Clusterin, an Abundant Serum Factor, Is a Possible Negative Regulator of MT6-MMP/MMP-25 Produced by Neutrophils*

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MT6-MMP/MMP-25 is the latest member of the membrane-type matrix metalloproteinase (MT-MMP) subgroup in the MMP family and is expressed in neutrophils and some brain tumors. The proteolytic activity of MT6-MMP has been studied using recombinant catalytic fragments and shown to degrade several components of the extracellular matrix. However, the activity is possibly modulated further by the C-terminal hemopexin-like domain, because some MMPs are known to interact with other proteins through this domain. To explore the possible function of this domain, we purified a recombinant MT6-MMP with the hemopexin-like domain as a soluble form using a Madin-Darby canine kidney cell line as a producer. Mature and soluble MT6-MMP processed at the furin motif was purified as a 45-kDa protein together with a 46-kDa protein having a single cleavage in the hemopexin-like domain. Interestingly, 73- and 70-kDa proteins were co-purified with the soluble MT6-MMP by forming stable complexes. They were identified as clusterin, a major component of serum, by N-terminal amino acid sequencing. MT1-MMP that also has a hemopexin-like domain did not form a complex with clusterin. MT6-MMP forming a complex with clusterin was detected in human neutrophils as well. The enzyme activity of the soluble MT6-MMP was inactive in the clusterin complex. Purified clusterin was inhibitory against the activity of soluble MT6-MMP. On the other hand, it had no effect on the activities of MMP-2 and soluble MT1-MMP. Because clusterin is an abundant protein in the body fluid in tissues, it may act as a negative regulator of MT6-MMP in vivo.

Cells are continuously communicating with the extracellular environment through the cell surface where molecules mediating signals exist. Proteolysis is an important part of the regulation of these transmembrane signals by activation, inactivation, or functional conversion of the molecules (1–3). However, our knowledge about the proteases involved in such processes remains limited compared with that of the intracellular signaling events and responsive molecules. Recently, a number of cell-associated proteases have been identified by cDNA cloning techniques and from the whole genome sequencing projects, and this has gradually opened the way to addressing the proteolytic events in the pericellular space.

One such example is MT6-MMP, the latest member of the MT-MMP subgroup in the MMP family (matrixins) (4, 5). Among the six members, MT6-MMP tethers to the cells through a covalent link to the glycosylphosphatidylinositol (GPI) in plasma membranes (6, 7) like MT4-MMP (8), whereas other MT-MMPs are integrated into the plasma membrane through a C-terminal transmembrane sequence (MT1, MT2, MT3, and MT5-MMP) (3, 9). MT6-MMP is expressed almost specifically in neutrophils (polymorphonuclear leukocytes, PMN) (4, 7), although some brain tumors also express it (5). Neutrophils express other MMPs such as MMP-8 (neutrophil collagenase) and MMP-9 (gelatinase B) as well (10), but these are also expressed in other tissues and cell types (2). Given this specificity in its expression, MT6-MMP seems to play a pivotal role in the neutrophil function. It is also interesting that stimulation of neutrophils with phorbol myristate acetate or interleukin-8 caused MT6-MMP to be released as a soluble enzyme from the cell surface or secretory vesicles (7). Because MMPs are responsible for the degradation of most of the components of the extracellular matrix, the MMPs produced by neutrophils are presumably important for invasion and migration of the cells to inflammatory sites and/or destruction of the host tissue. On the other hand, the substrates of MMPs are not restricted to the matrix components (11). Recent studies in the field have revealed their targets also include non-matrix type molecules, such as cell adhesion molecules, cytokines, growth factors, and receptors (2).

The proteolytic activity of MT6-MMP has been studied using recombinant catalytic fragments and was found to degrade type IV collagen, gelatin, fibrin, fibronectin, chondroitin sulfate proteoglycan, and dermatan sulfate proteoglycan but not types I–III collagens (7, 12). Although the substrate specificity of MMP is basically attributable to the structure of the catalytic cleft, the hemopexin-like domain is also known to modulate the activity by binding the substrates, interacting with other pro-

1 The abbreviations used are: MT-MMP, membrane-type matrix metalloproteinase; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; Mca, (7-methoxycoumarin-4-yl)acetyl; CHAPS, 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; PVPD, polyvinylidene difluoride; FN, fraction; PMN, polymorphonuclear leukocytes; GPI, glycosylphosphatidylinositol; MDCK, Madin-Darby canine kidney; mAb, monoclonal antibody.

