JLB

Article

Clusterin induces matrix metalloproteinase-9 expression via ERK1/2 and PI3K/Akt/NF-κB pathways in monocytes/macrophages

Young-Jun Shim, Byeong-Ho Kang,* Hye-Sook Jeon,* In-Sun Park,† Ki-Up Lee,‡ In-Kyu Lee,§ Gil-Hong Park,|| Kyung-Mi Lee,|| Pepper Schedin,¶ and Bon-Hong Min*,1*

*Department of Pharmacology and ||Biochemistry and BK21 Program in Biomedical Sciences, College of Medicine, Korea University, Seoul, Korea; †Department of Anatomy, College of Medicine, Inha University, Korea; ‡Department of Internal Medicine, University of Ulsan, College of Medicine, Korea; §Department of Internal Medicine and Biochemistry and Cell Biology, Kyungpook National University School of Medicine, Korea; and ¶Department of Medicine, Division of Medical Oncology, University of Colorado-Denver, Colorado, USA

RECEIVED MARCH 1, 2011; REVISED JUNE 13, 2011; ACCEPTED JUNE 22, 2011. DOI: 10.1189/jlb.0311110

ABSTRACT

Most solid tumor tissues possess a significant population of macrophages, which are known to be closely linked with tumor progression and metastasis. Clusterin has been reported to be overexpressed in various tumors and to have a tumor-promoting role. As clusterin induction and macrophage infiltration occur concurrently at the tumor site, it raises a possibility that clusterin may regulate the function of macrophages via facilitating ECM remodeling. Here, we demonstrate for the first time the expression of MMP-9 by clusterin in human primary monocytes as well as human and murine macrophage cell lines, THP-1, and Raw264.7. MMP-9 expression was accompanied by increased enzymatic activity, as revealed by gelatin zymography. The MMP-9 activity promoted by clusterin was found to be dependent on the activation of ERK1/2 and PI3K/Akt but not p38 or JNK pathways. Inhibition of PI3K activity did not affect the activation of ERK1/2 and vice versa, indicating that the two pathways were independently operated to stimulate MMP-9 activity. Moreover, clusterin facilitated nuclear translocation of NF-κB p65 along with IκB-α degradation and phosphorylation, which was critical for MMP-9 expression. As NF-κB is a central regulator of inflammation, clusterin may provide a molecular link between inflammation and cancer via up-regulating NF-κB and MMP-9. Collectively, these data highlight a novel role of clusterin as a stimulator for MMP-9 expression in macrophages, which may contribute to the tissue reorganization by serving as a modulator for ECM degradation. *J. Leukoc. Biol.* 90: 761–769; 2011.

Abbreviations: 3D=three-dimensional, LRP-2=LDLR-related protein-2, MMP-9=matrix metalloproteinase-9, N-IgG=normal IgG, NP-40=Nonidet P-40, rCLU=recombinant clusterin, siRNA=small interfering RNA, Sp1=specificity protein 1

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

Clusterin is a heterodimeric, disulfide-linked protein of 75–80 kDa, expressed ubiquitously in a wide variety of tissues and found in all biological fluids [1]. Since its initial discovery as a secretory glycoprotein with cell-aggregating activity in vitro [2], clusterin has been implicated in several diverse physiological processes, including sperm maturation (sulfated glycoprotein-2), lipid transport (apolipoprotein J), complement regulation (complement lysis inhibitor), cell interactions (clusterin), and morphologic transformation or apoptosis (testosterone-repressed prostate message-2) [1, 3]. Clusterin functions primarily through two isoforms: a glycosylated, secreted form and a nonglycosylated, intracellular form [4, 5]. The secretory, glycosylated form of clusterin has been studied extensively and most likely functions as an extracellular chaperone [6]; however, its physiological relevance still remains to be elucidated.

Up-regulation of clusterin mRNA and protein has been reported in various tissues undergoing significant patho-physiological modifications, such as inflammation, cancer, and tissue regeneration after injury [5, 7–11]. In particular, up-regulation of clusterin is seen with tumor progression, correlating with high grade and metastasis in gastric cancer [12], prostate cancer [13], breast carcinoma [14], and lung carcinoma [15]. Moreover, the fact that ectopic overexpression of clusterin in human breast cancer MCF-7 cells increased invasion to the lung suggests that this secretory glycoprotein might play an important role in the metastatic spread from the primary tumor site [16]. Clusterin is also increased dramatically in the rodent mammary gland following weaning [17], in the regressing ventral prostate following finasteride treatment [18], in the recovery from ischemic renal injury [19], and in ventricular myocytes during myosin-induced myocarditis [11], presum-

1. Correspondence: Department of Pharmacology and BK21 Program in Biomedical Sciences, College of Medicine, Korea University, 126-1, Anam-Dong 5-Ga, Seongbuk-Gu, Seoul 136-705, Korea. E-mail: bhmin@korea.ac.kr

ably as a protective mechanism from tissue damage. Similarly, we observed the concurrent up-regulation of clusterin at the time of tissue regeneration after pancreatic injuries [10, 20]. Up-regulation of clusterin with cancer metastasis, inflammation, and tissue regeneration processes suggests that clusterin may function as a modulator of tissue remodeling under conditions where degradation of ECM and cell migration actively occur.

MMPs, a family of zinc-dependent enzymes capable of degrading ECM components, have been implicated in the progression and metastases of tumors and tissue remodeling [21, 22]. Among the members of MMPs, MMP-9 was shown to be increased in malignant cancers and play an important role in tumor invasion [23]. Interestingly, inflammatory and stromal cells within the tumor mass, rather than tumor cells themselves, were shown to express MMPs [22, 23]. As clusterin induction and macrophage infiltration occur concurrently at the site of the above-stated pathologic conditions [5, 22, 24], it raises the question of whether clusterin secretion by macrophages modulates the microenvironment to one favorable to tissue organization and cancer metastasis. Indeed, data presented here demonstrate that clusterin increases MMP-9 expression at the mRNA and protein levels in monocytes and macrophages and that this induction is dependent on ERK1/2 and PI3K/Akt/NF-κB pathways. These data are consistent with clusterin-regulating ECM remodeling, required for tumor cell invasion, and may explain the high correlation index between the level of clusterin and cancer prognosis observed in human cancer patients.

MATERIALS AND METHODS

Cell culture

Murine macrophage cell line Raw264.7 was obtained from Korean Cell Line Bank (Seoul, Korea), and THP-1 human monocytic cell line was graciously obtained from Dr. Jesang Ko (Korea University, Seoul, Korea). Cells were grown in DMEM (Gibco-BRL, Manassas, VA, USA), supplemented with heat-inactivated 10% (v/v) FBS (Hyclone, South Logan, UT, USA) and 1% antibiotics-antimycotics (Gibco-BRL) in a 5% CO_2 humidified incubator at 37°C. Peripheral blood monocytes were isolated from healthy donors by Ficoll-Hypaque density gradient using the Vacutainer CPT cell preparation tube (Becton Dickinson, Franklin Lakes, NJ, USA) with sodium heparin (REF 362753), according to the manufacturer's protocol. Briefly, blood collected into CPT was centrifuged for 15 min at 1800 *g* at room temperature, and the mononuclear cell fraction was washed twice with 7 vol PBS. After centrifugation, the cells were cultured in RPMI 1640 containing 10% FBS. Nonadherent cells were removed after 3 h incubation at 37°C, and adherent monocytes were maintained with new media until the day of the experiment. Thioglycollate-elicited peritoneal macrophages were obtained from specific pathogen-free male C57BL/6 mice (Orient Bio, Sungnam, Korea) at 7 weeks of age by injection of 2.5 ml sterile 3% thioglycollate solution (BD Biosciences, San Jose, CA, USA) for 5 days before lavage with 10 ml ice-cold PBS. The peritoneal exudate cells were centrifuged at 1500 rpm and washed once with RPMI 1640 (without phenol red), supplemented with 10% endotoxin-free, heat-inactivated FBS (Hyclone), 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were resuspended in RPMI 1640 at a density of 2×10^6 cells/ml. Viability was >95%, as determined by trypan blue dye exclusion, and the cells were plated and incubated for 3 h in a 5% CO_2 humidified incubator at 37°C to allow macrophage adherence. The plates were then washed once with warm RPMI 1640 to remove nonadherent cells.

