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This information is current as of March 7, 2022.

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J Immunol 2006; 177:6471-6479; ;
doi: 10.4049/jimmunol.177.9.6471
<http://www.jimmunol.org/content/177/9/6471>

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Characterization and Functional Consequences of Underexpression of Clusterin in Rheumatoid Arthritis¹

Valérie Devauchelle,*[†] Abdellatif Essabbani,*^{§¶} Gonzague De Pinieux,^{||} Stéphane Germain,^{###} Léa Tourneur,*^{§¶} Sylvie Mistou,*^{§¶} Florence Margottin-Goguet,*^{§¶} Philippe Anract,^{††} Henri Migaud,^{§§} Dominique Le Nen,^{§§} Thierry Lequerré,^{¶¶} Alain Saraux,[†] Maxime Dougados,^{|||} Maxime Breban,*^{§¶||###} Catherine Fournier,*^{§¶} and Gilles Chiochia^{2*§¶||##}

We previously compared by microarray analysis gene expression in rheumatoid arthritis (RA) and osteoarthritis (OA) tissues. Among the set of genes identified as a molecular signature of RA, clusterin (*clu*) was one of the most differentially expressed. In the present study we sought to assess the expression and the role of CLU (mRNA and protein) in the affected joints and in cultured fibroblast-like synoviocytes (FLS) and to determine its functional role. Quantitative RT-PCR, Northern blot, in situ hybridization, immunohistochemistry, and Western blot were used to specify and quantify the expression of CLU in ex vivo synovial tissue. In synovial tissue, the protein was predominantly expressed by synoviocytes and it was detected in synovial fluids. Both full-length and spliced isoform CLU mRNA levels of expression were lower in RA tissues compared with OA and healthy synovium. In synovium and in cultured FLS, the overexpression of CLU concerned all protein isoforms in OA whereas in RA, the intracellular forms of the protein were barely detectable. Transgenic overexpression of CLU in RA FLS promoted apoptosis within 24 h. We observed that CLU knockdown with small interfering RNA promoted IL-6 and IL-8 production. CLU interacted with phosphorylated I κ B α . Differential expression of CLU by OA and RA FLS appeared to be an intrinsic property of the cells. Expression of intracellular isoforms of CLU is differentially regulated between OA and RA. We propose that in RA joints, high levels of extracellular CLU and low expression of intracellular CLU may enhance NF- κ B activation and survival of the synoviocytes. *The Journal of Immunology*, 2006, 177: 6471–6479.

The pathogenesis of rheumatoid arthritis (RA),³ the most frequent inflammatory chronic rheumatic disorder affecting 0.5–1% of the population (1), is still poorly understood. The disease is characterized by a symmetrical polyarticular joint inflammation that results in major changes in synovial tissue and joint destruction. The synovial cells from the lining and sublining layers are activated and hyperproliferative (2) and the tissue is infiltrated with many different inflammatory cell types such as macrophages, neutrophils, and B and T lymphocytes, contributing to pannus formation and neovascularization. RA is associated with

the production of a large array of cytokines and proteases, and activation of the complement cascade, all of which contribute to cartilage and bone resorption.

The mechanisms underlying inflammation and the immunological network leading to disease progress or chronicity are unknown. Therefore, a major challenge for research in this domain is the discovery of new pathophysiological and/or diagnostic targets. Using cDNA microarray technology to compare RA and osteoarthritis (OA) patients, we found molecular evidence for identifying RA patients based on a selected set of genes expressed in affected synovial tissue (3). Among the identified genes, clusterin (*clu*) appeared to be a potential pathophysiologically interesting gene because it has multiple functions related to apoptosis, inflammation, proliferation, and differentiation, all playing a role in the disease. CLU is a ubiquitous glycoprotein constitutively expressed in most mammalian tissues (4, 5). Notably, CLU is expressed in nonepithelial secretory cells that line fluid compartments, for instance in ovarian granulosa cells (5). This protein has a multiplicity of biological functions (6). Indeed, CLU has been described as a protein interacting with lipids, the complement membrane attack complex, TGF β s, and Igs. Moreover, the protein expression is related to apoptotic phenomena, tissue injury, or autoimmune damage. The ability of CLU to bind to the endocytic receptor Megalin (LRP-2) and to toxic substrates from extracellular spaces like unfolded proteins, cell debris, and immune complexes, brings the hypothesis that a main biological role of CLU is the clearance of toxic substrates (6).

