-Control (Clontech, Palo Alto, California) into pcDNA3.1-V5HisA. The construction of the expression vector for ADAM10 was previously described (Hikita et al. 2006). Expression vectors for ADAM9, ADAM17, ADAM19, and MMP7 were con-

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structed by inserting the cDNA subcloned from corresponding pcDNA3.1-V5HisA constructs (Hikita et al. 2006) along with V5 and His tag into pSG5 vector. Expression vectors for MT1-MMP, MT2-MMP, MT3-MMP, MT4-MMP, MT5-MMP, and MT6-MMP were generous gifts from Dr. Motoharu Seiki (Center for Experimental Medicine, Institute of Medical Science, The University of Tokyo). Expression vectors for TIMP-1, TIMP-2, and TIMP-3 were constructed by inserting cDNA coding in these TIMPs to the *Eco*RI and *Xba*I sites of pcDNA3.1-V5HisA. Short hairpin RNA (shRNA) plasmids for GFP, mouse or human ADAM10 and ADAM17, and mouse TIMP-1 were constructed using piGENE mU6 vector or piGENE hU6 vector (iGENE Therapeutics Inc., Tsukuba, Japan) according to the manufacturer's protocol. Target sites are listed in Table 1. Retroviral shRNA vectors were constructed as follows: cDNA fragments for shRNA including the mouse U6 promoter were subcloned from piGENE constructs by PCR using M13F and M13R primers and ligated into pCR-blunt II TOPO (Invitrogen), digested by *Eco*RI, and inserted in the *Eco*RI site of pMX-IRES-bsr (a generous gift from Dr. Sunao Takeshita).

Table 1. Target sequences for RNAi.

Gene	Sequence
GFP	5′-GCTACGTCCAGGAGCGCACCA-3′
Mouse *Adam10*	5′-GACATTATGAAGGATTATCTT-3′
	5′-GGGTCTGTCATTGATGGAAGA-3′
Mouse *Adam17*	5′-GCGACACACTTAGAAACATTA-3′
	5′-GGAACTCTTGGATTAGCTTAC-3′
Human *ADAM10*	5′-GACATTATGAAGGATTATCTT-3′
	5′-GGTCTCATGTACCTCCCAAAG-3′
Human *ADAM17*	5′-GCTCTCAGACTACGATATTCT-3′
	5′-GCTAGAGCAATTTAGCTTTGA-3′
Mouse *Timp1*	5′-GCAACTCGGACCTGGTCATAA-3′
	5′-GGAACGGAAATTTGCACATCA-3′
	5′-GCACAGTGTTTCCCTGTTTAT-3′

Note: GFP was used as a control.

Screening of TNF-α sheddases

Alkaline phosphatase activity in culture media was assayed by a previously described method except for the use of tTNF-SEAP instead of tRANKL-SEAP (Hikita et al. 2005). The method for Western blot analysis was also described previously (Yamamoto et al. 2002). Soluble TNF-α in culture media was precipitated at 85% saturation of ammonium sulfate (Mueller et al. 1999), dissolved in TNE buffer [10 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP-40], and subjected to SDS–PAGE. Soluble TNF-α was collected using Ni-NTA beads (Qiagen, Hilden, Germany) when it contained 6x His tag.

Determination of cleavage sites of TNF-α

pSG5-mTNFα-V5His was transfected to 293A cells. Seventy-two hours after transfection, 200 mL of culture medium was collected and soluble TNF-α was recovered using Ni-NTA beads. Samples were subjected to SDS–PAGE, transferred to polyvinylidene membrane, and stained with Coomassie Brilliant Blue. The band was excised and the N-terminal amino acid sequence was determined by Hokkaido System Science Co., Ltd. (Hokkaido, Japan).

ELISA

The concentration of soluble TNF-α in culture media was determined using a Murine TNF-α ELISA development kit (Peprotech, Rocky Hill, New Jersey) for mouse macrophages and NIH3T3 and UV‡2 cells. The amount of TNF-α released from primary cultured chondrocytes was determined as follows. Forty-eight hours after plating, culture media were replaced with those containing 3 μg/mL LPS. For some cells, recombinant human TIMP-1 or TIMP-3 was added to the media at a concentration of 100 nmol/L. Twenty-four hours later, the media were collected and the amount of TNF-α was determined by ELISA (QuantiGlo Chemiluminescent Sandwich ELISA kit, R&D Systems).

Real-time RT-PCR

The reaction mixture for real-time RT-PCR was prepared using SYBR Premix Ex *Taq* (TAKARA Biochemicals, Shiga, Japan) and analyzed using LightCycler (Roche Applied Science, Indianapolis, Indiana) according to the manufacturer's protocol. The sets of primers used are listed in Table 2.

Retrovirus infection

The procedure for the preparation of the retrovirus was described previously (Kitamura 1998). Spleen macrophages were incubated with culture media containing retrovirus supplemented with 4 μg/mL polybrane (Sigma-Aldrich Co.) and 200 ng/mL M-CSF for 6 h. Eighteen hours later, infected cells were selected by culturing in the media containing 1 μg/mL blasticidin S (Kaken Pharmaceutical Co., Tokyo, Japan) for 48 h.

Immunostaining

A skin biopsy specimen was obtained from a 57-year-old female patient with Henoch-Schönlein purpura, and a control specimen was acquired from a 73-year-old female who died of an unrelated cause. Immunostaining of the skin tissues was performed as follows. Four-micrometre-thick sections were prepared, deparaffinized in xylene, and rehydrated in graded concentrations of ethanol. After blocking endogenous peroxidase activity, antigen retrieval was performed by autoclaving sections for 30 min in Target Retrieval Solution (pH 6.0) (Dako Japan, Tokyo, Japan). Primary antibodies for ADAM10 and TNF-α were both purchased from Abcam (Cambridge, Masschusetts) and used at a concentration of 1/100. Color detection of those antibodies was performed by a commercially available kit (EnVision HRP kit, Dako Japan). Immunostaining of cartilage tissue was described before (Fukui et al. 2006). In brief, 6-μm-thick cryosections were prepared from osteoarthritic or control cartilage, fixed with ice-cold acetone, and digested with sheep testis hyaluronidase (type IV, Sigma-Aldrich Co.) for antigen retrieval. Osteoarthric cartilage was obtained at prosthetic surgery, and control cartilage was obtained from a nonarthritic knee joint of a donor who died of an unrelated disease. The anti-ADAM10 and anti-TNF-α antibodies were used at a concentration of 1:100 and visualized with a commercially available kit (ABC Staining System, Santa Cruz Biotechnology)

Table 2. Primer sequences for mouse genes and human genes used for real-time PCR.