Reagents and antibodies

Cell culture reagents were purchased from Life Technologies (Grand Island, NY, USA). Bacterial LPS (purified from *Escherichia coli*, serotype 0111: B4) and gelatin (from porcine skin) were from Sigma-Aldrich (St. Louis, MO, USA). The following inhibitors, PD98059 (MEK inhibitor), LY294002 (PI3K inhibitor), and SP600125 (JNK inhibitor), were products of Calbiochem (San Diego, CA, USA). Antibodies for clusterin (M-18; sc-6420), IκB-α and phospho-IκB-α (C-21; sc-371), NF-κB p65 (sc-109), lamin B (C-20; sc-6216), GAPDH (V-18; sc-20357), and protein A/G PLUS-agarose beads (sc-2003) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-specific or the total form of antibodies against ERK1/2, p38, JNK1/2, Akt (ser473), and MMP-9 (G657) was purchased from Cell Signaling Technology (Beverly, MA, USA). Anticlusterin mAb (1G8) for a clusterin-blocking experiment is generated by our laboratory. Intracellular and secretory forms of rCLU were purchased from AdipoGen (Incheon, Korea).

Purification of clusterin

Clusterin was purified as described previously [25] from fresh, normal human plasma, which was precipitated using 12–23% polyethylene glycol (MW 3350; Sigma-Aldrich) overnight at 4°C. This precipitate was dissolved and subjected to DEAE-Sepharose and heparin-Sepharose column chromatography (GE Healthcare Life Sciences, Piscataway, NJ, USA). Clusterin-positive fractions were then applied to a clusterin mAb (1G8) affinity chromatography column. The anticlusterin mAb (1G8) was generated using human full-length rCLU expressed in *E. coli* as an antigen and covalently conjugated to cyanogen bromide-activated Sepharose 4B (Sigma-Aldrich). Eluted proteins were dialyzed against PBS and stored at −80°C prior to use. The endotoxin level of purified clusterin was below the detection level, as measured by the *Limulus* amoebocyte lysate kinetic turbidimetric assay (Endosafe, Charles River Laboratory, Korea).

Gelatin zymography

Raw264.7 cells (5×10^5 cells) were plated and cultured in DMEM containing 10% FBS overnight. After removing the medium, clusterin or LPS was treated in fresh, serum-free medium and cultured for 24 h. For the samples treated with specific kinase inhibitors (PD98059, LY294002, SP600125), preincubation was performed for 30 min prior to addition of clusterin. Spent media (500 μl) were collected and concentrated using an Amicon Ultra centrifugal filter device with 10 kDa cutoff (Millipore, Bedford, MA, USA). Equal volumes of conditioned media were separated under nonreducing conditions on 7.5% SDS-PAGE gel containing 3 mg/ml porcine gelatin (Sigma-Aldrich). After electrophoresis, gels were washed twice for 30 min each in washing buffer (2.5% Triton X-100) at room temperature. Gelatinolytic reactions were induced by incubating gels in substrate buffer (50 mM Tris, pH 8.0, 5 mM $CaCl_2$) at 37°C for 1 or 2 days while rocking. Gels were stained with 0.25% Coomassie blue and destained in 7% acetic acid and 30% methanol.

Western blot analysis

Raw264.7 cells were rinsed once with cold PBS and lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitor (Roche, Mannheim, Germany). After incubation for 15 min on ice, cell lysates were centrifuged at 13,000 rpm for 15 min. The equal amount of protein samples was resolved by 10% SDS-PAGE under reduced conditions and transferred to a nitrocellulose membrane (Millipore), which was blocked with TBST (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1% Tween-20) containing 5% skim milk for 30 min and then incubated with appropriate primary antibody overnight at 4°C. After the immunoblot was incubated with HRP-conjugated antimouse IgG (Zymed, San Fransisco, CA, USA) for 60 min at room temperature, the immunoreactive protein bands were visualized using the ECL detection system (Pierce, Rockford, IL, USA), followed by exposure to AGFA X-ray blue film (AGFA-Gevaert, Hortsel, Belgium).

Preparation of cytosolic and nuclear extracts

To prepare cytosolic and nuclear extracts, Raw264.7 cells, treated with clusterin for various time-points, were allowed to swell by adding 150 μl lysis buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.5% NP-40, 1 mM DTT, 0.5 mM PMSF, pH 7.9). After scraping into a microfuge tube, the nuclei were pelleted by centrifugation (1000 *g*, 10 min), and supernatant was stored at –20°C as the source of cytosolic protein extracts. Next, the pellets containing crude nuclei were resuspended in 50 μl of the extraction buffer (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF), incubated for 20 min on ice, and centrifuged at 13,000 rpm for 10 min to obtain the supernatant-containing nuclear extracts. Cell extracts were snap-frozen in liquid nitrogen and then stored at –20°C until use.

RNA isolation and RT-PCR

Total RNA was extracted from Raw264.7 cells treated with clusterin for various time-points by a single-step method using RNeasy mini kits (Qiagen, Chatsworth, NJ, USA), following the manufacturer's instructions. cDNA was reverse-transcribed from 200 ng total RNA using the SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA). The sequences of specific primers were as follows: MMP-9 sense, 5′-TTCTGCCCTAC-CCGAGTGGA-3′, and antisense, 5′-CATAGTGGGAGGTGCTGTCGG-3′; β-actin sense, 5′-TGGAATCCTGTGGCATCCATGAAAC-3′, and antisense, 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′. The PCR cycle consisted of 94°C for 45 s, 57°C for 1 min, and 72°C for 1 min. Samples were amplified for 25–33 cycles. The reaction was terminated by heating at 72°C for 7 min. The PCR products were separated by 1% agarose gel and visualized by ethidium bromide staining on an UV transiluminator.

MMP-9 gene promoter and luciferase assay

The MMP-9 promoter reporter plasmid (pGL2-MMP9-pro-Luc) was kindly provided by Dr. In Kyu Lee (Kyungpook National University, Daegu, Korea), and the MMP-9 promoter region was amplified by PCR, using proper primers designed according to the MMP-9 gene sequence. The primers were forward, 5′-ACATTTGCCCGAGCTCCTGAAG-3′, and backward, 5′-AGGGGCTGCCAGAAGCTTATGGT-3′. The amplified promoter fragment was cloned into the pGL4.14 vector, kindly gifted from Dr. Mirim Jin (Daejeon University, Daejeon, Korea). By direct sequencing, the sequence of the cloned promoter region was confirmed. Raw264.7 cells were transfected with the pGL4.14-MMP9 promoter luciferase construct with Lipofectamine 2000 and selected for the stable cell line with hygromycin (200 mg/ml). Mixed stable transfectants were treated with clusterin in the presence of MEK or PI3K inhibitors, and luciferase activity was measured according to the manufacturer's recommended protocol (Promega, Madison, WI, USA).

Statistical analysis

Nonparametric statistical analysis was performed by the Kruskal-Wallis test, followed by evaluation with the Mann-Whitney test with Bonferroni correction for multiple comparisons. Data were analyzed by the SPSS Version 12.0 software (SPSS, Chicago, IL, USA). The difference was considered statistically significant at values of $P < 0.05$.

