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Matrix Pathobiology

Interaction of Clusterin and Matrix Metalloproteinase-9 and Its Implication for Epithelial Homeostasis and Inflammation

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Uncontrolled increases of matrix metalloproteinase-9 (MMP-9) activity have been causally linked to epithelial barrier disruption and severe symptoms of inflammatory diseases such as dry eye (DE). The data presented here show that the anti-inflammatory, cytoprotective intracellular and extracellular chaperone protein clusterin (CLU) interacts with MMP-9 both inside and outside epithelial cells. CLU bound very strongly to active MMP-9, with an affinity constant K_D of 2.63 nmol/L. Unexpectedly, CLU had a much higher affinity for pro-MMP-9 than for active MMP-9 or pro-MMP-2, requiring the N-terminal propeptide domain of pro-MMP-9. The significance of the interaction between CLU and MMP-9 was demonstrated by the observation that CLU prevents stress-induced MMP-9 aggregation and inhibits MMP-9 enzymatic activity. Furthermore, CLU inhibited MMP-9-mediated disintegration of the tight junction structure formed between human epithelial cells. Additionally, CLU inhibited enzymatic activities of MMP-2, MMP-3, and MMP-7. Treatment with proinflammatory cytokines, which are known to increase MMP-9 transcription under inflammatory conditions, reduced the expression of CLU in human epithelial cells. Similarly, in a mouse model of human DE, inflammatory stress depleted CLU in the ocular surface epithelium but allowed MMP-9 to prevail therein. The present results thus provide novel insights into previously unrecognized mechanisms by which CLU maintains fluid-epithelial interface homeostasis, thereby preventing the onset of inflammatory conditions, especially where MMP-9 is actively involved. *(Am J Pathol 2012, 180: 2028–2039; DOI: 10.1016/j.ajpath.2012.01.025)*

The epithelial cell lining of structures that face the external environment, such as the airway and ocular surface, is frequently subject to chronic or acute inflammation. An inflammatory morbidity of the ocular surface, dry eye disease (DE), affects approximately 4.9 million Americans over the age of 50 years.[1] The burden to the patient is substantial, adversely affecting visual function and quality of life, with few therapeutic options.

The matrix metalloproteinases (MMPs) are a family of extracellular zinc-dependent endopeptidases whose activity is tightly regulated at multiple stages, including transcription, secretion, activation, activity, and clearance.[2,3] MMPs play diverse roles in maintenance and repair of the ocular epithelium.[4] MMP-9 is secreted as inactive pro-MMP-9, and the N-terminal propeptide of pro-MMP-9 blocks the catalytic domain by binding intramolecularly through the cysteine-zinc bridge between these two domains.[5] MMP-9, which is induced by damage to the corneal epithelium, coordinates multiple events in the process of corneal epithelial regeneration; either too little or too much MMP-9 leads to undesirable outcomes.[6,7] Stud-

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ies from knock-out mouse models have demonstrated that excessive MMP-9 activity is associated with abnormal corneal wound-healing processes.[6–8] Increased MMP-9 activity has also been shown to play a causal role in the destruction of the corneal epithelial barrier in DE disease,[9] and was strongly correlated with severe clinical symptoms in patients with DE.[10] Recently it has been suggested that increased MMP-9 affects cell proliferation,[11] which further emphasizes the damaging role of MMP-9 in inflammatory disease.

CLU (alias apolipoprotein J or Apo-J) has three isoforms with distinct subcellular locations. The secreted form (sCLU) is a heterodimeric glycoprotein (~80 kDa) composed of disulfide-linked α (34 to 36 kDa) and β (36 to 39 kDa) subunits derived inside cells from a presecreted, glycosylated peptide (psCLU) (~60 kDa); sCLU works as an extracellular molecular chaperone for numerous protein ligands.[12,13] sCLU and psCLU proteins have been suggested to play cytoprotective, anti-inflammatory roles in various organs (see reviews by Trougakos et al[13] and by Falgarone and Chiocchia[14]). Nuclear CLU (nCLU), a product of an alternative splicing of pre-mRNA, is correlated with the apoptotic response of cells to certain stimuli, including ionizing irradiation.[15]

sCLU facilitates the clearance of denatured proteins, protein aggregates, proteinases, and dead cell debris,[16–18] interacts with the cell surface receptor, low-density lipoprotein receptor-related protein 2 (LRP-2) (LRP-2/megalin/gp330),[19] protects the cells from oxidative stress,[20] and inhibits immune complement-mediated cytolytic activity.[21–23] psCLU protects from cell death by blocking the intracellular activity of Bax,[24] and sCLU protects some cell lines from TNF-α-mediated cell death.[25] psCLU also stabilizes I-κB and inhibits nuclear NF-κB translocation,[26–28] which is one of the key events involved in inflammation. Overexpression of CLU was shown to inhibit MMP-9 expression mediated by the NF-κB pathway.[28] Results from several genetic studies using CLU-deficient mice have supported the protective role of CLU during inflammation in various organs.[29–33]

Even though MMP-9 is a critical factor in ocular surface inflammation, molecular studies are required for better understanding of how its activity is regulated. With the present study, we show that CLU proteins interact with MMP-9 in unique ways, interfering with its biochemical activity and function. These data provide new insight into important roles of CLU in ocular surface homeostasis and in the prevention and treatment of inflammatory disease.

Materials and Methods

Cell Culture

Cells of the human embryonic kidney cell line HEK293 and the human breast cancer cell line MCF-7 were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in a cell culture incubator at 37°C and 5% CO_2. Immortalized human corneal-limbal epithelial (HCLE) cells, kindly provided by Ilene Gipson (Harvard University), were grown in Gibco keratinocyte serum-free medium (Invitrogen, Carlsbad, CA), as described previously.[34]

MMP-9 cDNA Construction

The entire MMP-9 cDNA (coding amino acids 1 to 703) was reverse-transcribed and amplified from mouse muscle tissue. Primers MMP9.1 (5′-GGCGCCGAATTC-ATGAGTCCCTGGCAGCCCCTG-3′) and MMP9.2109 (5′-GGGCCCGTCGACTCAAGGGCACTGCAGGAGGTCGT-AGGTCA-3′) were used, incorporating EcoRI and SalI sites, respectively. This RT-PCR product was inserted into the yeast expression vector pGBKT7 and labeled pGB-M9. Using the pGB-M9 plasmid as a template, an N-terminus MMP-9 truncated PCR product missing the signal and propeptide domain (amino acids 109 to 703) was generated using MMP-9.325 (5′-GGCGCCGAATTC-CAAACCTTCAAAGGCCTCAAGTGGG-3′) and MMP9.2109 primers and labeled pGB-M9ΔPP. The glutathione *S*-transferase (GST)-tagged MMP-9 constructs were generated by ligation of the pGB-M9 and pGB-M9ΔPP EcoRI/SalI inserts into the pGEX 4T vector (Amersham; GE Healthcare, Piscataway, NJ).

Library Construction and Screening

Whole mouse cornea was excised and total RNA was isolated using TRIzol reagent (Invitrogen). The cDNA library was synthesized using 2 μg total RNA and random primers according to the protocol for the Matchmaker yeast two-hybrid system (Clontech, Mountain View, CA). This system uses an *in vivo* library construction method; the cDNA library and the linearized yeast expression vector pADT7-Rec were therefore simultaneously transfected into the AH109 pGB-M9ΔPP stable yeast cells. Positive colonies were selected on quadruple knock-out plates (ie, yeast minimal agar plates lacking tryptophan, leucine, histidine, and adenine). The histidine and adenine reporter genes were used to test for interactions. An additional screen involved the addition of X-α-galactosidase to the quadruple knock-out medium, whereby a positive interaction allowed for blue/white screening.