Gene	Forward	Reverse
Mouse		
Adam9	5′-GGATATGGAGGAAGCGTGGA-3′	5′-GCAACAAGGGGGACGATTAG-3′
Adam10	5′-AGCAACATCTGGGGACAAAC-3′	5′-TGGCCAGATTCAACAAAACA-3′
Adam17	5′-GTACGTCGATGCAGAGCAAA-3′	5′-GAAATCCCAAAATCGCTCAA-3′
Adam19	5′-GGTTCTGCTTGCTGGCTCTC-3′	5′-CCTTCTTGGCTTCCTCTTGTG-3′
Mmp7	5′-AGGCGGAGATGCTCACTTTG-3′	5′-GGTGGCAGCAAACAGGAAG-3′
Mmp14 (MT1-MMP)	5′-CCCAAGGCAGCAACTTCA-3′	5′-CAATGGCAGCTGAGAGTGAC-3′
Mmp16 (MT3-MMP)	5′-ATCATGGCCCCATTTTATCA-3′	5′-GCATTGGGTATCCATCCATC-3′
Mmp24 (MT5-MMP)	5′-TTGAGCAGGAGGAGGAGAAA-3′	5′-GAGTCACCTTCTGCCACACA-3′
Timp1	5′-ATCTGGCATCCTCTTGTTGC-3′	5′-CGTTGATTTCTGGGGAACC-3′
Timp2	5′-CACCCAGAAGAAGAGCCTGA-3′	5′-GTGACCCAGTCCATCCAGAG-3′
Timp3	5′-GCGTGTATGAAGGCAAGATGTA-3′	5′-GAGGTCACAAAACAAGGCAAGTA-3′
Actb (actin, beta)	5′-AGATGTGGATCAGCAAGCAG-3′	5′-GCGCAAGTTAGGTTTTGTCA-3′
Freyer et al. (1999)		
ADAM10	5′-TTTGAAGGATTCATCCAGACTC-3′	5′-ACACCAGTCATCTGGTATTTCC-3′
ADAM17	5′-AAGCTTGATTCTTTGCTCTCAG-3′	5′-TACTCGCTTTCGTTTTTACCAT-3′
TIMP1	5′-AGCGTTATGAGATCAAGATGACCA-3′	5′-GTTTTCCAGCAATGAGAAACTCCT-3′
TIMP2	5′-ATGATAGGTGAACCTGAGTTGCAG-3′	5′-CTATCCTAACCCCCATATCACTGG-3′
TIMP3	5′-AACTCCGACATCGTGATCCG-3′	5′-CGTAGTGTTTGGACTGGTAGC-3′
GAPDH	5′-AAAACCTGCCAAATATGATGAC-3′	5′-CAGGAAATGAGCTTGACAAAGT-3′

coupled with 3-amino-9-ethylcarbazole (AEC Liquid Substrate Chromogen, Dako Japan).

Results

Screening of MMPs and ADAMs as TNF-α sheddases

We constructed an expression vector encoding a fusion protein of secreted placental alkaline phosphatase (SEAP) with the C-terminally truncated form of TNF-α, which contained the stalk region, the transmembrane domain, and the intracellular domain of TNF-α (tTNF-SEAP) (Fig. 1A). Expression vectors of various MMPs or ADAMs were transfected to 293A cells together with tTNF-SEAP plasmids and the alkaline phosphatase activity of culture media was measured. To achieve sufficient levels of proteinase expression, proteinase expression vectors were transfected in 80-fold excess of the tTNF-SEAP expression vector. In this assay system, increased alkaline phosphatase activity in the medium indicates an increase in TNF-α shedding. ADAM9, ADAM10, ADAM17, ADAM19, MMP7, MT1-MMP, MT3-MMP, and MT5-MMP exhibited tTNF-SEAP shedding activities (Fig. 1B). In contrast, MMP1, MMP2, MMP3, MMP9, MMP11, MMP13, MMP23, MMP28, MT2-MMP, MT4-MMP, and MT6-MMP failed to cleave tTNF-SEAP (data not shown).

ADAM10 and ADAM17 are major TNF-α sheddases in 293A cells

To confirm the capacity of these proteinases to cleave full-length TNF-α, they were expressed in 293A cells together with full-length TNF-α, which was C-terminally tagged with myc. In this experiment, expression vectors for proteinases and those for TNF-α were transfected at an equal molarity. Soluble TNF-α was recovered from culture media by ammonium sulfate precipitation and subjected to Western blotting analysis. When TNF-α was overexpressed

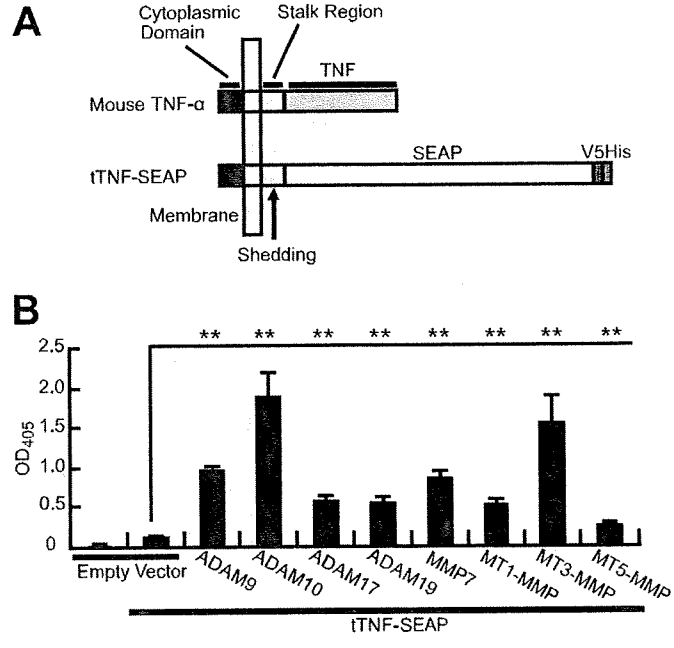


Fig. 1. Screening of potential TNF-α sheddases, MMPs, and ADAMs. (A) Schematic representations of TNF-α and the tTNF-SEAP fusion protein. tTNF-SEAP is a fusion protein of SEAP with C-terminally truncated TNF-α, which contains the stalk region but lacks the TNF domain. The protein has a V5 and 6x His tag at the C terminus. Cleavage of tTNF-SEAP can be evaluated by alkaline phosphatase activity in culture supernatants. (B) Alkaline phosphatase activity of culture media of 293A cells transfected with tTNF-SEAP and expression vectors for ADAMs or MMPs. $^{**}P < 0.01$.

in 293A cells, soluble TNF-α cleaved by endogenous proteinase in the cells appeared in culture media (Fig. 2A). The N-terminal sequence of soluble TNF-α was determined (Fig. 2B), which agreed with the cleavage site reported pre-