RESULTS

Clusterin up-regulates the protease activity and expression of MMP-9 in monocytes and macrophages

As MMP-9 activity was shown to be increased in malignant cancers and play an important role in tumor invasion, we first examined whether clusterin could induce MMP-9 activity in human primary monocytes. When cells were incubated with 1 μg/ml clusterin for 24 h and subjected to gelatin zymography, significant enhancement of MMP-9 proteolytic activity was observed as shown in **Fig. 1A** (upper panel). In contrast, the activity of MMP-2 was unaltered by clusterin treatment, demonstrating that clusterin specifically increased MMP-9 activity. Up-regulation of MMP-9 activity was also confirmed in a human monocytic cell line, THP-1, and mouse primary macrophages and cell line Raw264.7 (Fig. 1A, upper panel). Therefore, in both species, clusterin induced the protease activity of MMP-9 without affecting MMP-2 activity. The relatively high basal expression of MMP-9 was observed in THP-1 cells, which is consistent with a previous result that endogenous TNF-α is responsible for constitutive MMP-9 expression and secretion in THP-1 cells. Increased MMP-9 activity was associated with its elevated protein level, as assessed by Western blot analysis (Fig.

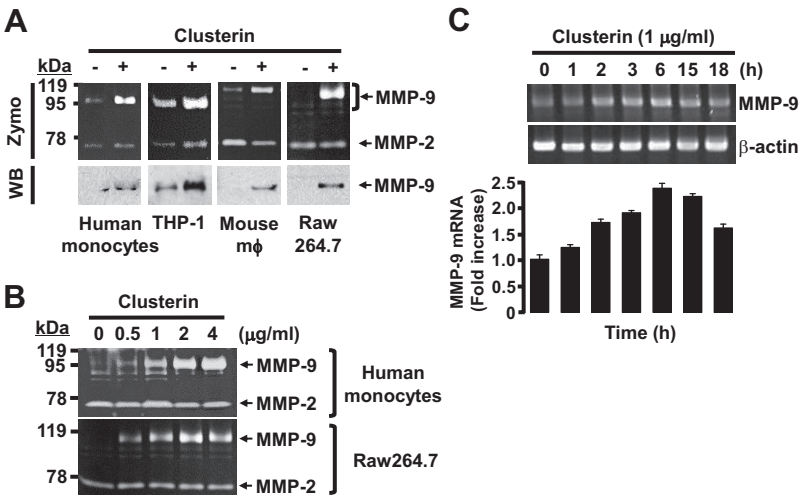

Figure 1. Clusterin induces MMP-9 expression in monocytes/macrophages. (A) Human primary monocytes, human monocytic cell line (THP-1), peritoneal mouse macrophages (ϕ), and murine macrophage cell line (Raw264.7) were suspended in DMEM containing 10% FBS and seeded onto 12-well culture plates at a density of 2×10^5 cells/well. After overnight incubation for attachment, cells were washed with PBS, and media were replaced with serum-free DMEM alone (–) or serum-free DMEM containing 1 μg/ml clusterin (+). Spent media were collected after 24 h, concentrated, and analyzed for MMP-9 by gelatin zymography (Zymo) and Western blotting (WB). The molecular size corresponding to the latent forms of MMP-9 in human and mouse represents 95 and 105 kDa, respectively. (B) Human primary monocytes and Raw264.7 macrophages were treated with serum-free DMEM containing clusterin at the indicated doses. Spent media were collected after 24 h, concentrated, and analyzed for MMP-9 activity by zymography. (C) Raw264.7 macrophages were cultured with clusterin (1 μg/ml) for the indicated times, and total RNA was isolated and subjected to RT-PCR. The bar graph represents fold changes of MMP-9 mRNA evaluated by densitometry. All experiments were repeated at least three times with independently derived sets of cultures, and the results shown are representative.

1A, lower panel). These data imply that clusterin-induced gelatinolytic activity is likely a result of increased production of MMP-9 in monocytes/macrophages. A dose-dependent stimulatory effect of clusterin on MMP-9 activity was confirmed further in Raw264.7 cells as well as human primary monocytes, as shown in Fig. 1B. In both cases, maximum expression of MMP-9 was observed at 2 μg/ml clusterin. The basal level of MMP-9 activity was also observed in the zymography gels without exogenous clusterin treatment. To address the question of whether the endogenous clusterin secreted from the cells could induce MMP-9 in an autocrine manner, we suppressed its expression in Raw264.7 cells using siRNA and monitored the MMP-9 activity by zymography. We found that the expression levels of clusterin in macrophage cell lines were not comparable with the exogenous clusterin (1 μg/ml). In addition, suppression of endogenous clusterin by siRNA did not affect MMP-9 activity (Supplemental Fig. 1). Therefore, we assume that the residual MMP-9 activity in gelatin gels without exogenous clusterin treatment is not a result of endogenous clusterin secreted in Raw264.7 cells. MMP-9 mRNA level was increased time-dependently after clusterin treatment, reaching a plateau at 6 h, as observed by RT-PCR analysis (Fig. 1C). Together, these data demonstrate that exogenously added clusterin induced the activity and expression of MMP-9 in human and mouse primary macrophages, as well as their cell lines.

Carbohydrate portion of clusterin is critical for the MMP-9-stimulating activity, rather than its 3D peptide conformation

Although unlikely, the observed up-regulation of MMP-9 by clusterin could have been a result of the presence of a bacterial endotoxin contaminant that occurred during the purification process of clusterin. To rule out this possibility, we mixed purified human clusterin in serum-free DMEM with its specific mAb (1G8) or N-IgG and performed immunoprecipitation using Protein A/G PLUS agarose beads (sc-2003, Santa Cruz Biotechnology) prior to treatment. As shown in **Fig. 2A**, clusterin-induced MMP-9 activity was blocked almost completely after immunoprecipitation with clusterin antibody but not with N-IgG. Evidence that the supernatant after immunoprecipitation with 1G8 resulted in near-complete depletion of clusterin, and that with control IgG did not is shown in Fig. 2B. Immunoprecipitated clusterin was found mostly in the pellet, indicating the specific interaction of clusterin and its antibody (Fig. 2B). Furthermore, although LPS caused activation of MMP-9 similar to clusterin, treatment of cells with polymyxin B, a well-characterized, pharmacological LPS antagonist [26], which suppressed the LPS-stimulated MMP-9 activity, did not abrogate the clusterin-induced MMP-9 activity (Fig. 2C). These data strongly suggest that the increased MMP-9 activity with clusterin treatment is not a result of the bacterial contamination that occurred during clusterin preparation. Furthermore, rCLU, generated in human embryonic kideny 293 cells, induced MMP-9 activity similar to that induced by clusterin purified from human plasma (Fig. 2D). Together, these data highlight the role of clusterin, and not contaminated bacterial components, in stimulating the expression and protease activity of MMP-9 in macrophages. As an initial study to exclude the potential contamination artifact, the purified clusterin was boiled for heat denaturation for 30 min to destroy its 3D conformation prior to the addition into the macrophage cultures. To our surprise, heat-denatured clusterin was still able to induce MMP-9 activity, although at slightly lower levels (**Fig. 3A**). These data demonstrate that the heat-stable component of clusterin, but not its 3D conformation, is sufficient to stimulate MMP-9 activity (Fig. 3A). As clusterin purified from human sera is heavily glycosylated, we next examined whether the heat-resistant carbohydrate moieties of clusterin are necessary for the induction of MMP-9. For this, a nonglycosylated rCLU of 50 kDa, obtained from *E. coli*, transfected with cDNA encoding mature human clusterin lacking signal peptide sequences, was applied to the macrophage cell cultures. In contrast to the secretory, glycosylated form of clusterin, nonglycosylated clusterin did not induce MMP-9 activity (Fig. 3B). These data demonstrate that the carbohydrate portion of clusterin may play an

