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Secretory clusterin inhibits osteoclastogenesis by attenuating M-CSF-dependent osteoclast precursor cell proliferation

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ABSTRACT

Secretory clusterin (sCLU)/apolipoprotein J is a multifunctional glycoprotein that is ubiquitously expressed in various tissues. Reduced sCLU in the joints of patients with bone erosive disease is associated with disease activity; however, its exact role has yet to be elucidated. Here, we report that CLU is expressed and secreted during osteoclastogenesis in mouse bone marrow-derived macrophages (BMMs) that are treated with receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF). CLU-deficient BMMs obtained from $CLU^{-/-}$ mice exhibited no significant alterations in OC differentiation in comparison with BMMs obtained from wild-type mice. In contrast, exogenous sCLU treatment significantly inhibited OC formation in both BMMs and OC precursor cultures. The inhibitory effect of sCLU was more prominent in BMMs than OC precursor cultures. Interestingly, treating BMMs with sCLU decreased the proliferative effects elicited by M-CSF and suppressed M-CSF-induced ERK activation of OC precursor cells without causing apoptotic cell death. This study provides the first evidence that sCLU reduces OC formation by inhibiting the actions of M-CSF, thereby suggesting its protective role in bone erosion.

1. Introduction

Clusterin (CLU) is a multifunctional glycoprotein that is ubiquitously expressed and secreted in various tissues [1,2] and upregulated in response to cellular stress [3,4]. CLU transcription gives rise to two splice variants: full-length CLU mRNA (which generates glycosylated secretory CLU [sCLU] [5]) and an alternative mRNA splice variant called "nonglycosylated nuclear CLU" (nCLU) [4,6]. While nCLU functions as a proapoptotic protein [1,7], sCLU possesses antiapoptotic functions [8]. It has been noted that sCLU is linked with diverse pathophysiological processes, including apoptosis, inflammation, proliferation, and differentiation [1,2]. sCLU is detected in virtually all human body fluids, and its level is closely associated with the activities of several diseases [9]. sCLU binds to amyloid-beta, thereby preventing the further formation of senile plaques in Alzheimer's disease patients [10]. Systemic lupus erythematosus patients demonstrate reduced sCLU expression in serum in comparison with healthy donors [11]. Moreover, sCLU expression in the cartilage and synovial tissues of osteoarthritis and rheumatoid arthritis patients is lower than healthy donors [12], indicating the potential biological role of sCLU in bone degenerative joint diseases. However, the exact role of sCLU is not fully understood.

Osteoclasts (OCs) are the specialized cells responsible for the resorption of old bone matrix and maintaining bone integrity in cooperation with bone-forming osteoblasts in the body [13]. OCs are formed by fusing hematopoietic monocyte/macrophage precursor cells in the presence of two regulators of OC differentiation [14]: macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) [15]. M-CSF transmits signals to monocyte/macrophage precursor cells through

Abbreviations: CLU, clusterin; sCLU, secretory clusterin; nCLU, nuclear clusterin; OC, osteoclast; OCPs, OC precursors; pOCs, pre-fusion osteoclasts; mOCs, mature osteoclasts; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of nuclear factor kappa-B ligand; BMMs, bone marrow-derived macrophages; TRAP, tartrate-resistant acid phosphatase; ELISA, enzyme-linked immunosorbent assay; BrdU, 5-bromo-2′-deoxyuridine; PI, propidium iodide; MNCs, multinucleated cells; ERK, extracellular signal-regulated kinase.

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the receptor for M-CSF, c-fms, which is responsible for expressing RANK (receptor activator of nuclear factor kappa-B) receptor [13–15]. M-CSF plays a role in the proliferation and survival of OC precursor cells, mainly by activating extracellular signal-regulated kinase (ERK) and Akt [16–18]. In addition, RANKL binds to RANK, thereby activating the intracellular signaling pathways required for OC differentiation and biological function [19]. Excessive resorption and the increase in OC formation in inflamed joints appears to cause bone erosion [20]. Thus, targeting OC-induced joint destruction is critical when managing pathological progress of these diseases.