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Expression plasmids for proteins were transfected using FuGENE™ 6. Stable transfectants were selected in DMEM supplemented with 10% fetal bovine serum, 0.1 mg/ml kanamycin. After incubation for 72 h, the conditioned medium (CM) was collected, washed three times with PBS and replenished with serum-free DMEM. After incubation for 10 days, each single clone was selected by limiting dilution. Positive clones were amplified by washing with 2.5% Triton X-100-containing buffer for 1 h and then dialyzed against 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 5 mM MgCl₂, 50 μM ZnCl₂, and 0.01% Brij 35 at 4°C for 24 h and 15,000 rpm for 15 min. The supernatant was incubated with anti-FLAG M2 antibody or anti-human clusterin antibody. Other experiments were described as previously (16). The final preparation was frozen at −80 °C before use.

**Western Blot Analysis.** Samples were subjected to SDS-PAGE under reducing or non-reducing conditions (16), and transferred to PVDF membrane (Millipore, MA). After blocking with 10% fat-free dry milk in PBS for 1 h, the membrane was probed with primary antibody specific to each antigen. The membrane was further probed with horseradish peroxidase-conjugated anti-mouse IgG (Amersham Biosciences) and detected using ECL Plus (Amersham Biosciences) as described in our previous report.

**Gelatin Zymography.** Gelatin zymography was performed as described previously (19). The samples were mixed with SDS sample buffer without a reducing agent and separated on 7.5% acrylamide gels containing gelatin (0.8 mg/ml). The gelatin-containing gel was renatured by washing with 2.5% Triton X-100-containing buffer for 1 h and incubated for 12 h at 37°C. The gelatin remaining in the gel was stained with Coomassie Brilliant Blue R-250, and gelatinolytic activity was detected as clear bands against a blue background.

**EXPERIMENTAL PROCEDURES**

**Materials.** Dulbecco’s modified Eagle’s medium (DMEM), RPMI 1640 medium, FLAG peptide, monoclonal mouse anti-FLAG M2 antibody, and anti-FLAG M2-agarose were purchased from Sigma. Monoclonal mouse anti-human clusterin antibody was from Research Diagnostics, Inc. (Flanders, NJ). G418 (geneticin) and hygromycin B were from Invitrogen. Protease inhibitor mixture and FuGENE™ 6 were from Roche Applied Science. A quenched fluorescent peptide substrate, Mca-PLG-Dpa-AR-NH₂, was obtained from Peptide Institute, Inc. (Osaka, Japan). Soluble form of MT1-MMP was kindly provided by Dr. Y. Okada at Keio University (Tokyo, Japan). MMP-2 was purified as described previously (16). TIMP-1 and -2 were a kind gift from Dr. Kai (Department of Pharmaceutical Chemistry, Tokai University, Isehara, Japan).

**Cell Culture and Transfection—**Madin-Darby canine kidney (MDCK) cells and human breast carcinoma MCF-7 cells were purchased from the American Type Culture Collection (Manassas, VA). MDCK cells were maintained in DMEM (Sigma) supplemented with 10% fetal bovine serum and 0.1 mg/ml of kanamycin (Invitrogen) at 37°C in a humidified atmosphere of 5% CO₂. MCF-7 cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum and 0.1 mg/ml kanamycin. For transfection, cells were seeded in 6-well plates at 1 × 10⁵ cells/well and transfection was performed after 16 h. Expression plasmids for proteins were transfected using FuGENE™ 6 (Roche Applied Science) according to the manufacturer’s instructions.

**Construction of Expression Plasmids.** cDNA of MT6-MMP was used as a template to generate DNA fragments for sMT6-F (Met₁ – Gly³⁵⁴) by PCR employing two primers: a forward primer (5’-AGT GAC TCA CGC TGC GGC TGC CTC GCT CTC GCC-3’) and a reverse FLAG insertion primer (5’-ACT CTC GAG TCA TCA CTT GTC ATC GTC GTC CTT CCA TGC GGC TGC GGC TCC G-3’). The fragments were subcloned into the pcDNA3.1 (+) expression vector (Invitrogen).