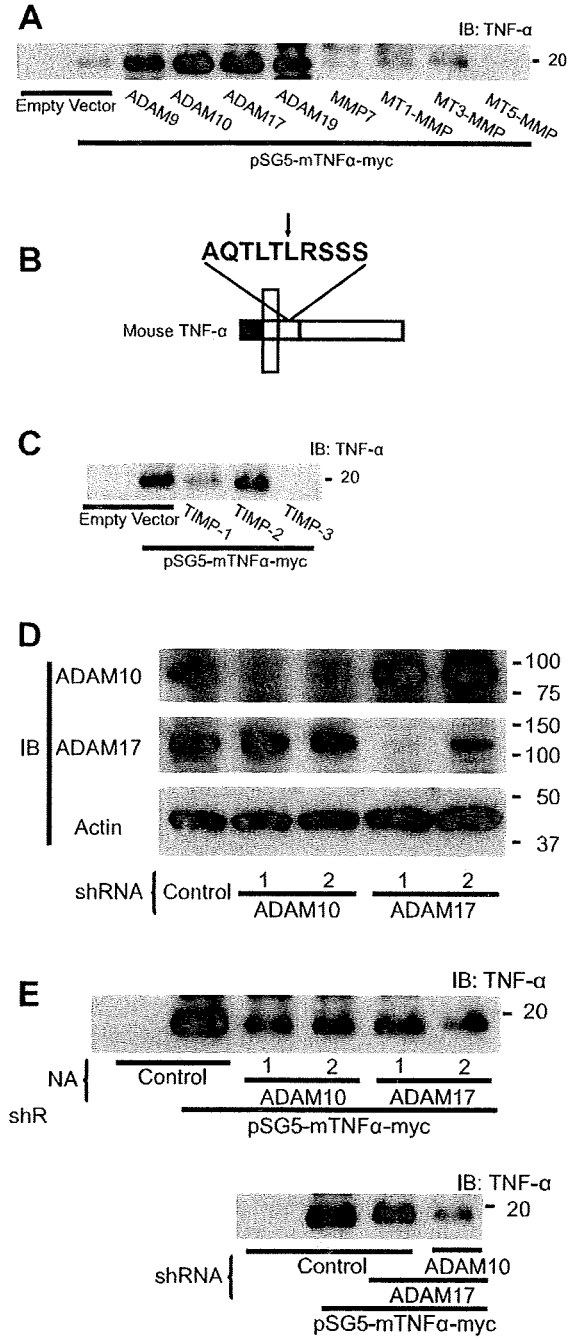


Fig. 2. Shedding of TNF-α in 293A cells. (A) Shedding of full-length TNF-α. 293A cells were transfected with pSG5-mTNFα-myc together with plasmids expressing MMPs or ADAMs. Seventy-two hours later, culture media were collected and proteins were precipitated with ammonium sulfate and subjected to Western blotting analysis using anti-TNF-α antibody. Soluble TNF-α was detected as a band of approximately 20 kDa. (B) Cleavage site of TNF-α in 293A cells. (C) Inhibition of TNF-α shedding by endogenous proteinases with TIMPs. 293A cells were transfected with pSG5-mTNFα-myc and pcDNA3.1-TIMP1-V5HisA, pcDNA3.1-TIMP2-V5HisA, or pcDNA3.1-TIMP3-V5HisA. Shedding of TNF-α was determined as described for Fig. 2A. (D) Suppression of ADAM10 and ADAM17 by RNAi. shRNA plasmids for ADAM10 or ADAM17 were transfected to 293A cells, and 48 h later, cell lysates were obtained and subjected to Western blotting analysis to evaluate suppression of endogenous ADAM10 or ADAM17. (E) Effects of RNAi for ADAM10 and ADAM17 on the shedding of TNF-α. 293A cells were transfected with pSG5-mTNFα-myc and shRNA plasmids for ADAM10 or ADAM17. Shedding of TNF-α was evaluated in the same manner.

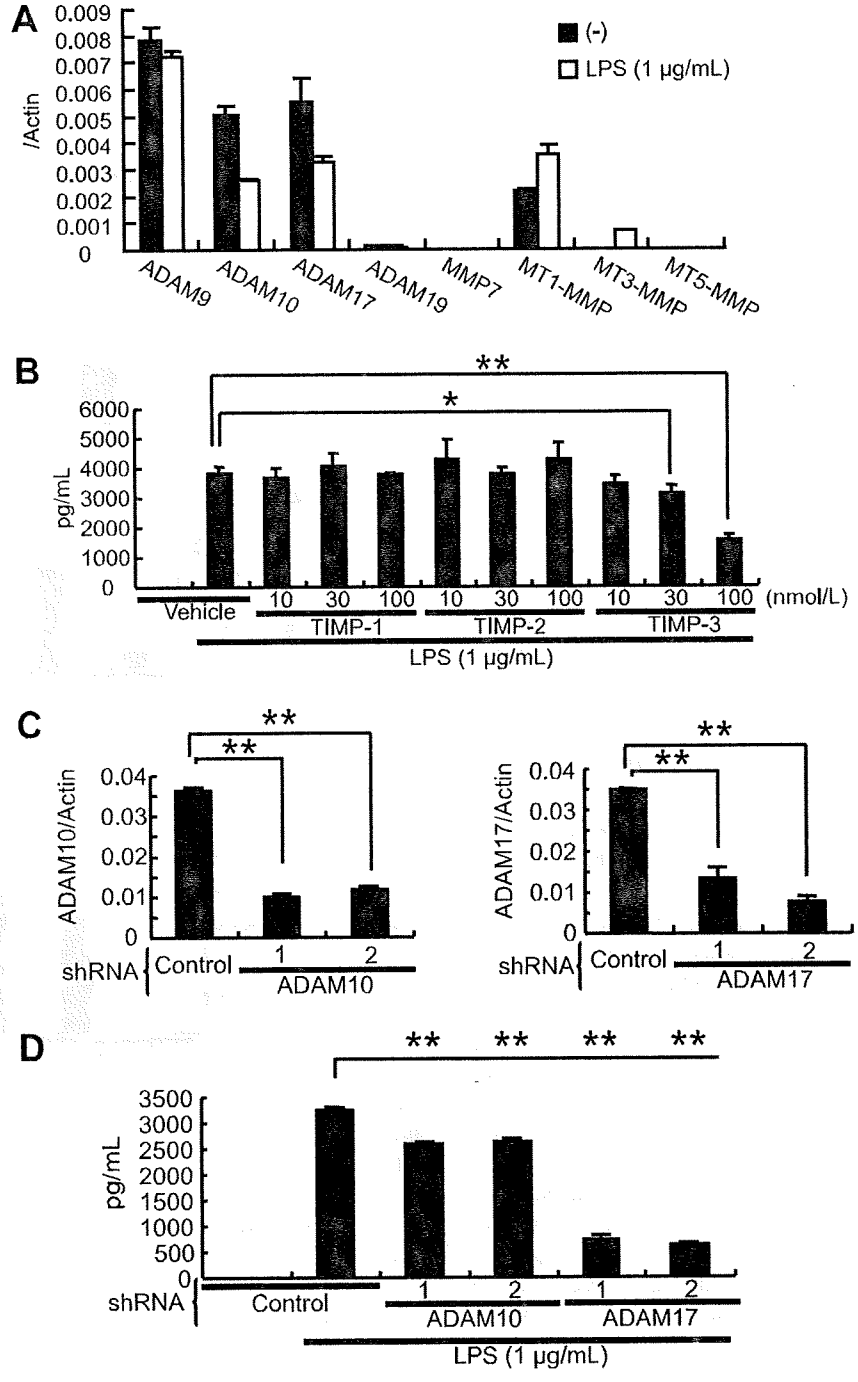
viously (Cseh and Beutler 1989). Among proteinases that had cleaved tTNF-SEAP, ADAM9, ADAM10, ADAM17, and ADAM19 showed shedding activities for full-length TNF-α (Fig. 2A). The cleavage of TNF-α by MMP7, MT1-MMP, MT3-MMP, and MT5-MMP was relatively low compared with the result of the screening using tTNF-SEAP (Fig. 1B). TIMPs are biologic inhibitors for metalloproteinases and four subtypes have been reported (Gomez et al. 1997). Spectra of inhibition are different among the subtypes, so TIMPs have been widely used for identification of endogenous proteinase(s). To identify the endogenous sheddase(s) in 293A cells, expression vectors for TIMP-1, TIMP-2, and TIMP-3 were cotransfected with an expression vector for full-length TNF-α to 293A cells. Overexpression of TIMP-1 and TIMP-3 decreased soluble TNF-α in culture media, while TIMP-2 had no apparent effect (Fig. 2C). This result ruled out the involvement of ADAM9 or ADAM19 in TNF-α shedding because the activities of these proteinases are not inhibited by any of those three TIMPs (Amour et al. 2002; Chesneau et al. 2003). Meanwhile, the reduction of soluble TNF-α by TIMP-1 and TIMP-3 suggested the involvement of ADAM10 in the shedding, since the activity of ADAM10 is inhibited by these TIMPs (Amour et al. 2000). The involvement of ADAM17 was also possible because ADAM17 is inhibited by TIMP-3 (Amour et al. 1998).