In our present study, we postulated that low sCLU in inflamed joints may be related to bone erosive phenotypes and is specific for OC cellular function. We show for the first time that sCLU inhibits OC formation by abrogating M-CSF-dependent ERK activation, which only occurs with exogenous sCLU treatment.

2. Materials and methods

2.1. Reagents and antibodies

Recombinant murine RANKL and M-CSF were purchased from Peprotech (London, UK). Secretory human clusterin (sCLU) was purchased from ProSpecbio (Ness-Ziona, Israel). Primary antibodies (Abs) targeting phospho-p44/42 MAPK (p-ERK), p44/42 MAPK (ERK), phospho-Akt (p-Akt), and Akt were purchased from Cell Signaling Technology (Danvers, MA). Polyclonal anti-clusterin Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-β-actin Ab and tartrate-resistant acid phosphatase (TRAP) kits were purchased from Sigma–Aldrich (St Louis, MO).

2.2. In vitro osteoclastogenesis assay

In vitro osteoclastogenesis assays were used to examine the effects of sCLU on OC differentiation. Bone marrow-derived macrophages (BMMs) were prepared from the OC precursor cells, as described by Chang et al. [21]. Briefly, in order to harvest BMMs, cells were extracted from the femur and tibiae of a 6-week-old C57/BL6 and CLU-deficient mice and incubated in α-MEM supplemented with 10% fetal bovine serum (FBS) and M-CSF (30 ng/mL). BMMs were cultured in osteoclastogenic media, and α-MEM was supplemented with 10% FBS, 100 ng/mL RANKL, and 30 ng/mL MCSF in the presence or absence of CLU for the indicated times in a 37 °C humidified incubator containing 5% CO_2. The cells were then fixed, stained for tartrate-resistant acid phosphatase (TRAP) using an acid phosphatase kit (Sigma–Aldrich), and observed under a light microscope. TRAP-positive multinucleated cells ($TRAP^+$ MNCs) with >3 nuclei were considered mature OCs [22].

2.3. Total RNA extraction, cDNA synthesis, and RT-PCR

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesized from total RNA using the RevertAid First strand cDNA Synthesis kit (Thermo Scientific, EU). mRNA expression was analyzed using RT-PCR, as previously described [23].

2.4. Cell proliferation and apoptosis assay

Cell proliferation was determined using the 5-bromo-2′-deoxyuridine (BrdU) incorporation assay (Roche, Indianapolis, IN) according to the manufacturer's instructions. To evaluate the stage of apoptosis, cells treated with 0, 1, or 5 μg/mL CLU were stained with Annexin V-FITC in Annexin V staining buffer for 15 min at room temperature, counterstained with 50 mg/mL propidium iodide (PI) (BD Biosciences, San Diego, CA), and analyzed using FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA). All analyses were performed in triplicate.

2.5. Immunoblotting assay

BMM cells (5×10^5 cells/well) were seeded onto 6-well plates and pretreated with or without sCLU (5 μg/mL) for 1 h prior to stimulation with M-CSF (30 ng/mL). Cells were lysed, and the protein lysate was resolved using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). Nonspecific interactions were blocked with 5% BSA for 1 h, and membranes were probed with the indicated primary antibodies overnight at 4 °C. Membranes were incubated with the appropriate HRP-conjugated secondary antibodies, and antibody reactivity was visualized using an enhanced chemiluminescence western blotting system (Millipore).

2.6. Statistical analysis

All quantitative experiments were performed in triplicate, and the data are shown as the mean ± standard deviation (SD) of 1 representative experiment. Statistical significance was determined using the student *t* test, and 95% confidence was defined as $p < 0.05$.