Recently, it became evident that the different functions of CLU depend on its final maturation and localization. The predominant form is a secreted heterodimeric protein of 80 kDa (secreted CLU (sCLU)) (7) produced by translation of the full-length single mRNA (8). sCLU is derived from a pre-sCLU protein of 60 kDa

*Institut Cochin, Département d'Immunologie, Paris, France; [†]Service de Rhumatologie, Hôpital La Cavale Blanche, Brest, France; [‡] Institut National de la Santé et de la Recherche Médicale (INSERM), Unité 567, Paris, France; [§]Centre National de la Recherche Scientifique, Unité Mixte de Recherche 8104, Paris, France; [¶]Université Paris Descartes, Faculté de Médecine René Descartes, Unité Mixte de Recherche et de Service 8104, Paris, France; ^{||}Service d'Anatomo-pathologie, Hôpital Cochin, Paris; ^{|||}INSERM Unité 36, Collège de France, Paris, France; ^{###}Service d'Hématologie Biologique A, Hôpital Européen Georges Pompidou, Paris, France; ^{††}Service d'Orthopédie Hôpital Cochin, Paris, France; ^{§§}Service d'Orthopédie Hôpital Roger Salengro, Lille, France; ^{¶¶}Service d'Orthopédie Hôpital La Cavale Blanche, Brest, France; ^{|||}INSERM Unité 519, Rouen, France; ^{|||}Institut de Rhumatologie, Hôpital Cochin, Paris, France; and ^{###}Hôpital Ambroise Paré, Boulogne-Billancourt, France

Received for publication July 28, 2005. Accepted for publication August 3, 2006.

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¹ This work was supported in part by Institut National de la Santé et de la Recherche Médicale, the Société Française de Rhumatologie, the Association de Recherche sur la Polyarthrite Rhumatoïde, and by Wyeth Lederle Laboratories.

² Address correspondence and reprint requests to Dr. Gilles Chiochia, Département d'Immunologie, Pavillon Hardy A1, 27 rue du Faubourg Saint-Jacques, 75674 Paris Cedex 14, France. E-mail address: chiochia@cochin.inserm.fr

³ Abbreviations used in this paper: RA, rheumatoid arthritis; OA, osteoarthritis; CLU, clusterin; sCLU, secreted CLU; nCLU, nuclear CLU; SF, synovial fluid; siRNA, small interfering RNA; QT-PCR, quantitative RT-PCR; FLS, fibroblast-like synoviocyte.

targeted to the endoplasmic reticulum and glycosylated. A nuclear form of CLU (nCLU) was recently reported to be the resulting product of an alternative splicing of exon II (9). The exact role of nCLU is still not fully elucidated although it seems to interact with Ku70, a DNA double-strand break repair protein (10). Finally, two functions of CLU have been recently attributed to cytoplasmic forms of the protein, in particular, in NF- κ B signaling and juxtanuclear aggregates (11, 12).

Collectively, these results made CLU a highly pathophysiologically interesting gene in a disease such as RA. Based on this concept, we have conducted a series of experiments focused on this gene to elucidate CLU expression, regulation, and function in synovial tissues, synovial cell culture, and biological fluids. We suggest that restoring intracellular CLU expression in synoviocytes could be beneficial in RA.

Materials and Methods

Patients and sample collection

Patients with RA who fulfilled the criteria of the American College of Rheumatology (13) and patients with OA were included in the study. Healthy controls were patients undergoing knee arthroscopy for traumatic ligament lesions (legal authorization CCPRB-HN from Centre Hospitalier Universitaire de Rouen). All the samples were obtained with informed consent of the patients.

Synovial tissue (ST), from patients with RA and from OA patients who underwent knee replacement surgery, was dissected out and samples were either immediately processed for RNA or protein extraction or stored at -70°C in RNeasy lysis buffer (Qiagen) or treated for cell culture. Synovial fluid (SF) was freshly centrifuged and the supernatant was stored in aliquots at -70°C until use.

RNA preparation

Total RNA was extracted in RLT RNA extraction buffer (RNeasy kit; Qiagen) and treated with DNase I. The integrity of the RNA was assessed by gel and RT-PCR and concentration was measured by absorbance at 260 nm. Reference RNA was prepared from different cell lines (Jurkat, U937, THP-1, HaCat).