For yeast plasmid isolation, positive colonies were inoculated into an overnight culture of yeast agar peptone dextrose (YAPD) medium, and plasmid isolation was performed on saturation using a yeast lysis method as follows. Briefly, pelleted yeast cells were mixed in a vortex mixer in equal volume yeast lysis buffer (2% Triton X-100, 1% SDS, 0.1 mol/L NaCl, 10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA) and phenol-chloroform-isoamyl alcohol (25:24:1) with acid-washed glass beads. The clarified supernatant was ethanol-precipitated in the presence of NaOAc. The resulting DNA complex was transformed into XL1-blue bacterial cells, and colonies were selected on ampicillin plates (to select for the pADT7-library clone). The resulting bacterial colonies were lysed via the standard alkaline lysis protocol and the plasmids were sequenced. The DNA sequences were translated based on the reading frame given for the pADT7-Rec vector, and both the protein sequence and the DNA sequence were subjected to GenBank BLAST searches (*http://www.ncbi.nlm.nih.gov/blast*).

The DNA sequences were translated into protein sequences using a translation software tool (*http://www.ncbi.nlm.nih.gov/projects/gorf*). Dual searches were performed to confirm that the protein sequences translated matched their DNA sequences; mismatching clones were discarded.

GST Pull-Down Assay

The GST-tagged MMP-9 constructs were generated by ligation of the pGB-MMP-9 EcoRI/SalI inserts into the pGEX 4T vector (Amersham; GE Healthcare). The pGEX 4T MMP-9 constructs were transformed into the *E. coli* strain BL21(DE3) (Novagen; EMD Chemicals, Gibbstown, NJ) for expression. The bacterial pellet was prepared from a 100-mL culture that was grown to mid-log phase, induced with 0.4 mmol/L isopropyl β-D-thiogalactopyranoside for 3 hours, and then centrifuged. The pellet was resuspended and lysed by sonication in ST buffer (50 mmol/L Tris pH 7.4, 100 mmol/L NaCl, 1 μg/mL leupeptin, 1 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L NaF), and GST-MMP-9 protein was purified via incubation with glutathione-coated beads (Amersham; GE Healthcare) overnight at 4°C. On the next day, the beads were extensively washed in ST buffer. To confirm production of GST-MMP-9 protein, proteins bound to the beads were resolved on a denaturing 8% SDS-PAGE gel. The gel was subjected to Coomassie Brilliant Blue staining or transferred to polyvinylidene difluoride membrane and immunoblotted using a rabbit polyclonal antibody to MMP-9 (Triple Points Biologics, Forest Grove, OR). With a TNT quick coupled transcription/translation system (Promega, Madison, WI), the pADT7-CLU clone was used to generate a CLU-HA fusion protein. Beads coated with GST-MMP-9 or GST were incubated with equal amounts of CLU-HA overnight at 4°C in immunoprecipitation (IP) buffer (50 mmol/L Tris pH 7.4, 100 mmol/L NaCl, 0.1% Triton X-100, 1 μg/mL leupeptin, 1 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L NaF) in the presence or absence of 1 mg/mL BL21 bacterial soluble extract (to decrease nonspecific background). On the next day, the beads were washed three times in IP buffer, before the proteins were eluted from the beads into SDS-PAGE loading buffer, which was subjected to Western blotting using a mouse HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Generation of Expression Vectors for MMP-9 and CLU

The pcDNA3.1(+) expression vector (Invitrogen) was used to insert PCR fragment generated using pGB-M9 as a template with the gene-specific primers containing EcoRI sites (forward primers) or NotI sites (reverse primers): mouse CLU with a C-terminal Myc tag (CLU-Myc), 5′-GATCGAATTCATGAAGATCTCCTGCTGT-3′ and 5′-CGATGCGGCCGCTCACAGGTCCTCCTCTGAGATCAGCTTCTGCTCTTCCGCACGGCTTTTCCT-3′; mouse MMP-9 with a C-terminal HA tag (MMP-9-HA), 5′-GATCGAATTCATGAGTCCCTGGCAGCC-3′ and 5′-CGATGCGGCCGCTCAAGCGTAATCTGGAACATCGTATGGGTAAGGGCACTGCAGGGT-3′. The DNA sequence of cloned DNA was confirmed by DNA sequencing.

Surface Plasmon Resonance Analysis

The SensíQ Pioneer system (ICx Nomadics, Oklahoma City, OK) was used for all surface plasmon resonance measurements at a constant temperature of 25°C. The running buffer for the SensíQ Pioneer system was 20 mmol/L HEPES pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, and 0.01% Tween-20. An ICx COOH5 sensor chip, which comprises a high-capacity charged polysaccharide for amine coupling of target molecules, was used for all experiments. For kinetic analysis of CLU binding MMP-9, recombinant catalytic domain of MMP-9 was immobilized to the COOH5 sensor by direct amine coupling. Amine coupling of MMP-9 was performed according to the manufacturer's protocol, in which MMP-9 is diluted in acetate buffer (pH 4.3). A solution of CLU-His (R&D Systems, Minneapolis, MN) was prepared in running buffer, and a serial doubling dilution series was prepared according to the SensiQ Pioneer protocol with a top concentration of 100 nmol/L. Each concentration in the series was injected for 3 minutes over three sensor channels that contained either MMP-9 or a blank reference channel. Dissociation of each injection was monitored for 6 minutes. Kinetic analysis of all response curves was performed using Qdat analysis software (ICx Nomadics and BioLogic Software, Campbell, Australia). The binding data were zeroed and referenced by subtracting the response of the reference channel and a buffer blank injection. A simple 1:1 kinetic model was fitted to the data, where the rate constants of association (k_a) and dissociation (k_d) were globally constrained for the assay.

Immunoprecipitation Assay

HEK293 cells were grown in subconfluent culture in a six-well plate and were transfected with 2.5 μg of pcDNA3.1(+)/MMP-9-HA, pcDNA3.1(+)/MMP CLU-Myc, or both, using Lipofectamine LTX according to the manufacturer's instruction (Invitrogen). At 1 day after transfection, whole-cell extracts were prepared using radioimmunoprecipitation assay buffer, incubated with anti-HA antibody to immunoprecipitate MMP-9-HA and partners. The complexes were resolved on a denaturing 8% SDS/PAGE gel for immunoblotting to detect CLU-Myc proteins with anti-Myc antibody.