3. Results

3.1. CLU expression in osteoclast lineage cells during osteoclastogenesis

Given that various cells express CLU (and its expression is induced by cell differentiation) [24], we investigated if OC lineage cells, including bone marrow-derived macrophages (BMMs), prefusion osteoclasts (pOCs), and mature osteoclasts (mOCs), express CLU during osteoclastogenesis (Fig. 1A). CLU mRNA expression was observed in BMMs and maintained in pOCs. mOCs exhibited

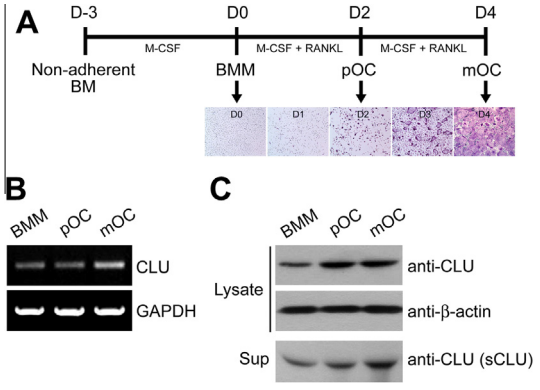

Fig. 1. CLU expression during osteoclastogenesis. (A) Schematic illustration of osteoclast (OC) differentiation. Non-adherent mouse bone marrow cells (BMs) were cultured for 3 days in the presence of M-CSF (30 ng/mL) to produce the committed bone marrow-derived macrophages (BMMs) (i.e., OC precursor cells). BMMs were cultured for 2 days with M-CSF (30 ng/mL) and RANKL (100 ng/mL) to produce prefusion OCs (pOCs). After culturing for additional 2 days, pOCs were fused to form mature OCs (mOCs) as shown as TRAP-positive multinucleated cells ($TRAP^+$ MNCs). (B) Total RNA was then isolated, and CLU mRNA expression levels in BMM, pOC, and mOC were evaluated using RT-PCR during osteoclastogenesis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. (C) Supernatants and cell lysates from BMM, pOC, and mOC were collected and analyzed using immunoblotting to quantify CLU protein levels. β-Actin was used as the internal control.

a modest increase in CLU mRNA expression in comparison with BMMs (Fig. 1B). Similarly, CLU protein expression was modestly increased in differentiated OCs (pOCs and mOCs) in comparison with undifferentiated cells (BMMs). In addition, we found that CLU proteins were secreted into the culture media (Fig. 1C).

3.2. Endogenous CLU demonstrates no significant effect on osteoclastogenesis

To evaluate the role of endogenous CLU in OC differentiation, we compared osteoclastogenic potential between BMMs isolated from CLU-deficient mice and their wild-type (WT) littermates. CLU protein expression was detected in BMMs from WT mice, but the absence of expression was confirmed in $CLU^{-/-}$ mice (Fig. 2A). Murine BMMs differentiate into multinucleated mature OCs after culturing for 4 days in osteoclastogenic medium containing M-CSF and RANKL (Fig. 2B). In addition, BMMs prepared from $CLU^{-/-}$ mice demonstrated no significant differences in the formation of TRAP-positive multinucleated cells ($TRAP^+$ MNCs) in comparison with corresponding WT cells (Fig. 2B and C). These findings demonstrated that endogenous CLU has no direct effect on OC differentiation.

3.3. Exogenous treatment with sCLU inhibits OC differentiation

To investigate the effects of secretory CLU (sCLU) on OC differentiation, BMMs were treated with various concentrations of exogenous sCLU on day 0 (D0) or pOC on day 2 (D2) of osteoclastogenesis (Fig. 3A). BMMs were induced to differentiate into OCs by cultivation in osteoclastogenic medium containing M-CSF and RANKL in the presence or absence of sCLU. As shown in Fig. 3B, BMM cells differentiated into numerous TRAP-positive OCs in the control group. However, the formation of OCs was significantly inhibited by sCLU treatment at 1 and 3 μg/mL, respectively. OC formation was almost completely abrogated in a dose-dependent manner in BMMs by treatment with 5 μg/mL sCLU (D0) (Fig. 3C). Although pOC treatment (D2) with sCLU resulted in less inhibition of OC formation than at D0, the number of OCs significantly decreased in a dose-dependent manner in the CLU-treated group (Fig. 3D). Collectively, these results suggest that sCLU negatively regulates OC differentiation.