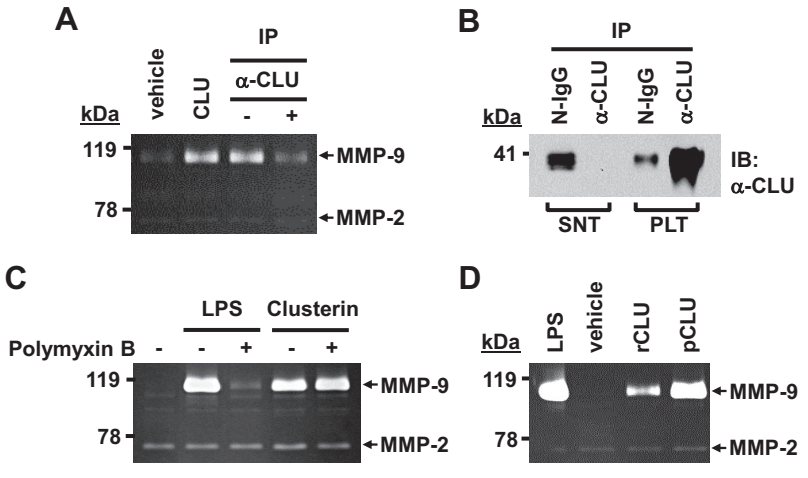

Figure 2. Clusterin-induced MMP-9 expression is LPS-independent. (A) Raw264.7 cells (12-well plate: 2×10^5 cells/well) were incubated with serum-free DMEM (1 ml) containing 1 μg purified human clusterin (CLU) and the supernatants resulting from immunoprecipitation (IP) with mouse anticlusterin mAb (+, α-CLU; 1G8) or mouse N-IgG (–, α-CLU) to deplete purified human clusterin in serum-free DMEM. Following 24 h incubation, spent media were analyzed for MMP-9 by zymography. (B) The clusterin-blocking experiment was performed by immunoprecipitation as mentioned in *A*, and the supernatant (SNT) and resuspended pellet (PLT) after immunoprecipitation were analyzed for clusterin by immunoblotting (IB). (C) Cells were preincubated with 10 μg/ml polymyxin B for 30 min and then treated with LPS (100 ng/ml) or clusterin (1 μg/ml) for 24 h. Spent media were analyzed for MMP-9 activity by zymography. (D) Cells were exposed with 1 μg/ml rCLU or purified clusterin (pCLU) for 24 h, and the spent media were analyzed for MMP-9 activity by zymography. All experiments were repeated at least three times with independently derived sets of cultures, and the results shown are representative.

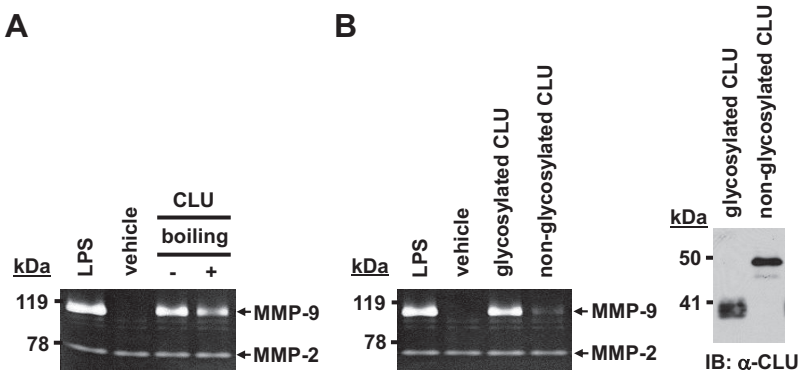

Figure 3. Effect of glycosylation of clusterin on MMP-9 expression. (A) Raw264.7 cells (12-well plate: 2×10^5 cells/well) were exposed in serum-free DMEM containing native (–) or heat-denatured (+) clusterin (1 μg/ml) for 24 h, and the spent media were analyzed for MMP-9 activity by zymography. (B) Cells were incubated for 24 h in serum-free DMEM, containing fully glycosylated clusterin or nonglycosylated clusterin, purified from human serum or *E. coli*, respectively, and the spent media were analyzed for MMP-9 activity by zymography (left panel). Both clusterins used in this experiment were confirmed by immunoblotting using the anticlusterin antibody (α-CLU) under reducing conditions (right panel). All experiments were repeated at least three times with independently derived sets of cultures, and the results shown are representative.

important role for its stimulatory effect on MMP-9 expression in macrophages.

ERK1/2 activation is required for the clusterin-induced MMP-9 production

We next investigated the intracellular signaling process required for the induction of MMP-9 by clusterin. In mammalian cells, MAPKs were shown to activate MMP-9 upon various stimuli [27]. Similarly, we found in murine pancreatic islet cells and astrocytes that clusterin induces cell proliferation through ERK1/2 activation [10, 26]. In this regard, we first examined the activation of MAPKs to identify the intracellular signal transduction pathways mediating the clusterin-induced MMP-9 expression and activity. Raw264.7 cells were exposed to clusterin at 1 μg/ml for various incubation times, and the phosphorylation statuses of ERK1/2, p38, and JNKs were examined by Western blot analysis using their phospho-specific antibodies. As shown in **Fig. 4A** (top panel), clusterin induced rapid phosphorylation of ERK1/2, which was clearly evident at 15 min after adding clusterin. This activation lasted for up to 60 min and then slowly declined, but it was still above the basal level at 2 h after clusterin addition. We also found that clusterin promoted the phosphorylation of JNK in a time-dependent manner (Fig. 4A, bottom panel). However, the phosphorylation of p38 was not observed in Raw264.7 cells by the treatment of clusterin (Fig. 4A, middle panel). Equal loading of the protein for each blot was ensured, as judged by the immunodetection of its total protein level. To confirm the involvement of ERK1/2 activation in the macrophages during clusterin-induced MMP-9 induction, we examined the MMP-9 gelatinase activity induced by clusterin in the presence or absence of PD98059, a MEK inhibitor. By gelatin zymography, we observed that clusterin-promoted MMP-9 activity was dose-dependently diminished by PD98059, whereas the inhibitor (50 μM) alone had no effect on MMP-9 activity (Fig. 4B, upper panel). The specific effect of PD98059 on clusterin-induced ERK1/2 phosphorylation was analyzed further by immunodetection against phospho-ERK1/2. Clusterin-induced ERK1/2 phosphorylation was also abrogated by PD98059 (Fig. 4C), which concurs with decreased MMP-9 gelatinase activity, as observed by zymography (Fig. 4B, upper panel). In contrast, treatment with SP600125, an inhibitor of JNK, did not affect the gelatinase activity of MMP-9 (Fig. 4B, lower panel). Thus, although clusterin stimulated the phosphorylation of JNK, it did not appear to be involved in the activation of MMP-9. Collectively, these data demonstrate that in Raw264.7 cells, the MMP-9 activity induced by clusterin is mediated through the activation of ERK1/2.

Clusterin activates Raw264.7 cells to induce MMP-9 expression through the PI3K/Akt/NF-κB pathway

As it has been shown that the secretory form of clusterin activates the PI3K/Akt pathway in the MAT-LyLu prostatic cell and the ARPE-19 retinal pigment epithelial cell [28, 29], and the PI3K/Akt pathway is involved in the MMP-9 activation by

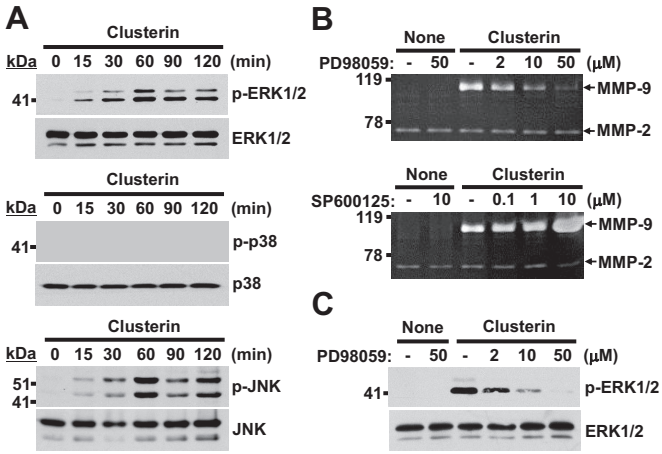

Figure 4. Activation of ERK1/2 is essential for clusterin-induced MMP-9 expression. (A) Raw264.7 cells (six-well plate: 1×10^6 cells/well) were treated with clusterin (1 μg/ml) in serum-free DMEM. At the indicated time-points, cell lysates were prepared, and phosphorylated (p) and total forms of ERK1/2, p38, and JNK were evaluated by immunoblotting. (B) Cells were preincubated with the indicated doses of PD98059 or SP600125 prior to clusterin stimulation, and the spent media after 24 h were analyzed for MMP-9 activity by zymography. (C) Cells were treated with 1 μg/ml clusterin for 60 min in the absence or presence of PD98059 (2, 10, and 50 μM). Cell lysates were prepared and analyzed for ERK1/2 activation by immunoblotting using an antiphospho-ERK1/2 or anti-ERK1/2 antibody. All experiments were repeated at least three times with independently derived sets of cultures, and the results shown are representative.