Real-time PCR analysis

Real-time PCR was conducted using a LightCycler system (Roche Diagnostics) according to the manufacturer's instructions. Reactions were performed in a 20- μl volume with 0.5 μl of primers, 2 μl of LightCycler FastStart reaction mix SYBR Green I (Roche Diagnostics), and adequate concentrations of MgCl_2 . β -Actin was chosen as housekeeping gene for relative quantification to normalize target gene expression.

The forward and reverse primers used were: β -actin forward, 5'-GGG TCA GAA GGA TTC CTA TG (237 bp) (57°C); β -actin reverse, 5'-GGT CTC AAA CAT GAT CTG GG-3'; CLU (α -chain) forward, 5'-GCG AAG ACC AGT ACT ATC TG-3' (201bp) (57°C); CLU (α -chain) reverse, 5'-TTT GGT GGT ATT CCT GCA GC-3'; nCLU forward, 5'-TGCCGCT GACCGAAATGTC-3' (210 bp) (65°C); nCLU reverse, 5'-GAGCTCC TTCAGCTTGTCTC-3'.

Each primer efficiency was tested using corresponding annealing temperatures and MgCl_2 concentrations (2–7 mM). Primer efficiency was calculated using the standard curve method ($E = 10^{-1/\text{slope}}$, where E represents the primer efficiency). Each standard curve was determined with multiple dilutions steps and replicates and stored as a coefficient file used for the analysis. For PCR quantification, the LightCycler Relative Quantification software, using the so-called coefficient files according to the manufacturer's recommendations, performs an efficiency-corrected calculation. Final results were measured using the software Rel-Quant (Roche Diagnostics).

Northern blot

Total RNA was electrophoresed in 1.2% agarose gel in a formaldehyde running buffer system. Northern transfer onto Genescreen plus Nylon membrane was conducted by passive blotting. Prehybridization and hybridization were conducted in Hybrisol II. Probes were added at a concentration of 12×10^6 cpm/ml hybridization mix and incubated with the membrane at 65°C for 2 h. The membrane was washed twice in SSC at 65°C for 15 min. Autoradiography was performed at -80°C using Kodak X-OMAT film. We accounted for possible differences in band intensities

due to difference in RNA loading by using the relative intensity of the 18S ribosomal band in each sample to normalize the respective probe value.

In situ hybridization

Paraffin section preparation, probe labeling by in vitro transcription of human *clu* full-length cDNA was cloned in pDEST26 mammalian expression vector (Invitrogen Life Technologies), and in situ hybridization, were performed as previously described by Le Jan et al. (14).

Immunohistochemistry

Immunohistochemical staining using polyclonal anti-CLU Ab (polyclonal H330; Santa Cruz Biotechnology) was performed on archival formalin-fixed, paraffin-embedded synovial tissues, using the Alkaline Phosphatase technique (ChemMate Detection kit; DakoCytomation). Briefly, sections were deparaffinized and rehydrated. Epitope retrieval was performed in citrate buffer (pH 6) using a water bath for 40 min at 98.7°C with cooling for 20 min before immunostaining. Tissues were incubated with the primary anti-CLU Ab at a 1/150 dilution for 25 min and then exposed to biotinylated secondary linking Ab for 25 min, streptavidin alkaline phosphatase complex for 25 min. Finally, the slides were incubated with a Fuchsin-type chromogen "Chromogen Red" for 10 min, and with Mayer's hematein as counterstain for 1 min. All incubations were performed at room temperature. Sections were washed between incubations with Tris-buffered saline buffer (pH 7.6). In each case, appropriate positive and negative controls were used throughout.

Synovial cell culture

For synoviocyte culture, synovial pieces were finely minced and digested with 4 mg/ml collagenase-dispase (Sigma-Aldrich) in PBS plus Dulbecco's medium (Invitrogen Life Technologies) for 4 h at 37°C . Cells were resuspended in complete medium. After 48 h, nonadherent cells were removed. At confluence, adherent cells were trypsinized and expanded in complete medium until third passage. At this stage, synovial cell cultures were almost exclusively (90–95%) fibroblast-like cells and there were <3% contaminating T and B lymphocytes, NK cells, and macrophages.