Immunofluorescence Staining

HCLE cells (5000 cells/well, for 2 days) and MCF-7 cells (25,000 cells/well for 4 days) were grown in a 16-well chamber slide before immunocytochemical analysis. The HCLE cells were washed once with PBS and then fixed in ice-cold methanol for 10 minutes. Next, the cells were washed three times with PBS, followed by blocking with 1% bovine serum albumin and 0.25% Triton-X 100 in PBS

for 1 hour at room temperature. The cells were then incubated at room temperature for 45 minutes with both goat anti-CLU (1:50) (Santa Cruz Biotechnology) and rabbit anti-MMP-9 (1:50) (Abcam, San Francisco, CA) antibodies in the blocking solution. After the primary antibody incubation, cells were washed three times with PBS for 5 minutes each and then were incubated at room temperature for 1 hour with both donkey Alexa Fluor 488 anti-goat IgG (1:2000) and goat Alexa Fluor 594 anti-rabbit IgG (1:2000) secondary antibodies. Cells treated with the secondary antibodies alone were used as a negative control. Subsequently, the cells were washed three times with PBS for 5 minutes each and rinsed once with distilled water. The cells were then covered with a drop of Vectastain containing DAPI (Vector Laboratories, Burlingame, CA) and coverslipped. For ZO-1 degradation experiments, MCF-7 culture was exposed to 25 ng of catalytic domain of MMP-9 in the presence or absence of 6 μg of recombinant CLU in fetal bovine serum-free Dulbecco's modified Eagle's medium for 4 hours in a CO_2 incubator in duplicate for each set. The cells were processed as above for fluorescent staining with anti-ZO-1 (1:50) and Alexa Fluor 488 anti-goat IgG antibodies (1:500).

The cells were imaged on an UltraVIEW ERS spinning disk laser confocal microscope (PerkinElmer, Waltham, MA) at magnification $\times$400, with Z-stacking at 0.5-μm intervals. A total of 12 images per each set were obtained randomly, in Z-stacking, and the general densities of ZO-1 were quantified with PerkinElmer Volocity software version 5.4. For Western blotting, 40 μg of whole-cell extract was loaded onto 8% denaturing SDS/PAGE gel. Antibody for β-actin (1:5000) was purchased from Abcam.

MMP Inhibition Assay

Recombinant mouse CLU with C-terminal $(His)_6$ tag (sCLU), MMP-2, and MMP-7 were purchased from R&D Systems (Minneapolis, MN); MMP-9 was purchased from AnaSpec (Fremont, CA). The recombinant catalytic domain of human MMP-9 (residue 112 to 445) was purchased from AnaSpec and from ProtEra (Sesto Fiorentino, Italy). A gelatinase-specific synthetic inhibitor, SB-3CT, was obtained from Biomol International (Plymouth Meeting, PA). MMPs were mixed with CLU in TSCB buffer (50 mmol/L Tris-HCl pH, 7.5, 150 mmol/L NaCl, 5 mmol/L $CaCl_2$, 0.002% Brij-35) for 30 minutes before addition of substrate, and were incubated for 3 hours at room temperature in the presence or absence of 0.02 mmol/L *p*-aminophenylmercuric acetate (APMA). The APMA was added only when pro-MMPs were used. MMP activity was measured using a 5-FAM/QXL520 fluorescence resonance energy transfer (FRET) peptide as a substrate (excitation, 490 nm; emission, 520 nm) (AnaSpec), according to the manufacturer's protocol. Relative fluorescence units (RFU) were obtained by subtracting the values of APMA- or enzyme-omitted reactions from those of test samples, and then considering the fluorescence unit of PBS (vehicle) sample as 100%. Enzyme reactions were performed in a black 96-well plate, containing combinations of pro-MMP-2 (10 ng), catalytic domain of MMP-3 (20 ng), pro-MMP-7 (5 ng), catalytic domain of MMP-9 (5 ng), and sCLU (2.5 μg or 0.6 μmol/L, or otherwise as indicated) in 50-μL reaction volume, where pro-MMPs were activated by APMA-induced processing of the pro-MMPs. As MMP substrates (0.4 μmol/L), 520 MMP FRET substrate I, QLX520-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-Lys(5-FAM)-NH_2, was used for MMP-2, MMP-3, and MMP-9, and 520 MMP FRET substrate XIV, QXL520-γ-Abu-Pro-Cha-Abu-Smc-His-Ala-Dab(5-FAM)-Ala-Lys-NH_2, was used for MMP-7 reactions; both were purchased from AnaSpec. Fluorescence was monitored using a Victor^3 V multilabel counter (PerkinElmer). For the gelatin digestion, a 50-μL mixture of gelatin (20 μg) and CLU (2 μg) was incubated with or without active MMP-9 (70 ng) for 2 hours at 37°C. The reaction products were run on a 12% denaturing SDS/PAGE gel for Coomassie Brilliant Blue staining.

Gelatin Zymography

Zymography to analyze the relative amounts of MMPs was performed according to the procedure described by Gordon et al.[35] Briefly, samples were loaded onto an 8% PAGE gel containing 0.1% gelatin (Sigma-Aldrich, St. Louis, MO). Gels were run for 45 minutes at 200 V. Gels were then incubated in 2.5% Triton-X for 1 hour at room temperature on a rocker. Gels were thoroughly washed with distilled water and incubated overnight in renaturing buffer (10 mmol/L $CaCl_2$, 50 mmol/L Tris-HCl pH 7.5) at 37°C. The next morning, gels were briefly washed and then stained (25 g Coomassie Brilliant Blue, 150 mL isopropanol, 50 mL acetic acid, 300 mL distilled deionized water) for 1 hour at room temperature on a rocker. Gels were then destained in distilled water until bands were clearly visible. Gels were then scanned for densitometric analysis using ImageJ software version 1.45 (NIH, Bethesda, MD).

MMP Enrichment Assay

To make various processed forms of MMPs, pro-MMPs were incubated with 1 mmol/L APMA in TSCB buffer at 37°C for 4 hours.[36] CLU-His (2.5 μg) was incubated with a mixture of 30 to 100 ng of unprocessed and processed pro-MMP-2 and pro-MMP-9 in TSCB buffer for 1 hour at 4°C, followed by additional incubation for 1.5 hours with anti-His tag antibody-conjugated agarose beads. The beads were washed three times with the binding buffer and then were dissolved in 1$\times$ SDS sample loading buffer. Relative amounts of bound MMPs were assessed from the gelatin zymography. To perform CLU binding domain analysis, CLU-His coupled to beads was preincubated with enough pro-MMP-9 to saturate all of the CLU molecules on the beads (confirmed by detection of a great quantity of unbound pro-MMP-9 after the preincubation); the beads were then purified and divided into two aliquots. These beads were incubated with and without 1 mmol/L APMA at room temperature for 1 hour in TSCB buffer on the rotator. The bead fraction was spun down and the supernatant was separated. The

beads were washed with TSCB and resuspended in 1× SDS sample loading buffer. Equivalent volumes of supernatant and bead fractions were subjected to gelatin zymography.

MMP Aggregation Assay

The pro-MMP-2 and MMP-9 (5 ng/tube), individually or in combination, were incubated in TSCB buffer containing 0.04% nonionic detergents (Brij-35, NP-40, or *n*-octyl glucopyranoside) in the presence of CLU-His (1.25 μg/tube) or PBS (vehicle control) at room temperature for 80 minutes, followed by microcentrifugation for 2 minutes at 21,000 × *g* to separate the soluble and insoluble fractions, which were resolved in zymography. Densities of the bands were quantified using ImageJ analysis. Relative solubility was calculated by dividing the density of soluble band with the combined densities of soluble and insoluble bands in each set of reactions.