**FLAG epitope (DYKDDDDK)-tagged MT1-MMP (MT1F) and MT6-MMP (MT6F) were constructed as described previously (6) and subcloned into the pCEP4 expression vector (Invitrogen). The MT6F plasmid was used as a template to generate DNA fragments for MT6F6CAT and MT6F6FEP. MT6F6CAT and MT6F6FEP were catalytic domain (Tyr¹⁰⁹-Gly³⁴⁶)-deleted and hemopexin-like domain (Cys¹¹⁷-Cys¹⁵⁸)-deleted mutants of MT6F, respectively. All the mutant constructs were generated by PCR employing the overlap extension method as described previously. All PCR products were confirmed by DNA sequencing.

**Establishment of Stable Cell Lines of sMT6-F.** The expression vector for sMT6-F was transfected into MDCK cells using FuGENE™ 6 (Roche Applied Science). At 2 days after transfection, the cells were selected in DMEM supplemented with 10% fetal bovine serum, 0.1 mg/ml kanamycin (Invitrogen), and 800 μg/ml G418. After culture for 10 days, each single clone was selected by limiting dilution. Positive clones were then used for protein purification.

**Purification of sMT6-F.** The stable sMT6-F transfectant was cultured in a cell factory (Nunc, Rochester, NY) until confluent. The cells were washed three times with PBS and replenished with serum-free DMEM. After incubation for 72 h, the conditioned medium (CM) was collected, clarified by centrifugation, and then concentrated using amomum sulfate to a final saturation of 0.80%. The precipitated protein was collected by centrifugation and then dissolved and dialyzed against Tris-buffered saline (TBS: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl) containing 0.05% Brij 35. This fraction was then applied to an anti-FLAG M2-agarose column, and the column was washed with the same buffer. Specific elution was carried out using FLAG peptide (100 μg/ml).

**Western Blot Analysis.** Samples were subjected to SDS-PAGE under reducing or non-reducing conditions (16), and transferred to PVDF membrane (Millipore). After blocking with 10% fat-free dry milk in PBS for 1 h, the membrane was probed with primary antibody specific to each antigen. The membrane was further probed with horseradish peroxidase-conjugated anti-mouse IgG (Amersham Biosciences) and detected using ECL Plus (Amersham Biosciences) as described in our previous report.

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**N-terminal Amino Acid Sequencing of Purified sMT6-F and Its Binding Proteins.** The samples were subjected to SDS-PAGE under reducing conditions and transferred to PVDF membrane (Millipore). After staining of the membrane with Coomassie Brilliant Blue R-250, the stained bands were excised, and the Beckman Coulter LF3000 amino acid sequencer was used.

**Gel Permeation Chromatography.** The sMT6-F-clusterin complex was separated by gel permeation chromatography. The complex-containing fractions were pooled and then dialyzed against 50 mM Tris-HCl (pH 7.5) buffer. After 12 h dialysis, the pooled fraction was denatured using 6 M urea. The denatured complex was applied to a Q-anion exchange column (Bio-Rad). The dissociated clusterin was eluted with the same buffer containing 0.1 M NaCl, whereas clusterin-free sMT6-F was eluted with buffer containing 0.2 M NaCl. Subsequently, the separated sMT6-F and clusterin were individually dialyzed against 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 5 mM CaCl₂, 50 μM ZnCl₂, and 0.01% Brij 35 at 4°C for 2 and 12 h, respectively. The final preparation was frozen at −80°C before use.

**Assay of Enzyme Activities.** Purified enzyme was assayed using a fluorescence-quenched peptide substrate (Mc-PLG-Dpa-AR-NH₂) (20). Purified enzyme (0.7 nM) was incubated with substrate (2 μM) in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 5 mM CaCl₂, and 0.05% Brij 35 at 37°C for 1 h. The concentration of enzyme was determined by active site titration with TIMP-2.