LPS [30], we next examined whether the PI3K/Akt pathway is associated with MMP-9 production in response to exogenous clusterin. Clusterin (1 μg/ml) was added to the culture of macrophages for various incubation times, and the serine 473 phosphorylation status of Akt was examined by Western blot analysis using a phospho-specific antibody. As shown in **Fig. 5A**, phosphorylation of Akt was evident at 15 min following clusterin stimulation and increased for up to 2 h. To assess whether the PI3K pathway, an upstream signaling component of Akt, is involved in the phosphorylation of Akt for MMP-9 activation in macrophages by clusterin, an inhibitor of PI3K, LY294002, was added to the cell culture prior to clusterin stimulation. MMP-9 gelatinolytic activity induced by clusterin was abolished by LY294002 at 50 μM, but the inhibitor alone had no effect on MMP-9 activity in the same condition (Fig. 5B). Similarly, the Akt phosphorylation, enhanced by clusterin stimulation, was also attenuated by LY294002 (Fig. 5C), suggesting that Akt signaling is required for induction of MMP-9 activity.

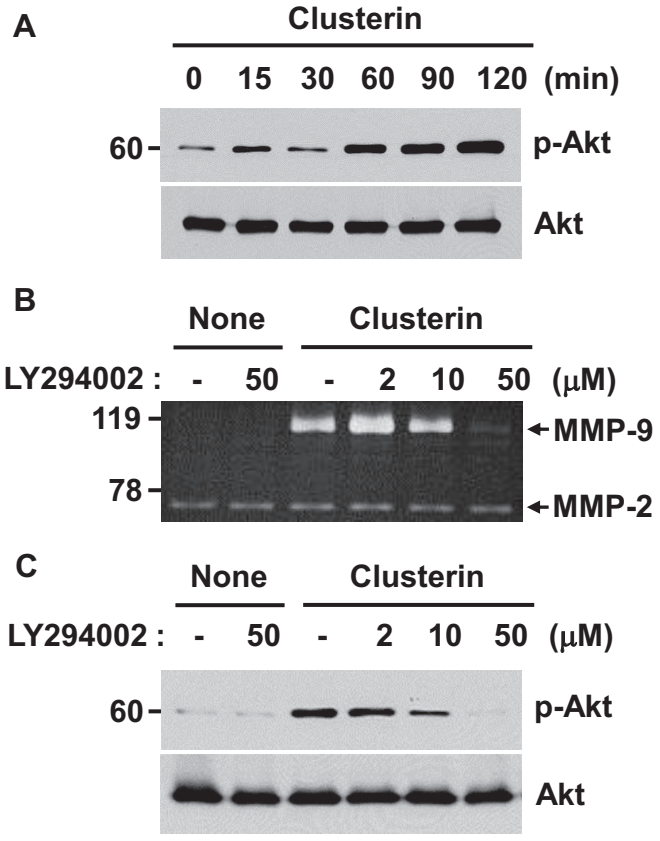

Figure 5. Clusterin-induced MMP-9 up-regulation requires activation of the PI3K/Akt pathway. (A) Raw264.7 cells (six-well plate: 1×10^6 cells/well) were treated with clusterin (1 μg/ml) in serum-free DMEM. At the indicated time-points, cell lysates were prepared, and phosphorylated and total forms of Akt were identified by immunoblotting. (B) Cells were preincubated with the indicated doses of LY294002 prior to clusterin treatment, and the spent media, after 24 h, were analyzed for MMP-9 activity by zymography. (C) Cell lysates were analyzed for Akt activation by immunoblotting using an antiphospho-Akt (ser 473) or anti-Akt antibody. All experiments were repeated at least three times with independently derived sets of cultures, and the results shown are representative.

Recent studies show that MMP-9 activity can be regulated by NF-κB signaling via PI3K and Akt pathways [30], thus we further analyzed the nuclear translocation of NF-κB as well as IκB-α phosphorylation and degradation. Raw264.7 cells were cultured in the presence of clusterin (1 μg/ml) for various incubation times, and the cell lysates were fractioned into cytosolic and nuclear fractions and immunoblotted for IκB-α and NF-κB, respectively, to assess the protein levels. Phosphorylation status of IκB-α was also examined in the cytosol fraction by Western blot analysis using the phospho-specific antibody. As shown in **Fig. 6A**, total IκB-α was decreased gradually, whereas its phosphorylated form was evidently increased at 5 min following clusterin treatment and lasted up to 2 h. In addition, the nuclear translocation of NF-κB p65 was increasingly observed, as assessed by the immunodetection of the NF-κB p65 protein in the nucleus, which is in correlation with the results of IκB-α degradation and phosphorylation. To assess whether the clusterin-induced NF-κB activation is a downstream event of PI3K/Akt or ERK1/2 signaling pathways, cells were stimulated with clusterin in the presence or absence of LY294002 as a PI3K inhibitor or PD98059 as a MEK inhibitor, and IκB-α protein degradation was monitored by Western blot analysis. Pretreatment of LY294002 abrogated clusterin-induced phosphorylation of Akt and degradation of IκB-α, but PD98059 did not give any alterations to Akt phosphorylation and IκB-α degradation (Fig. 6B), indicating that NF-κB is activated via the PI3K/Akt pathway. To further confirm whether NF-κB activation is actually involved in clusterin-induced MMP-9 production, we measured the effect of inhibition of NF-κB using a specific NF-κB inhibitor, helenalin. As shown in Fig. 6C, pretreatment with 20 μM helenalin completely abolished clusterin-induced MMP-9 activity. In addition, we observed that clusterin-stimulated phosphorylation of ERK1/2 or Akt was not suppressed by LY294002 or PD98059, respectively, although their phosphorylations were blocked by their own specific inhibitors (Fig. 6B). Taken together, these data demonstrate that two different pathways—ERK1/2 and PI3K/Akt/NF-κB—are independently involved in the activation of MMP-9 in Raw264.7 cells by clusterin.