RT-PCR of CLU and MMP-9 Induction

HCLE cells were stratified for 7 days according to the procedure described by Gipson et al[37] and were subjected to serum starvation for 6 hours before treatment with IL-1β, TGF-β2, and TNF-α (0 ng/mL each) and PMA (1 μmol/L) for 24 hours. cDNA synthesized from total RNA from each treatment was subjected to RT-PCR using primer sets of human MMP-9, CLU, and β-actin cDNA. Fold induction (versus untreated control) of gene expression were assessed by the ΔΔCt method using β-actin expression as a denominator. Primer sequences were as follows: β-actin, 5′-CATTGCCGACAGGATGCAGA-3′ and 5′-CTGATCCACATCTGCTGGAA-3′; MMP-9, 5′-TTGACAGCGACAAGAAGTGG-3′ and 5′-TCACGTCGTCCTTATGCAAG-3′; and CLU, 5′-ACCAGTACTATCTGCGGGTCA-3′ and 5′-CGTCACAGTGATGGGATCAG-3′.

Experimental DE Induction

The research protocol was approved by the University of Southern California, Department of Animal Resources and the Institutional Animal Care and Use Committee, and it satisfied the standards in the ARVO Statement for the Animals in Ophthalmic and Vision Research.

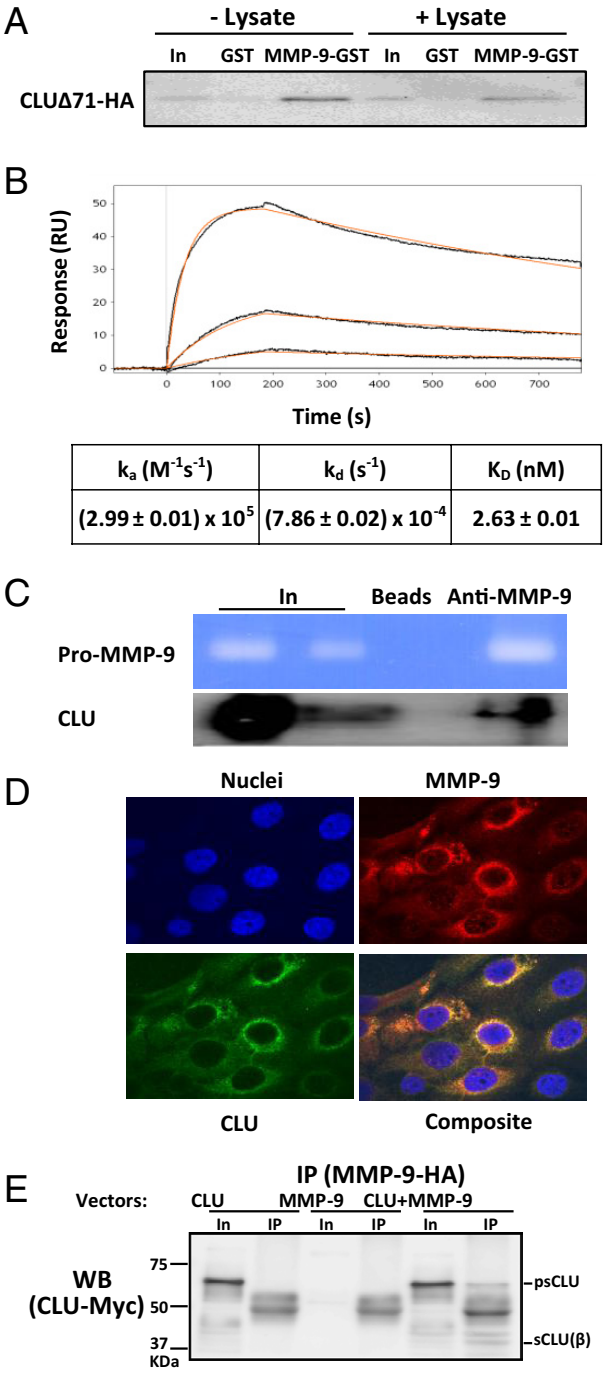
DE was experimentally induced in 8-week-old C57BL/6J female mice for 6 days according to the methods described by Corrales et al.[38] Briefly, scopolamine hydrobromide (Sigma-Aldrich) at 0.5 mg/0.2 mL in PBS was injected four times a day. An air draft was applied to the perforated cages for 18 hours per day in a vivarium with <40% humidity and with a temperature of approximately 80 ± 2°F. To measure tear production, we applied cotton threads to the ocular surface for 15 seconds and measured the amount of tear fluid uptake in millimeters.[9] For immunofluorescent staining, mice were euthanized and eyes were surgically excised, embedded into optimum cutting temperature compound, stored at −80°C, and used for frozen sectioning (10 μm thick). Rabbit Anti-CLU (1:50) (Abcam, San Francisco, CA) and goat anti-MMP-9 (1:100) (Santa Cruz Biotech, Santa Cruz, CA) were used as primary antibodies. Alexa Fluor 594 anti-rabbit IgG (1:2000) and Alexa Fluor 488 anti-goat IgG (1:2000) antibodies were used for immunofluorescent staining, followed by image analysis using a Zeiss LSM 510 multiphoton laser scanning confocal microscope (0.3 μm-interval Z-sectioning) and a 40× oil-immersion objective. Frozen sections treated with normal IgG or with the secondary antibodies were used as a negative control. Images from untreated and DE-induced samples were captured and processed with identical photomultiplier tube gain settings, allowing accurate comparison for every pair. The eyes were washed with 5 μL of PBS, 2.5 μL TRIzol (Invitrogen) was applied to the ocular surface, and epithelial cells were scraped from the ocular surface. From these cells we isolated total RNA and proteins according to the manufacturer's protocol, to perform quantitative RT-PCR and Western blotting. Protein extracts from individual eyes from each group were pooled to prepare protein extract in amounts sufficient for Western blotting. The same membrane was used for CLU and β-actin probing after stripping the membrane of proteins from the first blotting. Primers for mouse MMP-9 and GAPDH were as reported by Luo et al.[39] The primers for mouse CLU were 5′-CCAGACGTGGATTTCTTAAGAGAA-3′ and 5′-AGGATTGTTGGTTGAACAGTCC-3′. Relative expression of CLU and MMP-9 was calibrated by the expression of GAPDH.[39]

Results

CLU Interacts with MMP-9 in Vitro *and* in Vivo

To search for new MMP-9-interacting proteins, yeast two-hybrid screening was performed, for which we used mouse MMP-9 cDNA encoding the active form of MMP-9 as bait, resulting in the selection of 24 potential prey sequences including CLU cDNA (see Supplemental Table S1 at *http://ajp.amjpathol.org*). The yeast CLU clone that was isolated from the yeast assay contained CLU cDNA beginning at position 212 from the ATG site and thus lacked N-terminal 71 amino acids (CLUΔ71). To verify their interaction, we performed a GST pull-down assay using MMP-9-GST and CLU Δ71-HA fusion proteins, both expressed in a bacterial system (Figure 1A). MMP-9-GST-coated beads pulled down CLUΔ71-HA in this assay, even in the presence of bacterial lysate as a nonspecific competitor. Next, we purchased purified recombinant sCLU-His proteins that were expressed in mouse cells, to perform surface plasmon resonance assay, which indicated a very strong binding affinity between sCLU and the active domain of MMP-9, with an affinity constant (equilibrium dissociation constant K_D) of 2.63 nmol/L (Figure 1B). Next, because there are various known MMP-9 substrates, we tested whether CLU might be a novel enzymatic substrate of MMP-9; however, we found that CLU is not a substrate of active MMP-9 (data not shown).