**Reaction mixtures of enzyme and inhibitors were preincubated at 25°C for 15 min with TIMP-1, TIMP-2, and clusterin. The apparent inhibition constant Kᵥ (nM) was calculated by using Equation 1,

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Kᵥ = I/(V_/V₀ - 1)
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Purification of MT6-MMP

The translated pro-MT6-MMP has a hydrophobic amino acid stretch at the C terminus that acts as a signal for GPI anchoring. To prepare a soluble form of MT6-MMP, the hydrophobic stretch was deleted and substituted with the FLAG tag sequence (sMT6-F) (Fig. 1A). MDCK cells that stably express sMT6-F were prepared, and the conditioned medium containing the secreted sMT6-F was collected. The sMT6-F in the culture medium was detected as a band with an expected molecular size of 47 kDa under reducing conditions as demonstrated by Western blotting using anti-FLAG M2 monoclonal antibody (anti-FLAG mAb). sMT6-F was accumulated in the medium according to the time in culture without significant degradation (Fig. 1B). A weak band of 21 kDa presumably representing a degradation product was also detected depending on the culture conditions.

After concentration of the culture medium with ammonium sulfate, sMT6-F was purified using an agarose beads column conjugated with the anti-FLAG mAb. Most of the proteins did not bind to the column as shown in Fig. 2 (lanes 1 and 2). The proteins bound to the column were eluted with FLAG peptide and analyzed by SDS-PAGE under reducing conditions. Five major bands corresponding to 47, 45, 35, 27, and 21 kDa were detected in the elution fraction (lane 3). Among them, the 47- and 21-kDa bands had the FLAG sequence from the reactivity to the antibody, but the others did not (lane 4). SDS-PAGE under non-reducing conditions revealed four major bands of 73-, 70-, 46-, and 45-kDa proteins (lane 5). Both the 46- and 45-kDa proteins were detected by anti-FLAG mAb (lane 6) and showed gelatinolytic activity, whereas the 73- and 70-kDa proteins did not (lane 7, and also refer to Fig. 5 for better resolution). After concentration of the culture medium with ammonium sulfate, sMT6-F was purified using an agarose beads column conjugated with the anti-FLAG mAb. Most of the proteins did not bind to the column as shown in Fig. 2 (lanes 1 and 2). The proteins bound to the column were eluted with FLAG peptide and analyzed by SDS-PAGE under reducing conditions. Five major bands corresponding to 47, 45, 35, 27, and 21 kDa were detected in the elution fraction (lane 3). Among them, the 47- and 21-kDa bands had the FLAG sequence from the reactivity to the antibody, but the others did not (lane 4). SDS-PAGE under non-reducing conditions revealed four major bands of 73-, 70-, 46-, and 45-kDa proteins (lane 5). Both the 46- and 45-kDa proteins were detected by anti-FLAG mAb (lane 6) and showed gelatinolytic activity, whereas the 73- and 70-kDa proteins did not (lane 7, and also refer to Fig. 5 for better resolution). As the 73- and 70-kDa proteins were eluted from the column by FLAG peptide and were not detected in the preparations from the non-transfected MDCK cells (data not shown), they are presumably associating with sMT6-F.

RESULTS

Purification of MT6-MMP—The translated pro-MT6-MMP has a hydrophobic amino acid stretch at the C terminus that acts as a signal for GPI anchoring. To prepare a soluble form of MT6-MMP, the hydrophobic stretch was deleted and substituted with the FLAG tag sequence (sMT6-F) (Fig. 1A). MDCK cells that stably express sMT6-F were prepared, and the conditioned medium containing the secreted sMT6-F was collected. The sMT6-F in the culture medium was detected as a band with an expected molecular size of 47 kDa under reducing conditions as demonstrated by Western blotting using anti-FLAG M2 monoclonal antibody (anti-FLAG mAb). sMT6-F was accumulated in the medium according to the time in culture without significant degradation (Fig. 1B). A weak band of 21 kDa presumably representing a degradation product was also detected depending on the culture conditions.