Clusterin induces MMP-9 promoter activity via ERK1/2 and PI3K/Akt signaling

Next, we investigated whether clusterin activates transcription of the MMP-9 gene, in addition to its stimulatory effect on enzyme activity. To assess the effect of clusterin on MMP-9 promoter activity, we generated a stable cell line of Raw264.7 transfected with a WT MMP-9 promoter luciferase construct (pGL4-MMP9-pro-Luc). The cells were exposed to clusterin at 1 μg/ml for 24 h in the presence of an inhibitor of PI3K (LY294002) or by a MEK inhibitor (PD98059), and the luciferase activity was measured in the cell lysates. As shown in **Fig. 7**, clusterin increased MMP-9 promoter activity up to 2.5-fold, whereas pretreatment with a PI3K inhibitor (LY294002) or a MEK inhibitor (PD98059) significantly abolished the clusterin-induced promoter activity. Cumulatively, our data show that exogenous clusterin stimulates MMP-9 gene expression at

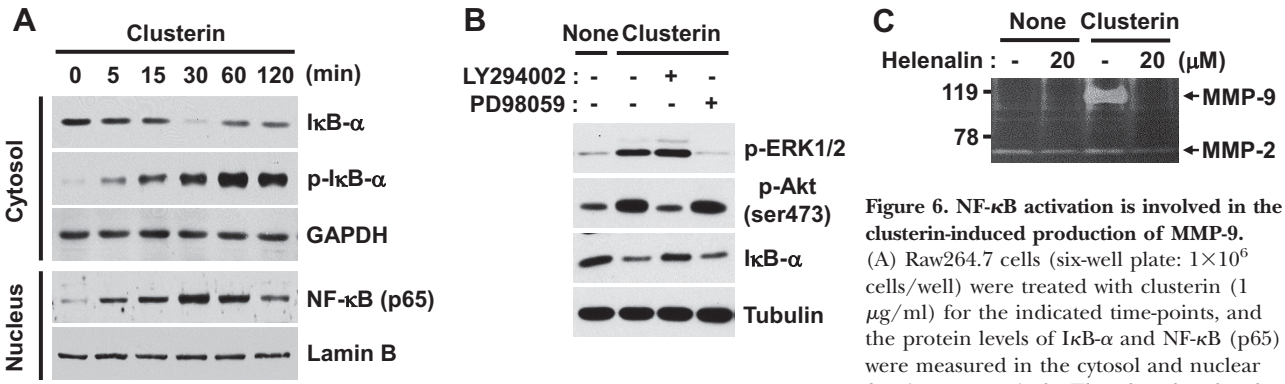

Figure 6. NF-κB activation is involved in the clusterin-induced production of MMP-9. (A) Raw264.7 cells (six-well plate: 1×10^6 cells/well) were treated with clusterin (1 μg/ml) for the indicated time-points, and the protein levels of IκB-α and NF-κB (p65) were measured in the cytosol and nuclear fraction, respectively. The phosphorylated form of IκB-α was evaluated in cytosol fraction. GAPDH and lamin B were used as an internal control for the equal loading of proteins from cytosolic and nuclear fractions, respectively. (B) Cells were treated with clusterin in the presence or absence of LY294002 (20 μM) or PD98059 (20 μM), and degradation of IκB-α protein and phosphorylations of ERK1/2 and Akt at 30 min after clusterin stimulation were assessed by immunoblotting. (C) The spent media from the cells pretreated with helenalin (a specific NF-κB inhibitor), followed by clusterin stimulation, were analyzed for MMP-9 activity by zymography. All experiments were repeated at least three times with independently derived sets of cultures, and the results shown are representative.

the transcription level as well as enzyme activity via ERK1/2 and PI3K/Akt pathways.

DISCUSSION

In this study, we present a novel role of secretory clusterin in stimulating the expression and activation of MMP-9 in macrophages and demonstrate that ERKl/2 and PI3K/Akt/NF-κB signaling pathways are critical to MMP-9 induction by clusterin. These data support the hypothesis that clusterin regulates ECM remodeling via increased MMP-9 expression in macrophages during tumor cell invasion, inflammation, and/or tissue remodeling. These data may provide a functional link between elevated clusterin levels observed in human cancer patients and poor prognosis.

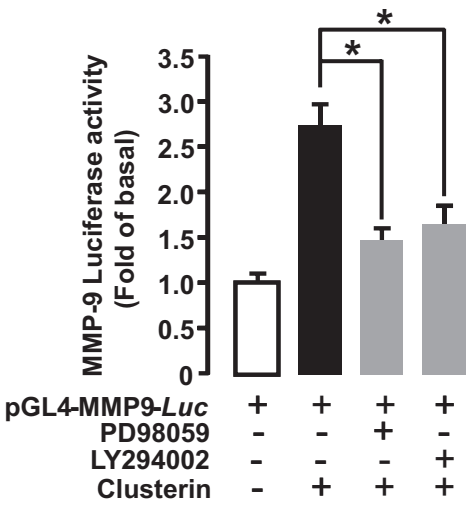

Figure 7. Involvement of ERK1/2 and PI3K/Akt pathways in clusterin-induced MMP-9 promoter activity. Raw264.7 cells transfected with the MMP-9-luc reporter gene (pGL4-MMP9-*Luc*) were suspended in serum-free DMEM and plated into 12-well plates at a density of 2×10^5 cells/well. After overnight culture, cells were pretreated with PD98059 (10 μM) or LY294002 (10 μM) for 30 min and then stimulated with 1 μg/ml clusterin for 24 h. Luciferase activity was determined in the cell lysates, as described in Materials and Methods. Results are presented as fold activations relative to the luciferase activity of the reporter alone from three separate experiments. $^*P < 0.05$ versus clusterin stimulation.

One of the most remarkable findings is that the MMP-9-promoting effect by clusterin was mediated by the heat-stable structure, rather than the heat-sensitive 3D conformation. Furthermore, glycosylation-deficient clusterin by peptide-N-glycosidase F treatment (Supplemental Fig. 2) and a nonglycosylated rCLU of 50 kDa obtained from *E. coli* were unable to stimulate MMP-9; hence, the carbohydrates portion of clusterin appears to be critical in triggering MMP-9 activation in macrophages. At present, the detailed mechanism of clusterin in stimulating membrane receptors or the identity of its receptor on macrophages is not clear. However, several possibilities could exist based on previous findings. First, transactivation of EGFR on the macrophages could have stimulated the expression and activity of MMP-9, similar to that seen in primary astrocytes [31]. Nonetheless, we found that EGFR did not get triggered following clusterin stimulation in the macrophages (data not shown). Second, LRP-2 (megalin), the only identified receptor for clusterin, was shown to be expressed at high levels in T lymphoid and erythroid cells. However, the expression of LRP-2 has been reported to be low in the granulocyte/macrophage lineage cells [32]. Rather, its related receptor, LRP-1, was shown to be involved in the activation of MMP-9 by activated α(2) macroglobulin via ERK1/2 and NF-κB pathways in macrophage cell lines [33]. Therefore, LRP-1 could have functioned as a potential receptor of clusterin in Raw264.7 macrophages. Third, as clusterin shares its cell adhesion properties and MMP-9 activity with fibronectin, it is tempting to speculate that clusterin might interact with integrins, for example, α5β1,

the receptor for fibronectin, to stimulate MMP-9 expression [34, 35]. An alternative possibility is that carbohydrates in the clusterin protein may bind to a TLR, similar to LPS, to activate macrophages for the secretion of MMP-9. Experiments are currently underway to delineate these possibilities.

Depending on the external stimulatory factors and receptors, distinct, internal signaling pathways could converge to modulate MMP-9 expression [34, 36, 37]. The proximal promoter of the MMP-9 gene contains multiple transcription factor-binding sites, including NF-κB, Sp1, and AP-1, which are differentially responsive to various stimuli [38]. For example, in lung carcinoma cells, fibronectin-integrin $\alpha5\beta1$ interaction increases MMP-9 expression via AP-1, but not NF-κB or Sp1 sites [34], whereas LPS-induced MMP-9 promoter activation requires NF-κB activation in Raw264.7 macrophages [39]. Here, we observed that ERK and PI3K/Akt/NF-κB signaling pathways operated to stimulate MMP-9 in macrophages by exogenous-added, glycosylated clusterin. Furthermore, clusterin-induced MMP-9 promoter activity was partially abolished by pretreatment with inhibitors to either pathway, ERK or PI3K/Akt/NF-κB. These data underscore that ERK1/2 and PI3K/Akt/NF-κB signaling pathways are activated independently for the production of MMP-9 in macrophages by clusterin, although further studies are required to identify transcription factors, activation sites, and upstream signaling molecules.