To clarify the involvement of these two ADAMs, shRNA vectors for ADAM10 and ADAM17 were constructed. Transfection of these constructs specifically reduced the expression of ADAM10 and ADAM17, respectively (Fig. 2D). Suppression of ADAM10 or ADAM17 resulted in decrease of soluble TNF-α in the culture media, and a further reduction was observed by simultaneous suppression of these two ADAMs (Fig. 2E). These results indicated that ADAM10 and ADAM17 are two major TNF-α sheddases in 293A cells.

Determination of endogenous sheddase(s) for TNF-α in macrophages

Macrophages are known to express several ADAMs (Verrier et al. 2004). To confirm the expression of proteinases that had shedding activities for tTNF-SEAP, RNA from macrophages with or without LPS treatment was analyzed by real-time RT-PCR. Considerable amounts of ADAM9, ADAM10, ADAM17, and MT1-MMP were expressed in macrophages with or without LPS stimuli, but the expression of ADAM19, which showed a shedding activity for full-length TNF-α, was considerably lower than that of the other four proteinases (Fig. 3A). Because ADAM10, one of the major TNF-α sheddases in 293A cells, is expressed in macrophages at a level comparable with ADAM17, we attempted to specify the endogenous sheddase(s) for TNF-α in macrophages. LPS was able to stimulate macrophages to release soluble TNF-α to culture media (Carswell et al. 1975). To identify the endogenous TNF-α sheddase(s), re-

Fig. 3. Shedding of TNF-α in mouse spleen macrophages. (A) Expression of MMPs and ADAMs in mouse spleen macrophages. After culturing for 16 h in the presence or absence of LPS (1 μg/mL), RNA was obtained and the expression of respective genes was analyzed by real-time RT-PCR together with that of β-actin. Results are shown by expression ratios against β-actin. (B) Inhibition of TNF-α shedding by TIMPs. Macrophages were cultured with or without 1 μg/mL LPS for 16 h together with recombinant TIMPs at the indicated concentrations and the concentration of soluble TNF-α in culture media was measured by ELISA. $*P < 0.05$; $**P < 0.01$. (C) RNAi for ADAM10 and ADAM17. Macrophages were infected with retroviruses containing shRNA sequence and infected cells were selected with 1 μg/mL blasticidin S for 48 h. RNA was extracted from cells and gene expression was analyzed by real-time RT-PCR. $**P < 0.01$. (D) Effects of RNAi for ADAM10 and ADAM17 on the shedding of TNF-α. Retroviruses containing shRNA sequence for ADAM10 or ADAM17 were infected to macrophages and infected cells were selected with 1 μg/mL blastcidin S for 48 h. Some cells were then treated with 1 μg/mL LPS. Sixteen hours later, soluble TNF-α in culture media was measured by ELISA. $**P < 0.01$.



combinant TIMP-1, TIMP-2, and TIMP-3 were added to culture media together with LPS and the concentration of soluble TNF-α in culture media was determined by ELISA. TIMP-3 suppressed shedding of TNF-α in a dose-dependent manner, while TIMP-1 and TIMP-2 failed to suppress it (Fig. 3B). This result suggested that ADAM17 might be the

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endogenous sheddase in macrophages, while ADAM10 could have little contribution to the shedding. To confirm this, we constructed retroviral vectors carrying shRNA for these proteinases. Suppression of ADAM10 or ADAM17 expression by those retrovirus vectors was confirmed by real-time RT-PCR (Fig. 3C). Using these vectors, the involvement of these two proteinases in TNF-α shedding was investigated in macrophages. RNAi for ADAM17 dramatically suppressed TNF-α shedding with LPS stimulation (Fig. 3D). On the other hand, suppression of ADAM10 showed a partial effect on the concentration of soluble TNF-α with LPS stimuli. These results confirmed the results of previous studies that ADAM17 is the major sheddase in macrophages (Bell et al. 2007; Horiuchi et al. 2007).

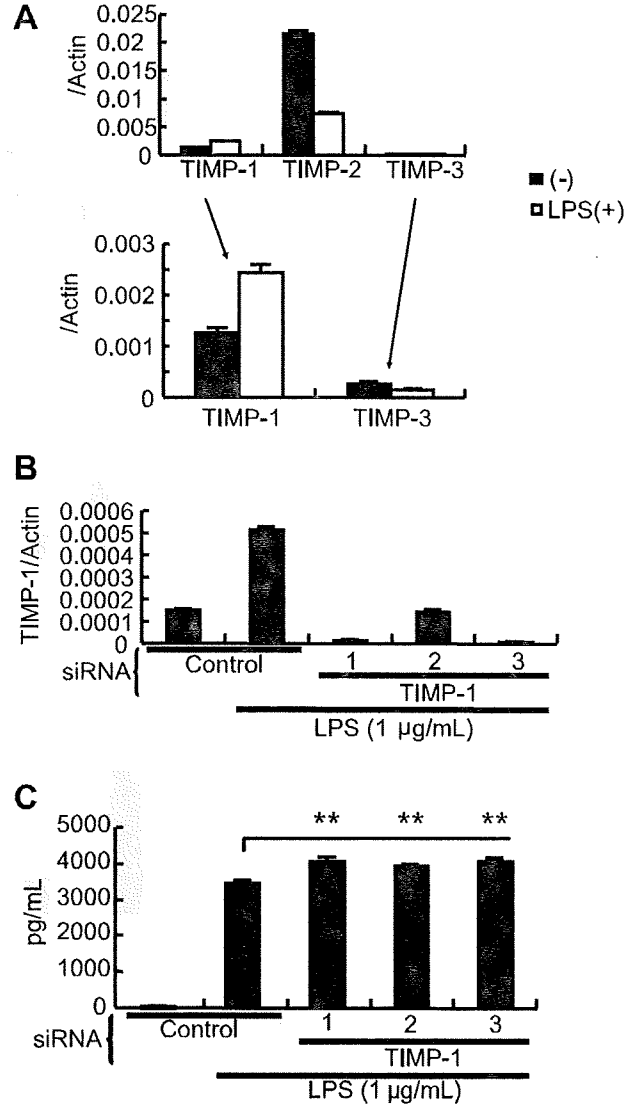
TIMP-1 inhibits ADAM10 activity in macrophages

Although ADAM10 played a significant role in TNF-α shedding in 293A cells, it was not a major TNF-α sheddase in macrophages in spite of its substantial expression. In an attempt to understand this seeming contradiction, we evaluated expression levels of ADAM10, ADAM17, TIMP-1, TIMP-2, and TIMP-3 in macrophages. In macrophages, ADAM10 was expressed at a level similar to that of ADAM17 (Fig. 3A). Meanwhile, TIMP-1 may be expressed more abundantly than TIMP-3, and that trend was augmented when the cells were stimulated with LPS (Fig. 4A). Considering the difference in inhibitory effects between these TIMPs, such a difference in their expression may account for the major role of ADAM17 in TNF-α shedding in macrophages. To confirm this speculation, we made retroviruses carrying shRNAs for TIMP-1 and evaluated their effects on the release of TNF-α (Fig. 4B). Suppression of TIMP-1 in macrophages by these constructs in fact increased shedding of TNF-α (Fig. 4C). Thus, the activity of ADAM10 was considered to be suppressed in macrophages by the abundance of endogenous TIMP-1.