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Identification of Canine Clusterin as the Protein Binding to sMT6-F—To analyze the polypeptide components of the bands detected under non-reducing conditions (Fig. 3A), they were extracted from the gel and examined further under reducing conditions (Fig. 3B). The 73-kDa protein in Fig. 3A was composed of two polypeptide chains of 45 and 35 kDa possibly linked with disulfide bonds (Fig. 3B, 2nd lane), and the 70-kDa protein also contained two polypeptides of 45 and 27 kDa (Fig. 3B, 3rd lane). The 46-kDa protein in Fig. 3A was separated into 27- and 21-kDa polypeptides (Fig. 3B, 4th lane). The smaller polypeptide has the FLAG tag from the reactivity to the antibody (Fig. 2, lane 5) and is thought to contain the C-terminal fragment of sMT6-F. The 27-kDa polypeptide presumably corresponds to the fragment containing the catalytic domain of sMT6-F, because the 46-kDa protein, from which this fragment is derived, retained the gelatinolytic activity (Fig. 2, lane 7, and also refer to Fig. 5 for better resolution). The 45-kDa protein in Fig. 3A was composed of a single polypeptide of sMT6-F (Fig. 3B, 5th lane). A shift of molecular mass from 45 kDa under non-reducing conditions to 47 kDa under reducing conditions was observed, and this reflects the disruption of the intramolecular disulfide bond in the hemopexin-like domain.

Then each polypeptide was extracted from the gel and subjected to N-terminal amino acid sequencing (Fig. 4). The N terminus of the 47-kDa polypeptide (108 amino acids from the translation start site) exactly matched the downstream sequence of the site of processing by furin-like enzymes that generate the mature form of MT6-MMP (4, 5), thus indicating that this is the intact sMT6-F. The 27-kDa polypeptide had the same sequence indicating that this is the N-terminal fragment of sMT6-F containing the catalytic domain. On the other hand, the N-terminal sequence of the 21-kDa polypeptide coincided with the sequence in the hemopexin-like domain that starts from the 348-amino acid position. Thus, the 46-kDa sMT6-F has a cleavage within the hemopexin-like domain, and the two polypeptide chains are connected to each other by a disulfide bond within the hemopexin-like domain ("nicked" sMT6-F).

The N-terminal amino acid sequence of the 45-kDa polypeptide derived from the 73- and 70-kDa proteins exactly coincided with the β subunit of the canine clusterin, whereas the sequences of the 35- and 27-kDa polypeptides corresponded to the α subunit of clusterin. Clusterin is known to be composed of α and β subunits connected by five disulfide bonds (22, 23). The difference in the molecular size of the two forms of the α subunit sharing the same N terminus may reflect the difference in modification or heterogeneity downstream.

sMT6-F Forming a Complex with Clusterin Has No Proteolytic Activity—To analyze the nature of the clusterin-sMT6-F complex, the eluate from the affinity column was applied to a Superdex 200 gel permeation column. The applied sample was eluted forming three major peaks corresponding to molecular sizes larger than the 669-kDa marker proteins, 540 and 62 kDa, respectively (Fig. 5A). Collected fractions were analyzed further by SDS-PAGE under non-reducing conditions (Fig. 5B). The fraction with the highest molecular weight peak (FN 53) contained an unknown protein of several hundred kilodaltons and a small amount of sMT6-F. The second peak (FN 57–70) with a broad range of 560–215 kDa contained two forms of clusterin and two forms of sMT6-F. However, the intensity of the intact sMT6-F band (45 kDa) was more abundant than the nicked molecule (46 kDa) in this fraction. The third peak contained only sMT6-F alone presumably as a monomer from the estimated molecular size, and the nicked molecule was predominant in this fraction compared with the intact form.
Cluster Inhibits the Activity of MT6-MMP

To examine the difference in proteolytic activity of sMT6-F between the clusterin complex and the free form, the activity of the clusterin-containing (FN, 57 and 60) and -free fractions (FN, 80 and 83) was analyzed using the fluorescence-quenched peptide substrate. The fractions free of clusterin showed significant proteolytic activity (Fig. 5C), but the activity of the clusterin-containing fraction was negligible. On the other hand, all fractions showed almost equal gelatinolytic activity in zymography (Fig. 5D), suggesting that the activity of sMT6-F is inhibited as a result of clusterin binding.