Similar to our findings, recent data have shown that clusterin exhibited MMP-9 gelatinase activity in the mouse mammary tumor cell line, BRI-JM01 cells [40]. Therefore, although clusterin-expressing cells in the tumor tissues were mostly found in the inflammatory or stromal cells, tumor cells themselves also had the capability to up-regulate MMP-9 by clusterin. In contrast, clusterin was shown to inhibit promoter activity of MMP-9 in primary vascular smooth muscle cells stimulated with TNF-α [41]. Therefore, the role of clusterin appears to vary depending on cell type, stimulus, and/or the tissue microenvironment.

The presence of the intracellular form of clusterin might also contribute to opposing functions of clusterin with respect to MMP-9 induction. For example, clusterin expression was lacking in rheumatoid arthritis patients, resulting in enhanced IκB degradation and prolonged activation of NF-κB [42]. Later, it was found that only the presecretory, intracellular form of clusterin, but not the secretory form, interacted with phospho-IκB-α, where it acted as a stabilizer of IκB and inhibited NF-κB signaling [43]. In contrast, secretory clusterin appeared to stimulate NF-κB expression in murine NK cells, as it facilitated production of IFN-γ in response to IL-2 [44]. Our data also present the activation of NF-κB via the IκB-α degradation and translocation of NF-κB by secretory clusterin, which leads to the stimulation of MMP-9 activity. These data highlight the contradictory function of clusterin depending on its isoform types—secretory versus intracellular. Identification of tissue-specific receptors and delineation of signaling pathways in the particular cell types on the given stimulus will greatly help to understand the diverse roles of clusterin in vivo.

In conclusion, our data demonstrate that exogenous secretory clusterin can stimulate MMP-9 mRNA and protein expression in monocytes and macrophages via ERK1/2 and PI3K/Akt/NF-κB pathways. As NF-κB is a central regulator of inflammation, clusterin may provide a molecular link between inflammation and cancer via up-regulating NF-κB and MMP-9. Collectively, these data highlight a novel function of clusterin as a stimulator for MMP-9 expression in macrophages.

AUTHORSHIP

Y-J.S. performed experiments, analyzed the data, and wrote the paper; B-H.K. and H-S.J. performed the research; I-S.P., K-U.L., I-K.L., and G-H.P. designed the research and analyzed the data. K-M.L., P.S., and B-H.M. designed the research, analyzed the data, and wrote the paper.

ACKNOWLEDGMENTS

This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST; No. 2010-0028414) and Basic Atomic Energy Research Institute (BAERI) grant from the National Nuclear R&D Program from the Korean Ministry of Education, Science and Technology (20100017420). K-M.L. was supported by a GRL grant.

REFERENCES

1. Trougakos, I. P., Gonos, E. S. (2002) Clusterin/apolipoprotein J in human aging and cancer. *Int. J. Biochem. Cell Biol.* **34,** 1430–1448.
2. Blaschuk, O., Burdzy, K., Fritz, I. B. (1983) Purification and characterization of a cell-aggregating factor (clusterin), the major glycoprotein in ram rete testis fluid. *J. Biol. Chem.* **258,** 7714–7720.
3. Rosenberg, M. E., Silkensen, J. (1995) Clusterin: physiologic and pathophysiologic considerations. *Int. J. Biochem. Cell Biol.* **27,** 633–645.
4. Rizzi, F., Bettuzzi, S. (2010) The clusterin paradigm in prostate and breast carcinogenesis. *Endocr. Relat. Cancer* **17,** R1–R17.
5. Shannan, B., Seifert, M., Leskov, K., Willis, J., Boothman, D., Tilgen, W., Reichrath, J. (2006) Challenge and promise: roles for clusterin in pathogenesis, progression and therapy of cancer. *Cell Death Differ.* **13,** 12–19.
6. Stewart, E. M., Aquilina, J. A., Easterbrook-Smith, S. B., Murphy-Durland, D., Jacobsen, C., Moestrup, S., Wilson, M. R. (2007) Effects of glycosylation on the structure and function of the extracellular chaperone clusterin. *Biochemistry* **46,** 1412–1422.
7. Falgarone, G., Chiocchia, G. (2009) Chapter 8: clusterin: a multifacet protein at the crossroad of inflammation and autoimmunity. *Adv. Cancer Res.* **104,** 139–170.
8. Gleave, M., Chi, K. N. (2005) Knock-down of the cytoprotective gene, clusterin, to enhance hormone and chemosensitivity in prostate and other cancers. *Ann. N. Y. Acad. Sci.* **1058,** 1–15.
9. McLaughlin, L., Zhu, G., Mistry, M., Ley-Ebert, C., Stuart, W. D., Florio, C. J., Groen, P. A., Witt, S. A., Kimball, T. R., Witte, D. P., Harmony, J. A., Aronow, B. J. (2000) Apolipoprotein J/clusterin limits the severity of murine autoimmune myocarditis. *J. Clin. Invest.* **106,** 1105–1113.
10. Min, B. H., Kim, B. M., Lee, S. H., Kang, S. W., Bendayan, M., Park, I. S. (2003) Clusterin expression in the early process of pancreas regeneration in the pancreatectomized rat. *J. Histochem. Cytochem.* **51,** 1355–1365.
11. Swertfeger, D. K., Witte, D. P., Stuart, W. D., Rockman, H. A., Harmony, J. A. (1996) Apolipoprotein J/clusterin induction in myocarditis: a localized response gene to myocardial injury. *Am. J. Pathol.* **148,** 1971–1983.
12. Bi, J., Guo, A. L., Lai, Y. R., Li, B., Zhong, J. M., Wu, H. Q., Xie, Z., He, Y. L., Lv, Z. L., Lau, S. H., Wang, Q., Huang, X. H., Zhang, L. J., Wen, J. M., Guan, X. Y. (2010) Overexpression of clusterin correlates with tumor progression, metastasis in gastric cancer: a study on tissue microarrays. *Neoplasma* **57,** 191–197.
13. Miyake, H., Hara, I., Fujisawa, M., Gleave, M. E. (2006) The potential of clusterin inhibiting antisense oligodeoxynucleotide therapy for prostate cancer. *Expert Opin. Investig. Drugs* **15,** 507–517.
14. Redondo, M., Tellez, T., Roldan, M. J. (2009) The role of clusterin (CLU) in malignant transformation and drug resistance in breast carcinomas. *Adv. Cancer Res.* **105,** 21–43.
15. July, L. V., Beraldi, E., So, A., Fazli, L., Evans, K., English, J. C., Gleave, M. E. (2004) Nucleotide-based therapies targeting clusterin chemosensitize human lung adenocarcinoma cells both in vitro and in vivo. *Mol. Cancer Ther.* **3,** 223–232.