Because these results indicated *in vitro* interaction of MMP-9 and CLU, we then asked whether the interaction could occur in human epithelial cells. Because CLU proteins are present mainly as sCLU, we first tested whether



k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)
(2.99 ± 0.01) x 10^5	(7.86 ± 0.02) x 10^{-4}	2.63 ± 0.01

Figure 1. CLU binds to MMP-9 *in vitro* and *in vivo*. **A:** GST pull-down assay was performed using MMP-9-GST and CLUΔ71-HA in the presence (+) or absence (−) of bacterial lysate as a nonspecific competitor, followed by Western blotting with anti-HA antibody. **B:** Surface plasmon resonance assay was performed to measure specific binding of 100, 25, and 12.5 nM sCLU to immobilized catalytic domain of MMP-9. Association rate constant (k_a), dissociation rate constant (k_d), and equilibrium dissociation constant (K_D) were measured by fitting the kinetic model (orange curves) to the binding data. M, mol/L; nM, nmol/L. **C:** Conditioned medium from HCLE cell culture was subjected to IP with anti-MMP-9 antibody, followed by zymography and Western blotting to detect pro-MMP-9 and sCLU, respectively. In, whole conditioned medium as input. **D:** Colocalization of MMP-9 and CLU using specific antibodies. Nuclei were stained with DAPI. The images were acquired using a spinning disk confocal microscope with Z-sectioning at 0.5-μm intervals. Original magnification, ×400. **E:** Whole-cell extracts from the transfected HEK293 cells with the plasmid vectors expressing MMP-9-HA and CLU-Myc were subjected to IP with anti-HA antibody, followed by Western blotting (WB) with anti-Myc antibody. In, whole-cell lysate as input; IP, immunoprecipitated sample.

the interaction also occurs naturally in the extracellular environment. A conditioned medium from HCLE cell culture was subjected to IP assay with anti-MMP-9 antibody (Figure 1C). Protein sample from the IP contained both pro-MMP-9 and sCLU, suggesting their extracellular interaction. Next, we tested their intracellular interaction under confocal microscopy of HCLE cells immunostained with MMP-9-specific and CLU-specific antibodies (Figure 1D). Both proteins were distributed throughout cytoplasm, more in the perinuclear region, and the composite image indicated that they were mostly colocalized.

We then asked whether there is a physical interaction between the two proteins, and what kinds of CLU isoforms bind to pro-MMP-9 inside cells. Three different CLU isoforms are present in the cytoplasm: unprocessed nonglycosylated (~50 kDa) and glycosylated single peptide (psCLU, ~60 kDa), as well as processed glycosylated dimeric sCLU (~ 80 kDa).[13] We first attempted IP assay using the HCLE whole-cell extract, but could not detect any MMP-9 or CLU protein bands; perhaps the amounts of these proteins inside the cells were too low to support IP assay. In an alternative approach, HEK293 cells were transfected with MMP-9-HA and CLU-Myc expressing vectors, individually and in combination (Figure 1E). The results showed two protein bands, corresponding to the sizes of psCLU (~60 kDa) and sCLU β-subunit (~40 kDa), only in the IP lane of cotransfection. We also noticed a band (~50 kDa) in the same lane that was slightly stronger, compared with the other two IP lanes, which may correspond to a nonglycosylated single peptide of CLU. Unfortunately, because this band coincides with the position of the IgG heavy chain of IP antibodies nonspecifically cross-reacting with Western blotting antibodies, we could not conclusively identify the band. The combined results suggested that sCLU and psCLU interact with pro-MMP-9 and active MMP-9 both inside and outside the cells.

CLU Binds to Pro-MMP-9 with a Higher Affinity than Processed MMP-9

Because the results so far indicated that sCLU binds to both pro- and active MMP-9, we next compared the relative binding affinities for these MMP-9 isoforms. To do this, we performed an enrichment assay by incubating recombinant sCLU-His with a mixture of pro-MMP-9 and active MMP-9, followed by a His-tag pull-down step (Figure 2A). The pro-MMP-9 was prepared by treating it with APMA. The MMP-9 isoforms present in the input mixture and the final precipitate were visualized with gelatin zymography. In the input mixture, the processed MMP-9 was more abundant than pro-MMP-9. In contrast, there was greater enrichment of pro-MMP-9 in the anti-His beads sample, and comparison of relative densities of these bands using ImageJ analysis indicated approximately 10 times greater sCLU enrichment of pro-MMP-9 than of processed MMP-9.

Because we found that MMP-2 also binds to sCLU under the same binding assay conditions (data not shown), we next performed a similar experiment with MMP-2 isoforms included in the reaction (Figure 2B). The results indicated, notably, that sCLU showed no preference toward pro-

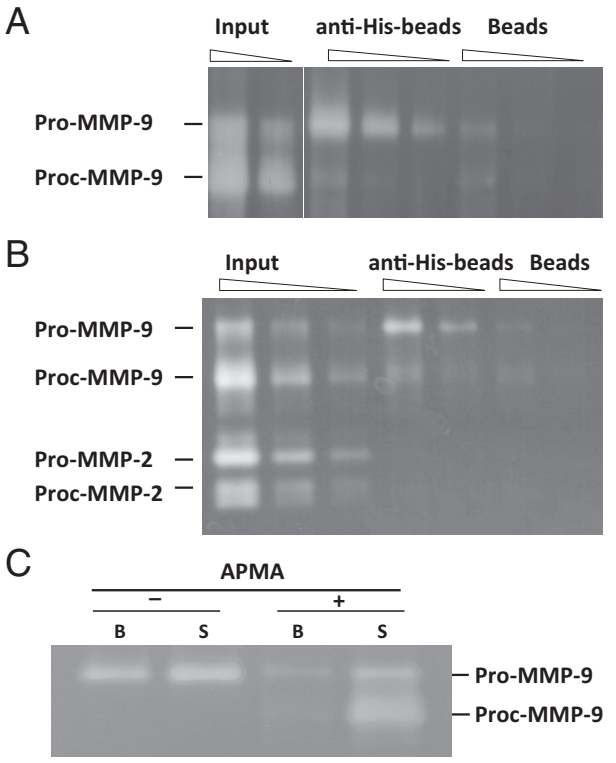


Figure 2. sCLU has a higher affinity for pro-MMP-9, mediated by the N-terminal propeptide domain. **A:** A mixture of pro-MMP-9 and APMA-processed MMP-9 (proc-MMP-9) was incubated with His-tagged sCLU, followed by pulling down with agarose beads coupled with His-antibody (anti-His-beads) or beads alone (Beads), and visualized with gelatin zymography. Serial dilutions of each sample were loaded to facilitate comparison among samples. **B:** The same experiment was performed as for **A**, but also including pro-MMP-2. **C:** ProMMP-9 that was prebound to CLU coupled to agarose beads was incubated in the presence (+) or absence (−) of APMA, before separation of bead (B lanes) and supernatant (S lanes) fractions, which were then resolved with zymography.

MMP-2, which belongs to the same MMP subfamily as MMP-9, indicating that pro-MMP-9 has a unique high affinity for sCLU. To better understand the molecular interaction between pro-MMP-9 and sCLU (Figure 2C), we prepared a complex of pro-MMP-9 and sCLU-His, in which sCLU-His was coupled to agarose beads, and then treated the complex with and without APMA, followed by separating by centrifugation into beads and supernatant fractions. We reasoned that if sCLU binds preferentially to the propeptide domain, the processed MMP-9 would be released from the beads and partitioned into the fraction S; otherwise, the processed enzyme would be in the bead fraction. Gelatin zymography of the supernatant and bead samples indicated that the majority of the processed MMP-9 was detected in the supernatant fraction of APMA-treated reaction, suggesting the direct involvement of the propeptide domain in their interaction, by directly binding to sCLU or mediating/stabilizing.