Dissociation of the Clusterin Complex—The clustor-sMT6-F complex was stable in the buffer containing high concentrations of NaCl (0.15 to 1 M) or 1% detergent (Triton X-100, n-octyl-β-D-glucoside, n-dodecyl-β-D-maltoside, CHAPS, and deoxycholic acid) (data not shown). The complex in the pooled fraction (FN, 55–73) was then treated with 6 M urea and subjected to anion exchange column chromatography. Elution was carried out with a urea-containing buffer with increasingionic strength. Clusterin was eluted with the buffer containing 0.1 M NaCl, whereas sMT6-F was eluted with 0.2 M NaCl (Fig. 6A). The 0.2 M NaCl fraction contained intact sMT6-F with minor contamination by the nicked molecule. To refold the denatured enzyme, the concentration of urea was gradually decreased by dialysis at 4 °C. The recovery of the enzyme activity during dialysis was monitored using the peptide substrate. The activity recovered rapidly according to the time of dialysis and reached a plateau after 1 h (Fig. 6C). The integrity of the enzyme was also checked by SDS-PAGE (Fig. 6B). Prolonged dialysis for more than 3 h caused auto-degradation of the enzyme. Thus, we used the 2-h dialyzed fractions as the refolded enzyme for subsequent experiments.

Cluster Is a Negative Regulator Specific to MT6-MMP—The refolded sMT6-F showed dose-dependent proteolytic activity against the peptide substrate (data not shown), and the activity was inhibited by TIMP-1 and TIMP-2 in a stoichiometric fashion as reported previously (Fig. 7A) (12). We then used the clusterin preparation isolated from the anion exchange column to examine the effect on the proteolytic activity after refolding (Fig. 7B). Increasing doses of clusterin inhibited the activity of sMT6-F in a dose-dependent manner, although the inhibition was considerably weaker than that by TIMPs. The K_inhib_value was 120 nM. On the other hand, it did not affect the activities of either MMP-2 or the soluble form of MT1-MMP. Thus, the inhibitory effect of clusterin is specific to sMT6-F and not a result of its nonspecific interaction with MMPs.

Complex Formation of Human Clusterin with GPI-anchored MT6-MMP—To confirm that human clusterin can form a complex with the GPI-anchored form of MT6-MMP, we expressed MT6-MMP having a FLAG tag downstream of the furin site (MT6F) (Fig. 8A) in human breast carcinoma MCF-7 cells that express clusterin constitutively. The FLAG tag MT1-MMP (MT1F) that was not inhibited by the canine clusterin was used as a negative control. Stable transfectants expressing either MT6F or MT1F were obtained (Fig. 8B). The cells were lysed, and then immunoprecipitation was carried out using anti-FLAG mAb. Clusterin in the precipitates was analyzed by Western blotting using anti-human clusterin antibody. Clusterin was specifically co-precipitated with MT6F but not with MT1F (Fig. 8C). Thus, the formation of a complex between MT6-MMP and human clusterin is not a nonspecific interaction.

To examine the importance of the ectodomains of MT6-MMP in forming a complex with human clusterin, deletion mutants lacking either the catalytic domain (MT6FdCAT) or the hemopexin-like domain (MT6FdPEx) were prepared (Fig. 9A) and expressed in the MCF-7 cells (Fig. 9B). As demonstrated in Fig. 9C, MT6FdCAT co-precipitated with clusterin but MT6FdPEx did not, indicating that the hemopexin-like domain is responsible for clusterin binding.

MT6-MMP Expressed in Neutrophils Forms a Complex with Clusterin—To confirm the complex formation with natural products, we purified human neutrophils and examined the endogenous MT6-MMP. For this study, a rabbit polyclonal antibody against recombinant sMT6-F was prepared, and its specificity was confirmed by Western blotting as in Fig. 10A. No cross-reactivity was detected with other MT-MMPs. The antibody also did not react with clusterin itself in MDCK and MCF-7 cells at all (data not shown).

The neutrophils collected were lysed and subjected to immunoprecipitation using the antibody. As demonstrated in Fig. 10B, a mature form of MT6-MMP (54 kDa) was precipitated as detected by Western blotting. An additional band of 42 kDa was also detected in the precipitate and presumably represents a degradation product. Thus, MT6-MMP is expressed in neutrophils as reported (7). Clusterin was detected in the precipitate indicating that a significant amount of endogenous MT6-MMP also forms a complex with clusterin. Although clusterin is reported to interact with immunoglobulin (24), it did not precipitate with the control IgG under our assay conditions.