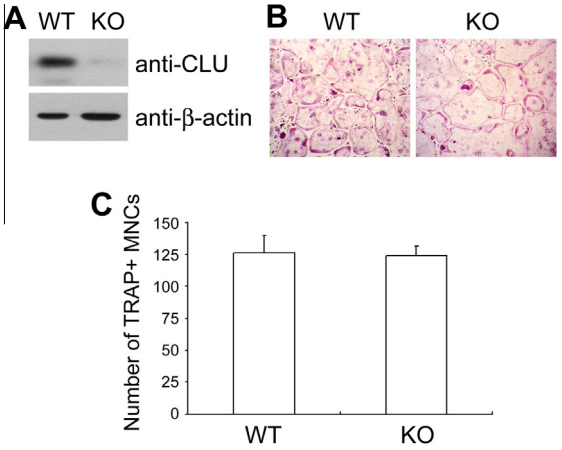

Fig. 2. Role of endogenous CLU in osteoclastogenesis. (A) $CLU^{-/-}$ mice were confirmed using an immunoblotting assay. β-Actin was included as the internal control. (B) BMMs for OC differentiation were prepared using $CLU^{-/-}$ and WT mice. Mouse BMMs were cultured for 4 days in the presence of RANKL (100 ng/mL) and M-CSF (30 ng/mL). Cells were then fixed with 4% PFA and stained for TRAP. (C) TRAP-positive multinucleated cells containing ⩾3 nuclei ($TRAP^+$ MNCs) were counted using a light microscope. Data are shown as the mean ± SD of triplicate experiments.

3.4. sCLU inhibits osteoclast precursor cell proliferation by suppressing M-CSF-induced activation of the ERK signaling pathway

During osteoclastogenesis, M-CSF supports the proliferation and survival of OC precursor cells and is required for RANKL-induced OC differentiation [25]. According to previous studies on sCLU function [1,2], we postulate that sCLU may inhibit OC differentiation by regulating cell death or the proliferation of OC precursor cells (i.e., BMMs) (Fig. 3). M-CSF was removed from the growth media to induce cell death, and sCLU-treated BMMs were stained with Annexin V-FITC/propidium iodide (PI) (Fig. 4A). The results show that approximately 22.1% of cells in the BMM control cultures were Annexin-V-positive (Fig. 4A, left panel). In comparison, approximately 18.4% and 15.2% of BMM cells treated with 1 or 5 μg/mL sCLU, respectively, were positive for Annexin-V (Fig. 4A, middle and right panel), indicating that sCLU does not significantly affect apoptotic cell death. Interestingly, sCLU treatment decreased M-CSF-dependent cell proliferation in a dose-dependent manner, as determined using the BrdU incorporation assay (Fig. 4B). These results demonstrate that the inhibitory effects of sCLU on OC differentiation might not arise from cytotoxicity, but from reducing OC precursor cell proliferation.

To identify the intracellular signaling molecules that mediate the M-CSF-dependent proliferation of OC precursor cells via sCLU, we investigated if sCLU influences M-CSF-activated ERK and Akt signaling [16,17]. As shown in Fig. 4C, M-CSF alone strongly induced ERK phosphorylation, but sCLU treatment dramatically inhibited M-CSF-induced ERK phosphorylation (Fig. 4C). In addition, there were no changes in Akt phosphorylation between the vehicle- and sCLU-treated cells (Fig. 4C). Taken together, these results reveal that sCLU specifically inhibits OC proliferation by affecting M-CSF-induced ERK activation, which could be the mechanism responsible for the anti-osteoclastogenic effects of sCLU.