CLU Prevents Stress-Induced MMP-9 Aggregation

Extracellular proteins are vulnerable to stress-induced structural alteration, leading to their aggregation, which can have harmful effects *in vivo*. We investigated whether MMP-9 aggregation could be caused by hydrophobic stress, generated by the treatment with nonionic detergents of different detergent properties (ie, Brij-35, NP-40, and *n*-octyl glucopyranoside), and whether aggregation could be prevented by adding sCLU in the reaction. We incubated pro-MMP-9 and pro-MMP-2 in the enzyme reaction buffer containing each of these detergents at 0.04% in the presence of sCLU or PBS as control (Figure 3). The soluble and insoluble fractions were separated by centrifugation and visualized with gelatin zymography. Brij-35 and NP-40 had little effect on the solubility of pro-MMP-2 and pro-MMP-9. In contrast, the solubility of these two MMPs was significantly reduced to <40% in the presence of *n*-octyl glucopyranoside, and these reductions were prevented by sCLU cotreatment.

CLU Inhibits the Enzymatic Activity and Function of MMP-9

To assess the functional aspects of the interaction between MMP-9 and CLU, we performed *in vitro* inhibition assays for various MMPs, including MMP-9 (Figure 4A). We performed FRET assay using catalytic domains of MMP-3 (stromelysin 1) and MMP-9 (gelatinase B) and APMA-processed MMP-2 (gelatinase A) and MMP-7 (matrilysin 1), as well as fluorescence-quenched peptide substrates. MMP-7 naturally lacks the C-terminal hemopexin domain. SB-3CT is a potent chemical inhibitor selective to MMP-9 and MMP-2, with inhibition constants in the submicromolar range.[40] sCLU strongly inhibited the activity of MMP-9 catalytic domain by 79% in a dose-dependent manner; it also inhibited the activity of APMA-processed MMP-9 dose-dependently and to a sim-

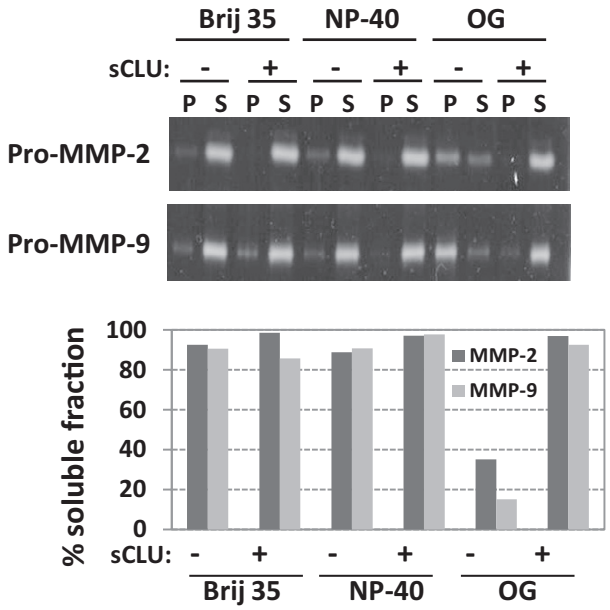


Figure 3. sCLU prevents detergent stress-induced MMP-2 and MMP-9 aggregation. Pro-MMP-2 or pro-MMP-9 was incubated in the buffer containing 0.04% of Brij-35, NP-40, or *n*-octyl glucopyranoside (OG) in the presence (+) or absence (−) of sCLU, and then the soluble (S) and insoluble (P) fractions, obtained by centrifugation, were resolved by gelatin zymography. Relative soluble fraction was calculated by ImageJ analysis.

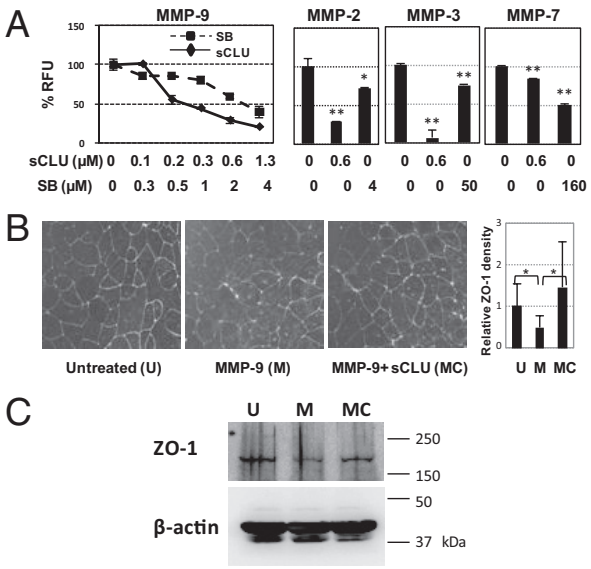


Figure 4. sCLU prevents activity and function of MMP-9. **A:** Enzymatic reactions of MMP-9, MMP-2, MMP-3, and MMP-7 were performed in the presence of sCLU or SB-3CT (SB). Relative fluorescence units (RFU) were obtained by setting PBS as 100%. Each reaction was performed in triplicate. Error bars indicate SE. $^{*}P < 0.01$; $^{**}P = 0.0001$ versus untreated control, Student's *t*-test. $n = 3$. **B:** Confocal fluorescence image analysis was performed for ZO-1 protein after exposing confluent MCF-7 cell culture to active MMP-9 alone (M) or in combination with sCLU (MC). The relative densities of ZO-1 were quantified over untreated (U) control, using Velocity software. $^{*}P < 0.01$. $n = 12$. **C:** Western blotting with protein extracts from the cells treated with MMP-9 and CLU was performed for ZO-1 detection (β-actin, control).

ilar extent (data not shown). In the present set of experiments, MMP-2 was also inhibited by 65%, MMP-3 by 93%, and MMP-7 by 17%, indicating that CLU can inhibit *in vitro* enzymatic activity not only of MMP-9 but also of various other MMPs.

We then examined the significance of the sCLU-mediated inhibition of MMP-9 activity at the cellular level. Recently, sCLU was shown to protect TJ structure from degradation caused by oxidative stress to endothelial cell culture.[41] Active MMP-9 is known to degrade the TJ structure formed in the apical surface of epithelia.[9] Therefore, by immunofluorescent staining of ZO-1, a TJ component protein, we tested whether CLU could affect the MMP-9-mediated destruction of TJ that formed in a confluent culture of human breast epithelial cancer cells (Figure 4B). Untreated control demonstrated typical continuous chicken-wire pattern of TJ. In contrast, the treatment of active MMP-9 caused many breakages in the TJ continuum and significantly reduced ZO-1 density by 51%, compared with control. However, cotreatment with sCLU prevented MMP-9 mediated ZO-1 degradation. We also performed Western blotting to confirm the above immunofluorescent staining results, showing that ZO-1 protein was decreased with MMP-9 treatment, which was abrogated by cotreatment with sCLU (Figure 4C).