16. Flanagan, L., Whyte, L., Chatterjee, N., Tenniswood, M. (2010) Effects of clusterin over-expression on metastatic progression and therapy in breast cancer. *BMC Cancer* **10,** 107.
17. Schedin, P., Mitrenga, T., McDaniel, S., Kaeck, M. (2004) Mammary ECM composition and function are altered by reproductive state. *Mol. Carcinog.* **41,** 207–220.
18. Astancolle, S., Guidetti, G., Pinna, C., Corti, A., Bettuzzi, S. (2000) Increased levels of clusterin (SGP-2) mRNA and protein accompany rat ventral prostate involution following finasteride treatment. *J. Endocrinol.* **167,** 197–204.
19. Rosenberg, M. E., Paller, M. S. (1991) Differential gene expression in the recovery from ischemic renal injury. *Kidney Int.* **39,** 1156–1161.
20. Kim, B. M., Han, Y. M., Shin, Y. J., Min, B. H., Park, I. S. (2001) Clusterin expression during regeneration of pancreatic islet cells in streptozotocin-induced diabetic rats. *Diabetologia* **44,** 2192–2202.
21. Cheung, C., Marchant, D., Walker, E. K., Luo, Z., Zhang, J., Yanagawa, B., Rahmani, M., Cox, J., Overall, C., Senior, R. M., Luo, H., McManus, B. M. (2008) Ablation of matrix metalloproteinase-9 increases severity of viral myocarditis in mice. *Circulation* **117,** 1574–1582.
22. Littlepage, L. E., Sternlicht, M. D., Rougier, N., Phillips, J., Gallo, E., Yu, Y., Williams, K., Brenot, A., Gordon, J. I., Werb, Z. (2010) Matrix metalloproteinases contribute distinct roles in neuroendocrine prostate carcinogenesis, metastasis, and angiogenesis progression. *Cancer Res.* **70,** 2224–2234.
23. Egeblad, M., Werb, Z. (2002) New functions for the matrix metalloproteinases in cancer progression. *Nat. Rev. Cancer* **2,** 161–174.
24. Inokuchi, C., Ueda, H., Hamaguchi, T., Miyagawa, J., Shinohara, M., Okamura, H., Namba, M. (2009) Role of macrophages in the development of pancreatic islet injury in spontaneously diabetic torii rats. *Exp. Anim.* **58,** 383–394.
25. Shin, Y. J., Kang, S. W., Jeong, S. Y., Shim, Y. J., Kim, Y. H., Kim, B. M., Kee, S. H., Park, J. J., Park, I. S., Min, B. H. (2006) Clusterin enhances proliferation of primary astrocytes through extracellular signal-regulated kinase activation. *Neuroreport* **17,** 1871–1875.
26. Smiley, S. T., King, J. A., Hancock, W. W. (2001) Fibrinogen stimulates macrophage chemokine secretion through Toll-like receptor 4. *J. Immunol.* **167,** 2887–2894.
27. Chakraborti, S., Mandal, M., Das, S., Mandal, A., Chakraborti, T. (2003) Regulation of matrix metalloproteinases: an overview. *Mol. Cell. Biochem.* **253,** 269–285.
28. Ammar, H., Closset, J. L. (2008) Clusterin activates survival through the phosphatidylinositol 3-kinase/Akt pathway. *J. Biol. Chem.* **283,** 12851–12861.
29. Kim, J. H., Jun, H. O., Yu, Y. S., Min, B. H., Park, K. H., Kim, K. W. (2010) Protective effect of clusterin from oxidative stress-induced apoptosis in human retinal pigment epithelial cells. *Invest. Ophthalmol. Vis. Sci.* **51,** 561–566.
30. Lu, Y., Wahl, L. M. (2005) Production of matrix metalloproteinase-9 by activated human monocytes involves a phosphatidylinositol-3 kinase/Akt/IKKα/NF-κB pathway. *J. Leukoc. Biol.* **78,** 259–265.
31. Shim, Y. J., Shin, Y. J., Jeong, S. Y., Kang, S. W., Kim, B. M., Park, I. S., Min, B. H. (2009) Epidermal growth factor receptor is involved in clusterin-induced astrocyte proliferation. *Neuroreport* **20,** 435–439.
32. Miharada, K., Hiroyama, T., Sudo, K., Danjo, I., Nagasawa, T., Nakamura, Y. (2008) Lipocalin 2-mediated growth suppression is evident in human erythroid and monocyte/macrophage lineage cells. *J. Cell. Physiol.* **215,** 526–537.
33. Cáceres, L. C., Bonacci, G. R., Sanchez, M. C., Chiabrando, G. A. (2010) Activated α(2) macroglobulin induces matrix metalloproteinase 9 expression by low-density lipoprotein receptor-related protein 1 through MAPK-ERK1/2 and NF-κB activation in macrophage-derived cell lines. *J. Cell. Biochem.* **111,** 607–617.
34. Han, S., Ritzenthaler, J. D., Sitaraman, S. V., Roman, J. (2006) Fibronectin increases matrix metalloproteinase 9 expression through activation of c-Fos via extracellular-regulated kinase and phosphatidylinositol 3-kinase pathways in human lung carcinoma cells. *J. Biol. Chem.* **281,** 29614–29624.
35. Silkensen, J. R., Skubitz, K. M., Skubitz, A. P., Chmielewski, D. H., Manivel, J. C., Dvergsten, J. A., Rosenberg, M. E. (1995) Clusterin promotes the aggregation and adhesion of renal porcine epithelial cells. *J. Clin. Invest.* **96,** 2646–2653.
36. Jin, Y. J., Park, I., Hong, I. K., Byun, H. J., Choi, J., Kim, Y. M., Lee, H. (2011) Fibronectin and vitronectin induce AP-1-mediated matrix metalloproteinase-9 expression through integrin $\alpha(5)\beta(1)/\alpha(v)\beta(3)$-dependent Akt, ERK and JNK signaling pathways in human umbilical vein endothelial cells. *Cell. Signal.* **23,** 125–134.
37. Li, H., Mittal, A., Paul, P. K., Kumar, M., Srivastava, D. S., Tyagi, S. C., Kumar, A. (2009) Tumor necrosis factor-related weak inducer of apoptosis augments matrix metalloproteinase 9 (MMP-9) production in skeletal muscle through the activation of nuclear factor-κB-inducing kinase and p38 mitogen-activated protein kinase: a potential role of MMP-9 in myopathy. *J. Biol. Chem.* **284,** 4439–4450.
38. Ray, A., Bal, B. S., Ray, B. K. (2005) Transcriptional induction of matrix metalloproteinase-9 in the chondrocyte and synoviocyte cells is regulated via a novel mechanism: evidence for functional cooperation between serum amyloid A-activating factor-1 and AP-1. *J. Immunol.* **175,** 4039–4048.
39. Rhee, J. W., Lee, K. W., Kim, D., Lee, Y., Jeon, O. H., Kwon, H. J., Kim, D. S. (2007) NF-κB-dependent regulation of matrix metalloproteinase-9 gene expression by lipopolysaccharide in a macrophage cell line RAW 264.7. *J. Biochem. Mol. Biol.* **40,** 88–94.
40. Lenferink, A. E., Cantin, C., Nantel, A., Wang, E., Durocher, Y., Banville, M., Paul-Roc, B., Marcil, A., Wilson, M. R., O'Connor-McCourt, M. D. (2010) Transcriptome profiling of a TGF-β-induced epithelial-to-mesenchymal transition reveals extracellular clusterin as a target for therapeutic antibodies. *Oncogene* **29,** 831–844.
41. Kim, H. J., Yoo, E. K., Kim, J. Y., Choi, Y. K., Lee, H. J., Kim, J. K., Jeoung, N. H., Lee, K. U., Park, I. S., Min, B. H., Park, K. G., Lee, C. H., Aronow, B. J., Sata, M., Lee, I. K. (2009) Protective role of clusterin/apolipoprotein J against neointimal hyperplasia via antiproliferative effect on vascular smooth muscle cells and cytoprotective effect on endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **29,** 1558–1564.
42. Devauchelle, V., Essabbani, A., De Pinieux, G., Germain, S., Tourneur, L., Mistou, S., Margottin-Goguet, F., Anract, P., Migaud, H., Le Nen, D., Lequerre, T., Saraux, A., Dougados, M., Breban, M., Fournier, C., Chiocchia, G. (2006) Characterization and functional consequences of underexpression of clusterin in rheumatoid arthritis. *J. Immunol.* **177,** 6471–6479.
43. Essabbani, A., Margottin-Goguet, F., Chiocchia, G. (2010) Identification of clusterin domain involved in NF-κB pathway regulation. *J. Biol. Chem.* **285,** 4273–4277.
44. Sonn, C. H., Yu, Y. B., Hong, Y. J., Shim, Y. J., Bluestone, J. A., Min, B. H., Lee, K. M. (2010) Clusterin synergizes with IL-2 for the expansion and IFN-{γ} production of natural killer cells. *J. Leukoc. Biol.* **88,** 955–963.

KEY WORDS:
MMP-9 · glycosylation · ECM remodeling · tumor invasion · inflammation