ADAM10 is the major TNF-α sheddase in NIH3T3 cells

The endogenous sheddase(s) for a substrate protein may differ from cell to cell. As shown above, the endogenous sheddase(s) could be determined by the expression of the proteinase(s) and its endogenous inhibitor(s). Thus, we next examined expression of ADAM10, ADAM17, TIMP-1, TIMP-2, and TIMP-3 in NIH3T3. Compared with macrophages, NIH3T3 cells expressed ADAM10 more abundantly than ADAM17 (Fig. 5A). In NIH3T3, the difference in expression between TIMP-1 and TIMP-3 was not so obvious as in macrophages (Fig. 5B). These data suggested that ADAM10 could have a greater contribution to TNF-α shedding in NIH3T3 than in macrophages. shRNA vectors for mouse ADAM10 and ADAM17 showed specific inhibition of respective proteinases in this cell line (Fig. 5C). These constructs were transfected with an expression vector for full-length TNF-α, and soluble TNF-α was collected from culture media using Ni-NTA beads. Although suppression of ADAM10 or ADAM17 resulted in reduction of soluble TNF-α, the reduction was more obvious with the suppression of ADAM10 than with that of ADAM17 (Figs. 5D and 5E). This result indicated that unlike in macrophages, ADAM10 is the major TNF-α sheddase in NIH3T3 cells.

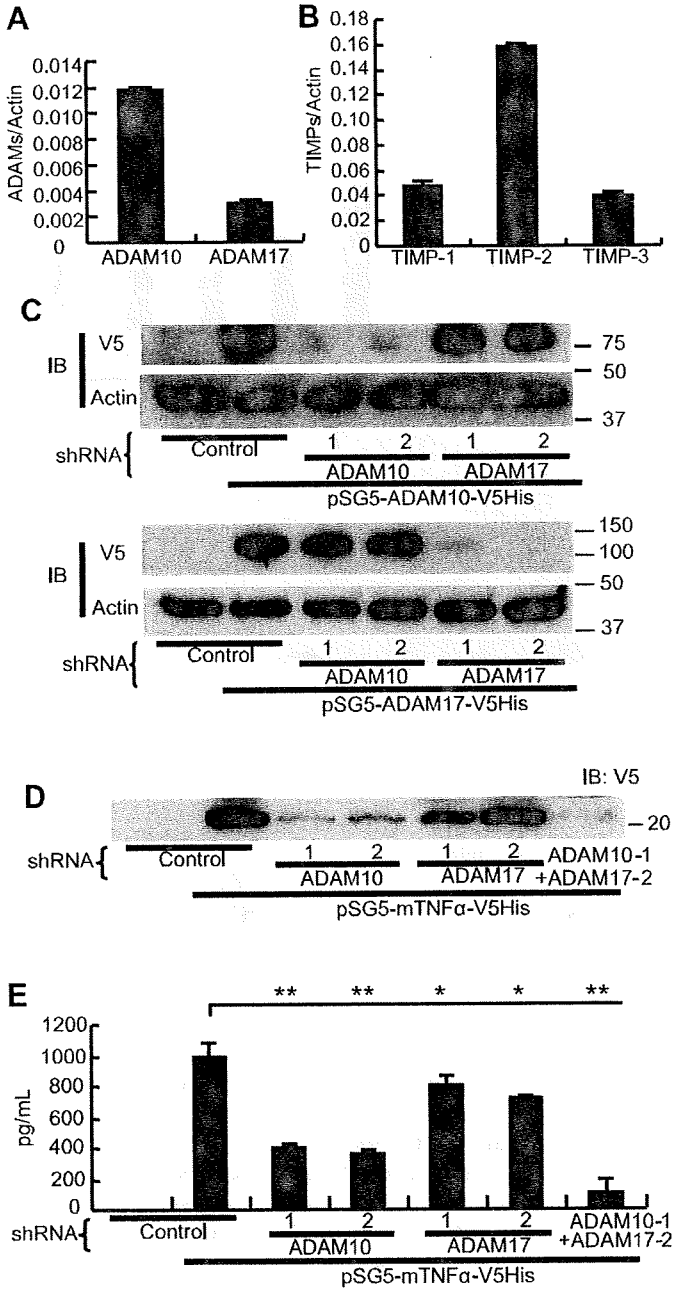
Fig. 4. Suppression of TNF-α shedding by TIMP-1 in mouse spleen macrophages. (A) Expression of TIMPs in mouse spleen macrophages. Expression of TIMP-1, TIMP-2, and TIMP-3 in macrophages was determined by real-time RT-PCR as described for Fig. 3A. Results are shown by expression ratios against β-actin. (B) RNAi for TIMP-1. Effect of retroviruses carrying shRNA constructs for TIMP-1 was confirmed in the manner described for Fig. 3D. Expression of TIMP-1 is shown by the ratios against that of β-actin. (C) Effects of RNAi for TIMP-1 on the shedding of TNF-α. TIMP-1 expression was suppressed and the change of TNF-α release into the media was evaluated as described for Fig. 3D.



Involvement of ADAM10 and ADAM17 in TNF-α shedding in vascular endothelial cell line

Vascular endothelial cells were known to release TNF-α with several stimulations such as high-mobility group protein-1, cell walls of *Streptococcus pneumoniae*, and LPS. Such release of TNF-α by endothelial cells is profoundly involved in several pathologies such as sepsis or bacterial meningitis (Freyer et al. 1999; Fiuza et al. 2003). To identify the endogenous sheddase(s) for TNF-α in vascular endothelial cells, we conducted experiments using a mouse vascular endothelial cell line, UV♀2. In this cell, both

Fig. 5. Shedding of TNF-α in NIH3T3 cells. (A and B) Expression of ADAMs and TIMPs in NIH3T3 cells. RNA was obtained from NIH3T3 and expression of ADAM10 and ADAM17 (Fig. 5A) and TIMP-1, TIMP-2, and TIMP-3 (Fig. 5B) was analyzed by real-time RT-PCR. Results are shown by ratios against β-actin expression. (C) RNAi for ADAM10 and ADAM17. pSG5-ADAM10-V5HisA or pSG5-ADAM17-V5HisA was transfected to NIH3T3 cells together with shRNA plasmids for the gene. Cell lysate was obtained 48 h after transfection and subjected to Western blotting analysis. (D and E) Effects of RNAi for ADAM10 or ADAM17 on the shedding of TNF-α. NIH3T3 cells were transfected with pSG5-TNF-α-V5HisA and shRNA plasmids for ADAM10 or ADAM17. Seventy-two hours later, soluble TNF-α in culture media was collected using Ni-NTA agarose and subjected to Western blotting analysis (Fig. 5D) or measured directly by ELISA (Fig. 5E). $*P < 0.05$; $**P < 0.01$.