DISCUSSION

In this study, clusterin was identified as a binding protein for human MT6-MMP. The interaction seemed to be specific to MT6-MMP because MT1-MMP did not bind clusterin. The MDCK cell line used to produce sMT6-F in this study is known to express endogenous clusterin constitutively (25) and has also
been used to produce other recombinant MMPs by us and others (7, 26, 27). We have expressed soluble forms of MT1-MMP and MT4-MMP and MMP-2 in the cells and purified the proteins. Neither MMP-2 nor the soluble form of MT1-MMP associated with the clusterin, but the soluble form of MT4-MMP, which is similar to MT6-MMP in amino acid sequence (45%) and GPI anchoring, also bound clusterin (data not shown). A soluble form of MT5-MMP and MMP13 was also expressed in MDCK cells and purified by other groups, but the association of purified clusterin at room temperature for 15 min, and the enzyme activities were analyzed using a fluorescence-quenched peptide substrate.

The deletion mutant either lacking the catalytic domain (MT6FdCAT) or the hemopexin-like domain (MT6FdPEX) was expressed in MCF-7 cells as a GPI-anchored form. MT6FdCAT co-precipitated with clusterin, whereas MT6FdPEX did not. Thus, the hemopexin-like domain is responsible for the clusterin binding. In a previous study, the catalytic fragment of MT6-MMP was also expressed in MDCK cells and purified without an association with clusterin (7). This is consistent with our finding that the hemopexin-like domain is responsible for clusterin binding. In addition to the intact sMT6-F, our preparation contained a molecule having a nick in the hemopexin-like domain. The nicked molecule was found less in the clusterin complex and more in the clusterin-free fractions, whereas the reverse was the case for the intact molecule. This phenomenon also indicates that the integrity or specific sequence of the hemopexin-like domain is important for the clusterin binding. Thus, it is not the result of nonspecific binding of clusterin to denatured proteins. However, it is still possible that partial denaturation in the region induces interaction with clusterin. Even in this case, this is a specific feature of MT6-MMP and MT4-MMP not found in other MMPs examined. In addition, clusterin forming a complex with MT6-MMP was detected in human neutrophils indicating that the two are interacting even in natural producer cells.

The separation of the complex by gel permeation column chromatography indicated that the main peak of the clusterin complex (540 kDa) is larger than the expected size of the stoichiometric complex (120 kDa). Thus, the complex is thought to be composed of a multimeric form of clusterin and sMT6-F.
Clusterin Inhibits the Activity of MT6-MMP

Fig. 8. Specific binding of human clusterin to MT6-MMP but not MT1-MMP. A, schematic presentation of MT6-MMP and MT1-MMP constructs used in this experiment. MT6F and MT1F have a FLAG epitope downstream of the furin motif, SP, signal peptide; Pro, proteptide; Furin Motif, furin cleavage site; Cat, catalytic domain; PEX, hemopexin-like domain; GPI signal, glycosylphosphatidylinositol anchor signal; TM, transmembrane domain; FLAG, FLAG epitope (DYKDDDDK). B, detection of MT6-F and MT1F expressed in MCF-7 cells. Plasmids for MT6F and MT1F, and an empty vector (mock) were stably transfected into MCF-7 cells. The hygromycin-resistant cell populations were subjected to Western blotting analysis using anti-FLAG M2 antibody. The asterisks indicate the nonspecific band. The upper arrow indicates MT1F, and the lower arrow indicates MT6F. C, immunoprecipitation (IP) of human clusterin with MT6-MMP and MT1-MMP. The cells expressing either MT6-MMP or MT1-MMP were lysed and subjected to immunoprecipitation experiments using anti-FLAG antibody. The proteins bound to the antibody was eluted with FLAG peptide and analyzed by Western blotting (WB) using antibodies for clusterin (upper panel) and anti-FLAG (middle panel). Conditioned medium was also analyzed for clusterin by Western blotting (lower panel).