4. Discussion

Here, we report the suppressive role of sCLU in OC differentiation, which may be correlated with protective functions against excessive bone erosion. Endogenous CLU deficiency had no effect on OC formation (Fig. 2). However, exogenous sCLU treatment significantly inhibited OC proliferation, and, following OC differentiation (Fig. 3), reinforced its pathological importance in the body fluids of patients with bone erosive disease. In general, extracellular glycosylated sCLU is believed to be a survival factor that demonstrates antiapoptotic function and thereby impacts diverse diseases, including cancer, autoimmunity, and cardiovascular disease [9]. On the other hand, sCLU is a negative regulator of smooth muscle cell proliferation [26], indicating its contradictory roles in different cell types. Here, there were no significant effects on apoptosis in OC precursor cells (Fig. 4A) or accompanying changes in Akt phosphorylation due to sCLU treatment (Fig. 4C). Instead, sCLU inhibited OC precursor cell proliferation by decreasing ERK activation (Fig. 4B and C). These data suggest that sCLU plays a major role in attenuating proliferation signals in OC precursor cells. The M-CSF-mediated ERK signaling cascade is critical for OC proliferation and survival [18,19], and inhibiting ERK suppresses OC formation [27]. Genetically disrupting ERK1 also reduces OC progenitor cells, M-CSF-mediated migration, and subsequent bone resorption [28]. In accordance with these studies, we conclude that sCLU inactivates the ERK1/2 signaling pathway in M-CSF-treated BMM and thereby decreases the proliferative capacity of OC precursor cells.

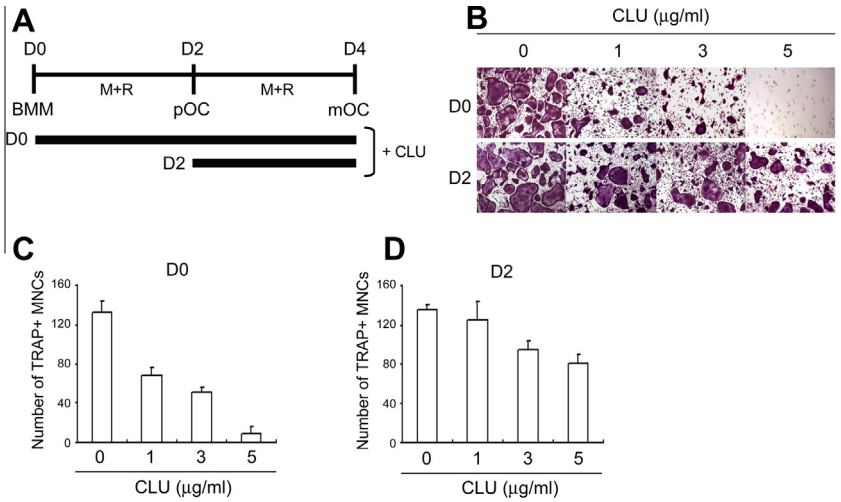

Fig. 3. Inhibitory effects of sCLU on OC formation. (A) Schematic illustration of sCLU treatment during OC differentiation. (B–D) Mouse BMMs were treated with various concentrations of sCLU on day 0 (D0) or pOC on day 2 (D2) in the presence of RANKL (100 ng/mL) and M-CSF (30 ng/mL) in order to differentiate into mOCs (B). Cells were then fixed with 4% PFA and subjected to TRAP staining. TRAP-positive multinucleated cells containing ⩾3 nuclei (TRAP$^+$ MNCs) differentiated from BMMs (C) or pOCs (D) were counted on day 4. Data are shown as the mean ± standard deviation of triplicate experiments.