ADAM10 and ADAM17 were expressed at substantial levels with the preponderance of ADAM10, similar to that in NIH3T3 (Fig. 6A). Meanwhile, the pattern of expression between TIMP-1 and TIMP-3 differed from that of NIH3T3 but resembled that of macrophages in that the expression of TIMP-1 was higher than that of TIMP-3 (Fig. 6B). Overexpression of TNF-α in UV♀2 resulted in release of soluble TNF-α into culture media (Fig. 6C). shRNA vectors for ADAM10 and ADAM17 suppressed TNF-α shedding, respectively, and transfection of both vectors showed an additive effect (Figs. 6C and 6D). Thus, it was considered that both ADAM10 and ADAM17 are endogenous sheddases for TNF-α in vascular endothelial cells. We next investigated the involvement of ADAM10 in the pathology of human vasculitis. In the acute phase of Henoch-Schönlein purpura, a form of allergic vasculitis, the serum TNF-α level is known to increase, and endothelial cells are assumed to be the source of TNF-α (Besbas et al. 1997; Ha 2005). In a small vessel of Henoch-Schönlein purpura, positive stain for ADAM10 and TNF-α was seen not only in infiltrated leukocytes but also along the endothelium of the vessel, suggesting that TNF-α could be released by ADAM10 in this pathology (Figs. 6E–6H).

TNF-α sheddase in osteoarthritic chondrocytes

TNF-α also plays an important role in the pathology of osteoarthritis (Fernandes et al. 2002). In osteoarthritis, chondrocytes secrete TNF-α, which induces catabolism in chondrocytes in an autocrine manner (Pelletier et al. 2001). In accordance with previous reports, immunostaining of osteoarthritic cartilage confirmed that osteoarthritic chondrocytes were expressing TNF-α (Fig. 7A), while it was little expressed in control cartilage (Fig. 7B). Osteoarthritic chondrocytes were intensely stained for ADAM10, which indicated a possibility that ADAM10 could be involved in TNF-α shedding in those chondrocytes (Fig. 7C). Interestingly, chondrocytes in control cartilage were also positive for ADAM10, although the staining tended to be weaker (Fig. 7D). We then evaluated the expression of ADAM10, ADAM17, TIMP-1, TIMP-2, and TIMP-3 in the chondrocytes within osteoarthritic cartilage. Similar to NIH3T3 and UV♀2 cells, ADAM10 and ADAM17 were expressed at comparable levels in those cells (Fig. 7E). Meanwhile, the expression of TIMP-1 could be more abundant than that of TIMP-3, showing a possibility that the enzymatic activity of ADAM10 might be more suppressed in the cells than that of ADAM17 (Fig. 7F). To investigate the significance of ADAM10 and ADAM17 in TNF shedding, we conducted experiments using cultured chondrocytes. Chondrocytes obtained from osteoarthritic cartilage secreted a small amount of TNF-α (1×10^6 cells released approximately 10 pg of TNF-α in 24 h). Stimulation with LPS increased the amount of released TNF-α three- to ninefold. This release of TNF-α was strongly inhibited by TIMP-3, but a significant reduction was also observed with TIMP-1 (Fig. 7G). These results indicate that ADAM17 could be the major endogenous sheddase for TNF-α in osteoarthritic chondrocytes. Although expressed at a comparable level, the activity of ADAM10 might be inhibited largely by endogenous TIMP-1 abundantly expressed in the cells.

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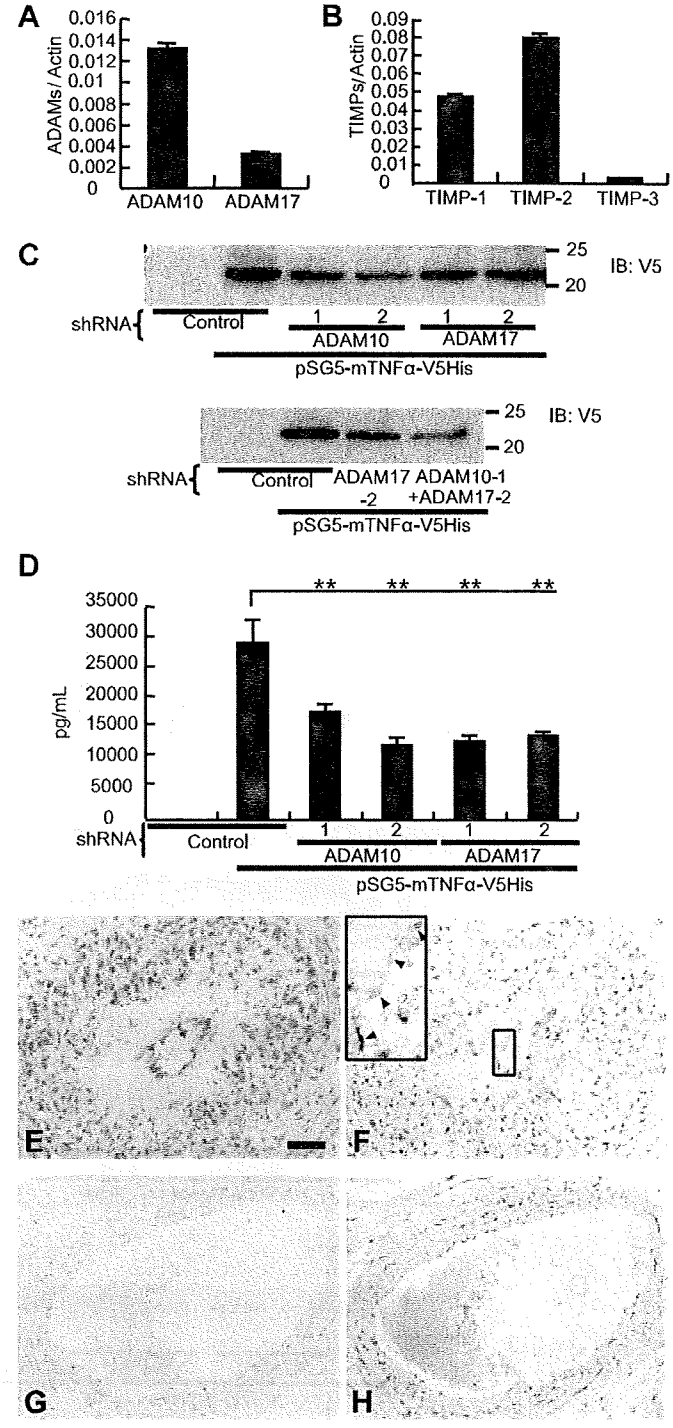