CLU Expression Is Down-Regulated by Proinflammatory Factors Such As Cytokines and Desiccating Stress

The inflammatory process involves increased activities of various proinflammatory cytokines, as well as of MMP-9.[42] We wanted to see how CLU expression is regulated under the inflammatory conditions in which MMP-9 is up-regulated. We first examined the transcriptional change in MMP-9 and CLU in HCLE cells exposed to IL-1β, TGF-β2, phorbol myristate acetate (PMA), or TNF-α, all of which are known to increase the production of MMP-9 (Figure 5A). In all four cases, the exposure increased MMP-9 expression and decreased CLU. Furthermore, we observed an intriguing correlation, in that the more MMP-9 was produced, the less CLU was expressed.

Because up-regulated MMP-9 is actively involved in inflammatory conditions such as DE,[9] we wanted to see how CLU and MMP-9 interact in an *in vivo* animal model. C57BL/6J mice have been broadly used as a model animal for human DE study. Desiccating stress is known to induce DE.[38,43] We housed mice in a warm, low-humidity vivarium for 6 days, and we regularly injected scopolamine hydrobromide to reduce tear production. First, we asked where MMP-9 and CLU are distributed in

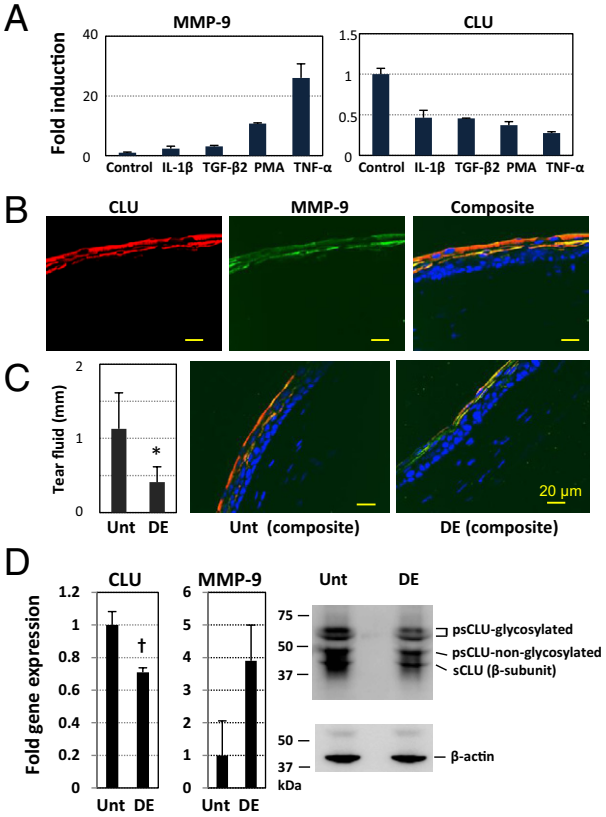


Figure 5. CLU is down-regulated in the corneal epithelial cells on cytokines and desiccating stress treatments. **A:** HCLE cells were treated by IL-1β, TGF-β2, PMA, and TNF-α. The expression of MMP-9 and of CLU was measured by RT-PCR, and fold induction was normalized using β-actin transcription. **B:** Immunolocalization of CLU (red) and MMP-9 (green) by fluorescence immunostaining with anti-CLU and anti-MMP-9 antibodies was performed using the frozen section of the eye from the untreated normal C67BL/6J mice. One of four representative eyes is shown. Nuclei (DAPI, blue) are shown only in composite images. **C:** DE was induced in mice by desiccating stress for 6 days, and the tear volumes were measured in untreated and treated mice. $^{*}P < 0.01$. $n = 10$ eyes. Distributions of CLU and MMP-9 in untreated (Unt) and DE-induced (DE) mouse eyes are shown. One of four representative eyes is shown. **D:** Quantitative RT-PCR and Western blotting were performed to confirm the changes in CLU and MMP-9 expressions in the ocular surface epithelium in untreated and DE mice. $^{\dagger}P = 0.01$. $n = 8$ eyes.

the corneal tissue sections from healthy mice (Figure 5B). MMP-9 is known to express minimally in the healthy ocular surface, so that it is rarely detected biochemically in the tear fluid,[9] but the immunofluorescent staining method allows its detection in the healthy cornea. In the healthy cornea, both MMP-9 and CLU were detected only in the anterior half of the corneal epithelium. Of note, spatial distributions of both CLU and MMP-9 proteins seem very similar, but CLU is distributed more anteriorly and MMP-9 more posteriorly. Strong colocalization in the ocular surface (Figure 5B) is consistent with our biochemical and cell culture results. After DE induction in the mice (Figure 5C), we first confirmed that the mice with DE had reduced tear volume, compared with the untreated mice. We next compared the expression and distribution of CLU and MMP-9 in the cornea in both mice groups. In acquiring the fluorescence images from these groups, we captured and processed images with identical photomultiplier tube gain settings for accurate comparison within pairs.[38] In the ocular surface from untreated control mice, CLU was prominent; however, its expression was markedly diminished after DE induction, whereas MMP-9 was increased and predominant along the apical layer of the epithelium. We also performed quantitative RT-PCR and Western blotting, to evaluate expression changes of CLU and MMP-9 at the transcriptional and post-transcriptional levels after DE induction, and confirmed the conclusions from the florescence image analysis (Figure 5D). Increase in MMP-9 expression has been reported previously for similar desiccating stress conditions.[38,39] Western blotting revealed various species of CLU, reflecting post-translational CLU processing, such as nonglycosylated nascent psCLU and intermediately and fully glycosylated psCLU, as well as mature sCLU molecules present in the ocular surface epithelium. Western blotting further confirmed the reduction of the amount of CLU proteins after DE induction.

Discussion

The expression and activation of MMP-9 is tightly controlled under normal conditions, but its regulation is altered in many inflammatory diseases. Dysregulated MMP-9 activity is associated with the destruction of TJs, a key constituent of epithelial barrier integrity.[9,44] MMP-9 is also able to proteolytically activate latent precursors of proinflammatory cytokines, such as interleukin-1β (IL-1β), transforming growth factor-β (TGF-β), and membrane-bound tumor necrosis factor-α (TNF-α).[45–47] Several proteins have known regulatory functions involving MMP-9. Tissue inhibitor of MMPs (TIMPs) interact with and inhibit the active site of MMP-9, and the C-terminal region of TIMP-1 is known to bind to the hemopexin domain of both pro-MMP-9 and processed MMP-9.[48,49] Extracellular matrix protein 1 (ECM1) also inhibits MMP-9 activity, through their interaction.[50] DNA repair protein Ku was shown to interact with the hemopexin domain of MMP-9 at the cell surface and to be involved in cellular invasion of collagen type IV matrices.[51] Neutrophil gelatinase-associated lipocalin (NGAL) was shown to complex with MMP-9 and protect it from degradation.[52]

Our observation that both bacterially expressed single-chain CLU peptide and psCLU bind to MMP-9 indicates that post-translational modifications of CLU, such as glycosylation and heterodimerization, may not be necessary for the CLU-MMP-9 interaction. Our present findings distinguish sCLU from TIMPs, in that sCLU has a far higher binding affinity (K_D = 2.63 nmol/L) for the catalytic domain of MMP-9 than do TIMP-1 and TIMP-2, which have affinities of K_D = 24 and 58 nmol/L, respectively, for the same domain; furthermore, sCLU prefers the N-terminal propeptide domain, whereas TIMPs have higher affinities for the C-terminal hemopexin domain over other domains of MMP-9.[53]

CLU is constitutively present at most fluid-tissue interfaces, and was proposed to protect those epithelial barrier from the surface-active components of the extracellular environment almost two decades ago.[54] The present study demonstrates previously unrecognized unique interactions of CLU and MMP-9 proteins, in that they strongly interact with each other both inside and outside cells, even before their processing and secretion. Inhibition of MMP-9 activity by sCLU results from the direct interaction of CLU with the catalytic domain of MMP-9. We also observed that sCLU inhibits the enzymatic activities of MMP-3 and MMP-7, which can activate pro-MMP-9.[55,56] MMP-3 was found exclusively in the eyes of patients with DE, and MMP-3 activity was implicated in the pro-MMP-9 activation in the tear fluid.[56] Our results thus suggest that sCLU can abrogate MMP-9 functions, directly and indirectly, by inhibiting the activities of both active MMP-9 and pro-MMP-9 activating enzymes.