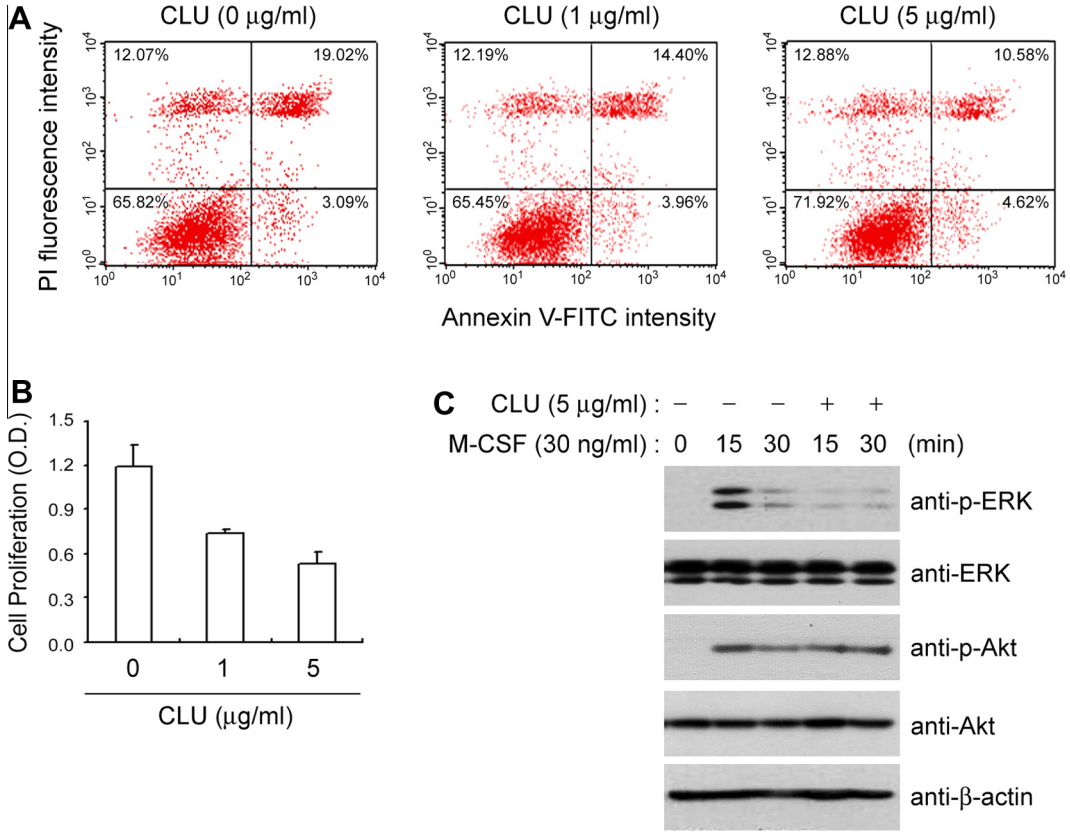

Fig. 4. Suppression of M-CSF-mediated ERK activation by sCLU. (A) The induction of apoptosis by sCLU was determined using flow cytometric analysis of Annexin V-FITC and PI staining. (B) CLU inhibited cell proliferation. BMMs were treated with exogenous sCLU, and cell proliferation was determined using BrdU ELISA. Data are the mean ± SD of triplicate experiments. (C) sCLU inhibits M-CSF-induced ERK phosphorylation. BMMs were pretreated with vehicle or sCLU (5 μg/mL) for 30 min, which was followed by M-CSF stimulation (30 ng/mL) for the indicated times. Cell lysates were prepared and phosphorylated, or the total forms of ERK and Akt were evaluated using immunoblotting analysis. β-Actin was included as the internal control.

Thus, this could be the underlying mechanism by which sCLU suppresses OC formation.

The mechanisms that describe how sCLU attenuates M-CSF-mediated ERK activation remain unknown. Direct receptor-mediated intracellular penetration of sCLU and intracellular ERK inhibition are possible. However, because sCLU is heavily glycosylated and does not appear to be capable of penetrating the plasma membrane [5], it is unlikely that it directly inhibits ERK activation. On the other hand, sCLU may indirectly inhibit the ERK pathway by hindering M-CSF-mediated signaling. Several lines of

evidence suggest that it is actually a family of abundant extracellular chaperons [29]. sCLU is associated with a wide spectrum of molecules, including cellular debris generated by apoptosis, necrosis, and leptin, and thereby mediates the clearance of extracellular proteins via endocytosis [30]. In the brain, sCLU binds to amyloid-beta and enhances clearance [31,32]. sCLU also binds to specific cell-surface receptors, such as low-density lipoprotein receptor, insulin-like growth factor 1 receptor, and low density lipoprotein-related protein 2 (LRP-2) [33]. Therefore, the potential interactions between sCLU, M-CSF, and c-fms may affect M-CSF-mediated intracellular signaling pathways; however, further investigations are needed to elucidate any associations with sCLU.

In conclusion, our present data demonstrate that sCLU is capable of inhibiting OC precursor cell proliferation by modulating ERK pathways, and, following OC formation, could be used to treat bone resorptive diseases.

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