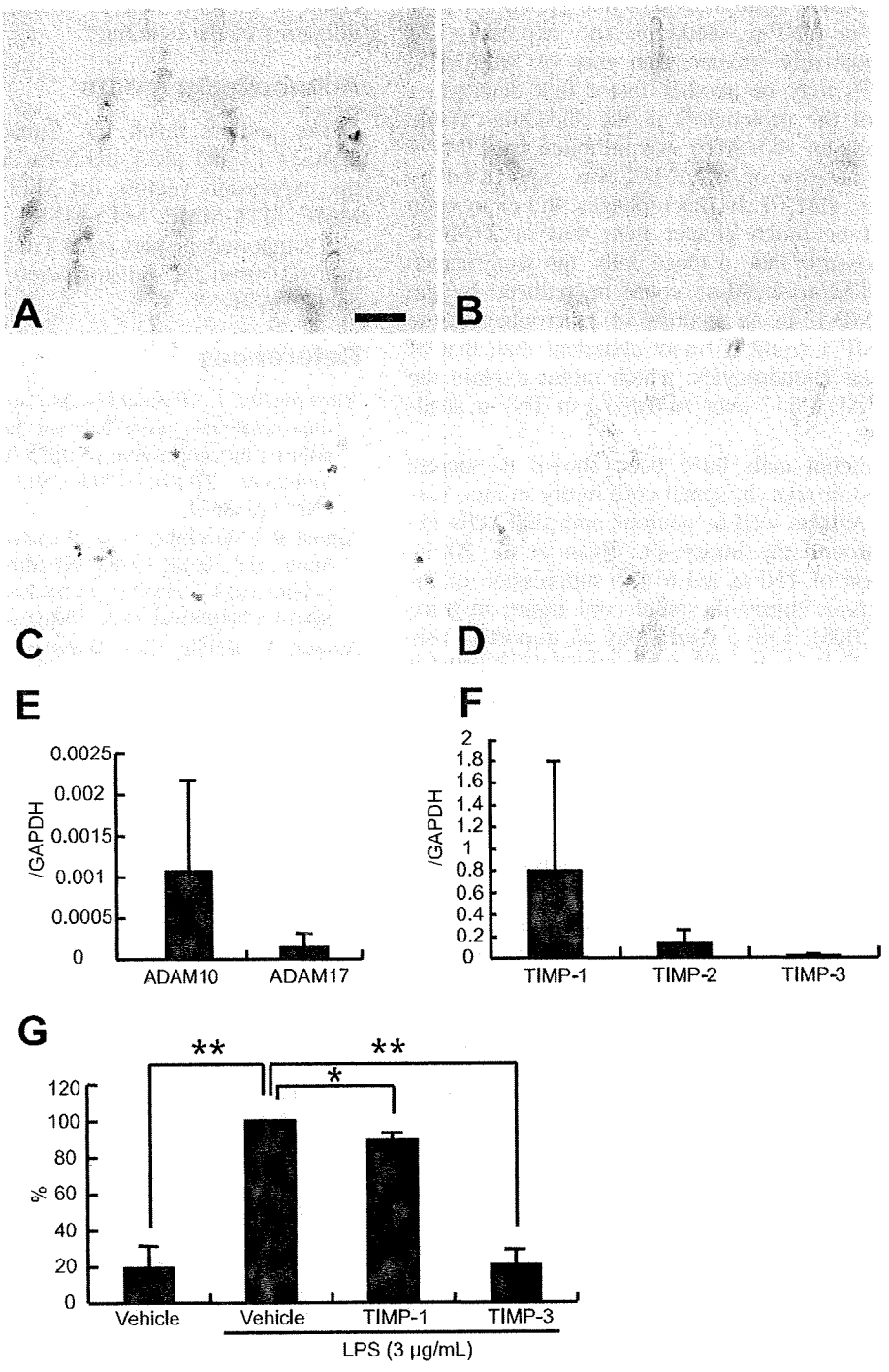
Fig. 6. Shedding of TNF-α in UV♀2 cells. (A and B) Expression of ADAMs and TIMPs in UV♀2 cells. RNA was obtained from UV♀2 cells and expression of ADAM10 and ADAM17 (Fig. 6A) and TIMP-1, TIMP-2, and TIMP-3 (Fig. 6B) was analyzed by real-time RT-PCR. Results are shown by ratios against β-actin expression. (C and D) Effects of RNAi for ADAM10 or ADAM17 on the shedding of TNF-α. UV♀2 cells were transfected with pSG5-mTNFα-V5His and shRNA plasmids for ADAM10 or ADAM17. Forty-eight hours later, soluble TNF-α in culture media was collected using Ni-NTA agarose and subjected to Western blotting analysis (Fig. 6C) or measured directly by ELISA (Fig. 6D). $^{**}P < 0.01$. (E–H) Expression of ADAM10 and TNF-α in vasculitic endothelium. Skin biopsy specimens obtained from a patient with Henoch-Schönlein purpura (Figs. 6E and 6F) and a control donor (Figs. 6G and 6H) were immunostained for ADAM10 (Figs. 6E and 6G) and TNF-α (Figs. 6F and 6H), respectively. In Fig. 6F, a higher magnification image of the boxed area is shown in the inset in which staining for TNF-α is indicated by arrowheads. Cross-sectional images of a blood vessel are shown. Bar = 50 μm.

Discussion

In this study, we employed a unique screening system to find possible sheddases for TNF-α. Although the system is not the one well established, we have already used a similar system to identify RANKL sheddases and found it to be a reliable method (Hikita et al. 2006). In this system, the result could be affected by expression levels of the proteinases. If proteinases are expressed at low levels, their capacity to cleave the substrate may be underestimated. To avoid this, in the present study, we transfected proteinase expression vectors in excessive amounts. Although there was some discrepancy between the result of the initial screening (Fig. 1B) and that of the second experiment with full-length TNF-α (Fig. 2A), it could not be related to the poor reliability of the screening but more likely to the difference in substrate proteins.

Currently, ADAM17 is considered to be the major sheddase for TNF-α (Black et al. 1997; Moss et al. 1997; Condon et al. 2001; Zheng et al. 2004; Bell et al. 2007; Horiuchi et al. 2007). However, besides ADAM17, several proteinases are known to cleave membrane-bound TNF-α. Haro et al. (2000) showed that MMP7 null macrophages failed to release soluble TNF-α, suggesting that MMP7 could be responsible for TNF-α shedding in macrophages. Purified proteinase 3 from activated neutrophils was also shown to have a shedding activity for TNF-α (Coeshott et al. 1999). ADAM19 could work as a sheddase for TNF-α when overexpressed in COS-7 and CHO cells (Chesneau et al. 2003). ADAM10 is also reported to have a shedding activity for TNF-α. Purified bovine ADAM10 and ADAM10 overexpressed in 293A EBNA cells processed TNF-α in vitro (Lunn et al. 1997). In contrast, when expressed in COS-7 cells or CHO cells, ADAM10 failed to release TNF-α (Zheng et al. 2004). Again, the lack of ADAM10 expression did not change TNF-α shedding in mouse embryonic fibroblasts (Zheng et al. 2004). In this study, we showed that not only ADAM17 but also ADAM9, ADAM10, and ADAM19 are capable of cleaving TNF-α in a cell-based assay. We also showed, for the first time, that endogenous ADAM10 is a major TNF-α sheddase in 293A cells, NIH3T3 cells, and UV♀2 cells. Our current result differed from those in previous reports in that endogenous ADAM10 can cleave TNF-α in certain types of cells. Since proteinases involved in protein shedding often differ among cell types (Schlöndorff et al. 2001; Hikita et al. 2006), the discrepancy between our current result and those reported previously could be ascribed to the difference in cell types used in the assay. Also, the difference in used constructs or substrate proteins could account for the discrepancy. In the report of Zheng et al. (2004), the expression vector for a fusion protein of alkaline phosphatase and TNF-α was used, while myc-tagged

Fig. 7. Shedding of TNF-α in articular chondrocytes. (A–D) Expression of TNF-α and ADAM10 in osteoarthritic and control cartilage. Histological sections of osteoarthritic and control cartilage were immunostained for TNF-α (Figs. 7A and 7B, respectively) and ADAM10 (Figs. 7C and 7D, respectively). Bar = 100 μm. (E and F) Expression of ADAMs and TIMPs in chondrocytes. RNA was obtained from human osteoarthritic chondrocytes and expression of ADAM10 and ADAM17 (Fig. 7E) and TIMP-1, TIMP-2 and TIMP-3 (Fig. 7F) was analyzed by real-time RT-PCR. Results are shown by ratios against GAPDH expression. (G) Effects of TIMP-1 and TIMP-3 on the secretion of TNF-α from primary cultured chondrocytes stimulated with LPS. $*P < 0.05$; $**P < 0.01$.