Fig. 9. Identification of the domain of MT6-MMP binding to human clusterin. A, schematic presentation of MT6-MMP and its mutants used in this study. SP, signal peptide; Pro, proteptide; Furin Motif, furin cleavage site; Cat, catalytic domain; PEX, hemopexin-like domain; GPI signal, glycosylphosphatidylinositol anchor signal; FLAG, FLAG epitope (DYKDDDDK). B, Western blotting of MT6-MMP and its mutants detected by anti-FLAG M2 antibody. An expression plasmid for MT6-MMP and that for mutants and an empty vector (mock) were stably transfected into MCF-7 cells. The hygromycin-resistant cell population was selected and used for analysis by Western blotting using anti-FLAG M2 antibody. The upper arrow indicates MT6F, the middle arrow indicates MT6FdPEx, and the lower arrow indicates MT6FdCAT. C, immunoprecipitation (IP) of human clusterin with MT6-MMP and its mutants. The transfectants were lysed and subjected to immunoprecipitation using anti-FLAG antibody. The proteins eluted from the precipitates with a FLAG peptide were analyzed by Western blotting (WB) using anti-clusterin or anti-FLAG antibodies (upper and middle panels). The conditioned medium was also analyzed by Western blotting using anti-clusterin antibody (lower panel).

to contain multiple clusterin and sMT6-F molecules, presumably due to the nature of clusterin to interact (22). The interaction between clusterin and sMT6-F seems stable and irreversible under physiological conditions, because no dissociation occurred in the buffer containing high concentrations of NaCl or detergents. However, it is still possible for dissociation to occur when a conformational change to clusterin or the hemopexin-like domain is induced by proteolytic cleavage or association with other proteins under certain conditions. The clusterin-sMT6-F complex did not show proteolytic activity against the synthetic peptide substrate, although the clusterin-free fraction did. However, gelatinolytic activities in the both fractions were detected by zymography, in which sMT6-F was dissociated from clusterin by detergent, suggesting that clusterin inhibits sMT6-F by forming a complex. The complex dissociated in the presence of 6 M urea and the components were separated by anion exchange column chromatography. Refolding of the denatured protein restored the proteolytic activity which was inhibited again on the addition of the purified and refolded clusterin. It is notable that the inhibitory activity of the purified clusterin is considerably weak compared with that in the complex. The refolding of clusterin may be too incomplete to restore full activity. It is not clear how clusterin binding to the hemopexin-like domain suppresses the catalytic activity. The formation of a high molecular weight aggregate may prevent the substrate from accessing the catalytic site or

effects of this protein is the ability to interact with a wide array of components in serum and on the cell surface including complements (31), immunoglobulins (24), lipids (32), β-amyloid peptide (33), and prion peptide (34) etc. It also binds to the Staphylococcus aureus cell surface (35). Because clusterin is abundant in serum and binds immunoglobulins, complements, and bacteria, it is reasonable to speculate that clusterin plays some role at the inflammatory site where neutrophils accumulate. It should be also noted that the level of clusterin in serum is about 10 times the K_{app}, value for MT6-MMP (36). Thus, it is reasonable to suppose that clusterin regulates neutrophil function by inhibiting MT6-MMP and prevents excessive destruction of the host tissue. Indeed, we ob-
Fig. 10. Detection of a clusterin-MT6-MMP complex in human PMNs. A, immunoreactivity of anti-MT6-MMP polyclonal antibody for MT-MMPs in human PMNs. WB; FLAG or biotinylated anti-MT6-MMP polyclonal antibody was subjected to immunoprecipitation (IP) with either rabbit IgG or anti-MT6-MMP polyclonal antibody. Precipitates were solubilized and analyzed by Western blotting using anti-clusterin antibody. The biotinylated antibody was visualized using avidin-conjugated horseradish peroxidase.

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observed that a substantial amount of MT6-MMP expressed in human neutrophils forms a complex with clusterin. Clusterin-deficient mice have been generated, and it was reported that excessive tissue damage in the clusterin null mice might be a reflection of the lack of the tissue-protective function of clusterin caused by inhibiting a certain class of MMPs at the site of inflammation. The expression of clusterin is also induced in brains with tumors. Because some brain tumors are reported to express MT6-MMP, the clusterin expressed there may also act to prevent tissue damage possibly caused by MT6-MMP. Thus, we propose that clusterin is a possible negative regulator of MT6-MMP produced by neutrophils and brain tumors.

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Clusterin, an Abundant Serum Factor, Is a Possible Negative Regulator of MT6-MMP/MMP-25 Produced by Neutrophils

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