It is also noteworthy that sCLU has far higher affinity for pro-MMP-9, but not pro-MMP-2, than the processed MMP-9, which is mediated by the N-terminal propeptide domain, implying a functional role of CLU in MMP-9 regulation. In this sense, one possibility is that sCLU bound to pro-MMP-9 via the propeptide domain may interfere with the activating process of pro-MMP-9 by, for example, sterically hindering the access of the cleavage site to activating enzymes (eg, plasmin, MMP-3, and MMP-7).

Although CLU is secreted through the endoplasmic reticulum-Golgi body pathway, it is also present as psCLU in cytosol compartments.[24,57] It has been suggested that the role of CLU is determined by its final location.[57] Increased CLU expression was reported to

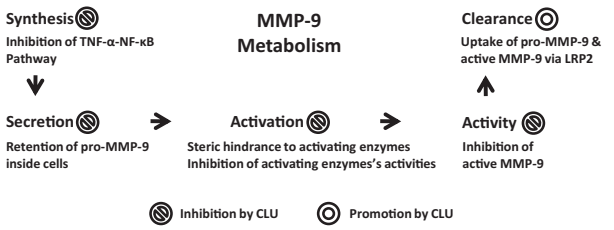


Figure 6. A model for the regulatory roles of CLU in MMP-9 metabolism inside and outside cells. psCLU and sCLU proteins interact with pro-MMP-9 and activated MMP-9 at multiple stages of MMP-9 metabolism inside and outside cells. CLU proteins may inhibit transcription, secretion, activation (processing), and/or activity of MMP-9, while also promoting the clearance of MMP-9 from the extracellular compartments.

repress TNF-α-induced MMP-9 expression in vascular smooth muscle cells.[28] psCLU prevents I-κB degradation, leading to inhibition of the NF-κB nuclear localization.[26] psCLU binds to Bax, a proapoptotic protein, and serves as its cytosol retention factor, functioning as a antiapoptotic component in cells under stress.[24,58] It is also shown that CLU can retrotranslocate into cytosol under certain cellular stress conditions.[57] MMP-9 is found in intracellular, pericellular, and extracellular compartments and even in the nucleus.[59] Given that CLU is a stress-induced, cytoprotective protein,[20] it would be worthwhile to determine whether, under certain conditions, increased CLU expression can affect some aspects of MMP-9 metabolism (eg, subcellular localization and secretion).

Secreted proteins are vulnerable to stress-induced denaturation and aggregation, which may be toxic to the cells and are to be eventually removed from their target sites.[60] Clearance of extracellular MMPs by cellular internalization has been suggested and observed for MMP-2, MMP-9, and MMP-13. MMP-2 forms a complex with thrombospondin-2 (TSP-2),[61] and MMP-9 does so with TIMP-1 *in vitro*,[18,62] to be internalized into the cells by recognizing a common cell surface receptor, LRP-2/megalin/gp330, on the cell surface. MMP-13 also involves a specific MMP-13 receptor and LRP receptor for internalization.[63] In this sense, it is noteworthy that CLU is also known to be internalized via the LRP-2 receptor.[19,64] Our present observation of the promotion of MMP-9 solubility by sCLU under detergent stress conditions leads us to speculate that CLU-MMP complex formation may contribute to MMP-9 solubility, which might otherwise be compromised by denaturing or aggregating stresses, and it may facilitate cellular MMP-9 internalization via LRP-2 receptors in a more regulated way, ending up with its degradation inside the cells. sCLU may work as an extracellular vehicle to deliver pro-MMP-9 to LRP-2, playing its stress-associated cytoprotective roles. In support of this idea, sCLU was observed to promote clearance of cellular debris via the cell surface receptor LRP-2.[19,60] LRP-2 is typically found on the apical surface of epithelium in several organs.[65,66] Thus, we envision that CLU may promote the internalization of activated and pro-MMP-9, leading to their clearance and degradation inside the epithelial cells.

CLU has anti-inflammatory functions *in vivo*. Low levels of CLU are correlated with the occurrence or severity of various inflammations in patients and in mouse models. Some inflammatory diseases (eg, rheumatoid arthritis and systemic erythematosus lupus) that present with DE as a pathological feature become more severe, in a mouse model, when CLU protein is deficient.[67,68] Increased MMP-9 activity was associated with keratinization of human keratinocytes.[69,70] CLU expression is markedly decreased in pathological, keratinized corneal epithelium from patients.[71] In a mouse injury model using C57BL/6J mice, CLU was recently found to play a critical role in promoting pancreas regeneration after injury and *in vitro* β-cell regeneration.[33] In human DE disease, elevated MMP-9 activity has been recognized as a main cause for derangement of corneal epithelial barrier function in DE.[9] In a mouse model of DE disease, MMP-9 expression was significantly enhanced on DE induction.[38] Our data indicate that CLU is localized at the superficial cell layer of the untreated healthy mouse corneal epithelium, where it is being bound to MMP-9; CLU thus appears to be confining MMP-9 molecules inside the mouse corneal epithelium. MMP-9 is up-regulated on DE induction and CLU expression is dramatically diminished, suggesting that down-regulation of CLU may be implicated in the onset of DE disease. CLU was recently reported to induce *in vitro* MMP-9 expression in monocytes/macrophages.[72] Given that the role of CLU appears to vary depending on cell types, stimulus, and/or the tissue microenvironment,[72] it would be worthwhile to investigate the *in vivo* function of CLU in the context of ocular surface homeostasis and DE, which also involves various immune cells as well as epithelial cells.

The complexity of inflammatory pathology in the ocular surface with DE presents a challenging problem, one with few therapeutic options. A key to successful treatment may be the identification and targeting of a critical, functional player in the inflammatory cycle constituted by cytokines, chemokines, mediators, and MMPs, all of which are produced by ocular surface epithelial cells and recruited immune cells.[42] From this point of view, MMP-9 could be a prime therapeutic target in that, in addition to its destructive enzymatic activity, it activates latent pro-inflammatory cytokines, which in turn up-regulate MMP-9 production and thereby critically contribute to sustaining of such morbid cycles in DE disease. Further elucidation of the roles of CLU proteins in the ocular surface using wild-type and CLU-deficient mice is warranted, and such studies may lead to prophylactic or therapeutic benefit in inflammatory diseases.

In summary, our results suggest that CLU modulates MMP-9 functions at multiple stages of its metabolism, including secretion, activation, enzymatic activity, and clearance (Figure 6), providing novel insight into a mechanism by which a number of intracellular and extracellular CLU-interacting proteins may be similarly regulated by CLU in the body. We also propose that the constitutive CLU expression in epithelia exposed to continuous external stress may serve as a previously unrecognized homeostasis mechanism, which works both inside and outside of the epithelial cells.

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