TNF-α was used in our assay. We often failed to detect alkaline phosphatase activity in the culture media of 293 cells when fusion proteins of the alkaline phosphatase and substrate proteins were overexpressed, possibly due to the interaction between the enzyme and the substrate proteins.

In this study, we confirmed that the major sheddase for TNF-α in macrophages is ADAM17. This result is consistent with those of two recent reports that dealt with ADAM17 conditional knockout mice and radiation chimeric mice reconstituted with leukocytes lacking functional ADAM17 (Bell et al. 2007; Horiuchi et al. 2007). ADAM10, which turned out to be the major TNF-α sheddase in the above-

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mentioned three types of cells, was known to play only a limited role in the release of TNF-α in macrophages. This discrepancy could be explained by the difference in expression levels between ADAM10 and ADAM17. Compared with macrophages, the expression of ADAM10 was more abundant than that of ADAM17 in NIH3T3 cells. Likewise, in UV♀2 cells in which both ADAM10 and ADAM17 play significant roles in TNF-α shedding, the expression of ADAM10 was relatively greater than that of ADAM17. Presence of TIMPs may be another factor that determines the significance of the proteinases in the shedding. While the enzymic activity of ADAM10 was inhibited by TIMP-1 and TIMP-3, the activity of ADAM17 was suppressed by TIMP-3 but not by TIMP-1. In macrophages, the expression of TIMP-1 could be much greater than that of TIMP-3. Therefore, it is possible that in those cells, the significance of ADAM10 in TNF-α shedding could be reduced by the abundance of TIMP-1. In an analogy of macrophages, the expression of TIMP-1 could be more abundant than that of TIMP-3 in articular chondrocytes, which might explain the predominance of ADAM17 over ADAM10 in TNF-α shedding in the cells.

Vascular endothelial cells have been shown to secrete TNF-α in vitro and in vivo. In spinal cord injury in rats, vascular endothelial cells as well as neurons and glial cells expressed TNF-α around the injury site (Yan et al. 2001). Since the inhibition of TNF-α led to the suppression of inflammation and tissue injury in spinal cord injury in mice (Genovese et al. 2008), TNF-α could play an important role in this pathology. Considering the result with UV♀2 cells, it seems possible in that condition that TNF-α is expressed by the endothelial cells and processed by both ADAM10 and ADAM17. The involvement of ADAM10 in the actual human vasculitis has been suggested by the result of immunostaining performed on the skin tissues from a patient with Henoch-Schönlein purpura. In the acute phase of this disease, TNF-α increases in serum and endothelial cells are likely the source of this cytokine (Besbas et al. 1997; Ha 2005). Therefore, the observation that endothelial cells express TNF-α and ADAM10 together convincingly indicates that ADAM10 could play a significant role in the release of TNF-α in the disease. Furthermore, the fact that human endothelial cells were intensely stained for ADAM10 (Fig. 6E) would suggest that those cells have a capacity to express the proteinase at significant levels. Considering this, ADAM10 could indeed be a major TNF-α sheddase in certain pathologies in which endothelial cells express TNF-α.

Although ADAM9 could process TNF-α and is abundantly expressed in macrophages, we could not find any evidence for its involvement in TNF-α shedding. The reason for this was not clarified in this study. Since ADAM9 is not suppressed by TIMPs (Amour et al. 2002), other endogenous inhibitor(s) for ADAM9 might have been expressed and prevented it from shedding.

The result of this study suggested a possibility that ADAM10 can be a major TNF-α sheddase in certain cell types or certain biological situations. As shown in macrophages, ADAM17 is the principal sheddase for TNF-α in certain cell types when ADAM10 activity is inhibited by TIMP-1. In fact, even in macrophages, ADAM10 can be a major sheddase when TIMP-1 expression is reduced for some reason. Thus, we have shown some evidence that both ADAM10 and ADAM17 can work as a sheddase for TNF-α. Their significance in the shedding may be determined by various factors, such as cell types, their expression levels, and the abundance of TIMPs. Since TNF-α is profoundly involved in various biological events, the present observation could be of some help in understanding the kinetics of mobilization of the cytokine.

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ORIGINAL PAPER

Aberrant $p16^{INK4a}$ methylation is a frequent event in colorectal cancers: prognostic value and relation to mRNA expression and immunoreactivity

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Abstract
Purpose Aberrant $p16^{INK4a}$ promoter methylation is common in colorectal cancer (CRC), but its clinicopathological significance remains controversial. The present study was therefore conducted to analyze $p16^{INK4a}$ methylation and its relationship to clinicopathological features, mRNA levels and immunoreactivity in a series of lesions.
Methods $p16^{INK4a}$ methylation was assessed for normal mucosa ($n = 30$) and CRC samples ($n = 212$) by methylation-specific real-time quantitative PCR, and $p16^{INK4a}$ expression by immunostaining in formalin-fixed paraffin-embedded specimens. In addition, fresh DNA ($n = 61$) was analyzed for relationships to $p16^{INK4a}$ mRNA by reverse-transcription PCR.
Results The $p16^{INK4a}$ methylation index of normal mucosa samples ranged from 0 to 2% (mean, 0.23%; median, 0.02%), while the values for tumor samples varied widely from 0 to 100% (mean, 25.7%; median, 7.1%), the difference being statistically significant ($P < 0.001$). Of 151 paraffin-embedded CRC tissue samples, 51 (34%), 54 (36%), and 46 (30%) were classified as low, intermediate, and high for aberrant methylation of $p16^{INK4a}$. High $p16^{INK4a}$ methylation was significantly associated with large tumor size ($P = 0.025$). Patients with higher methylation further showed more frequent recurrence as compared with the low-methylation group, and shortened cancer-related survival (Hazard ratio [HR], 3.379; $P < 0.001$) and recurrence-free survival (HR, 3.962; $P < 0.001$ on multivariate analysis). A significant inverse relationship was apparent between the $p16^{INK4a}$ methylation and immunoreactivity ($P = 0.017$). A similar tendency was also observed for the methylation status and the mRNA level ($P = 0.195$).
Conclusions We conclude that $p16^{INK4a}$ methylation results in transcriptional silencing and defines a group of CRCs with a poor prognosis.

Keywords $p16^{INK4a}$ · Methylation · Colorectal cancer · Prognosis

Introduction

Abnormal patterns of DNA hypermethylation are common in human tumors, promoter-associated CpG island regions being involved in many of the affected neoplastic cells (Baylin et al. 1998). In colorectal cancers, as with other tumors, aberrant promoter methylation frequently results in silencing of tumor suppressor genes such as APC (Derks et al. 2006; Iacopetta et al. 2006), O^6MGMT (Derks et al. 2006) and hMLH1 (Toyota et al. 1999; Shannon and Iacopetta. 2001; Van Rijnsoever et al. 2002; Ward et al. 2003; Iacopetta et al. 2006; Ogino et al. 2006). $p16^{INK4a}$ (p16) is located on chromosome 9p21 that exerts a negative effect on cell cycle progression at the G1/S checkpoint by

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