Western blot

Synovial tissues were dissected, carefully washed with PBS, and total proteins were extracted with lysis buffer (10 mM Tris-HCl, 150 mM NaCl (pH 7.8), 1% Nonidet P-40, 1 mM PMSF, 1 $\mu\text{g/ml}$ aprotinin, and 1 $\mu\text{g/ml}$ leupeptin), containing a mixture of protein phosphatase inhibitors (Calbiochem-Novabiochem). Sample protein concentrations were determined using a micro BCA protein assay reagent kit (Pierce), and 20 or 40 μg of total proteins from synovial tissue or synovial cells, respectively, were subjected to SDS-PAGE and transferred to nitrocellulose (NEN Life Sciences). The membrane was incubated with blocking buffer (TBS, 5% BSA), and probed overnight at 4°C with specific primary Abs anti-CLU (H330, sc8354) and anti-actin (C11, sc1615) manufactured by Santa Cruz Biotechnology. After washing, the membranes were incubated with secondary Ab, peroxidase-labeled anti-mouse IgG1 (Caltag Laboratories; 1/4000 dilution) or anti-rabbit IgG (Amersham Biosciences; 1/5000 dilution), respectively. The signal was detected using an ECL Western blotting detection system (Amersham Biosciences). Bands obtained were quantified by densitometry using biocapt and bio-profil bio software.

Transient CLU overexpression in synoviocytes

Following PCR amplification, the human CLU full-length cDNA was cloned in pENTRvector (Invitrogen Life Technologies) and subcloned directly into the pDEST26 mammalian expression vector (Invitrogen Life Technologies). Cells were transfected by using the Amaxa Nucleofector technology according to manufacturer's instructions. Transient CLU overexpression was assayed 24–48 h posttransfection.

Cell viability and DNA fragmentation analysis

The nucleosomal DNA degradation was analyzed as described by July et al. (15). After transfection, cells were harvested and viability was assessed by trypan blue exclusion technique and then lysed in a hypotonic lysing buffer containing 10 mM Tris (pH 7.5), 10 mM EDTA, and 0.5% Triton. After centrifugation at 12,000 rpm for 15 min, the supernatants, containing the fragmented DNA, were incubated with proteinase K for 3 h at 65°C . The DNA was extracted by the addition of phenol-chloroform (1 volume). Following centrifugation, the aqueous upper layer was treated with 2.5 M sodium acetate and 1 volume of isopropanol. The DNA precipitates were pelleted, air-dried, and resuspended in 10 mM Tris and 1 mM EDTA (pH 7.4). Following treatment with RNase A for 1 h at 37°C , the samples were

electrophoresed on a 2% agarose gel and the DNA visualized with ethidium bromide.

Small interfering RNA (siRNA) transfection

siRNA transfection of CLU-1 from (16) and CLU-2 and scrambled RNA duplexes was performed in third passage synoviocytes by using Lipofectamine 2000 (Invitrogen Life Technologies). Cells were seeded at 50% confluence 2 days before siRNA transfection in 6-well plates containing 4 ml of complete medium. The day before transfection, the cells were cultured in complete medium without antibiotics. Preliminary experiments using FITC-labeled C1 siRNA allowed to determine that mixture of 5 μ l of Lipofectamine and 150 pM siRNA allowed transfection efficiency of 90–100% at 48 h. Cells were cultured for 1 h before transfection in 1.5 ml of Opti-MEM without serum and antibiotics and then treated with the siRNA complexes. Five hours after starting the incubation, 2 ml of complete medium were added to the culture. Transfection was assayed after 24–72 h. C1 sense, 5'-(Fluo)rCrCrArgrArgrCUCrCrCrCUUrCUArCTT-3'; C1 antisense 5'-rgUrArgrArArgrgrgrCrgrArgrCUCUrggrTT-3'; C2 sense, 5'-rgUrCrCrCrgrCrAUrCrgUrCrCrgrCrArgUTT-3'; C2 antisense, 5'-rgrCUCrgrgrArCrgrAUrgrCrggrgrArCTT-3'; C4 scrambled sense, 5'-rgrCUCrCrCrCrArCrgrCUGrUrArCrATT-3'; C4 scrambled antisense, 5'-UrgUrArCrArgrCrgUrggrgrArgrArgrCTT-3'.

Cytokine assay

Human IL-6 and IL-8 were measured by an ELISA using commercially available reagents (BioSource International). A volume of 100 μ l of synovial cell culture supernatant was tested pure, and diluted 2- and 10-fold.

Immunoprecipitation

HeLa cells were grown in DMEM supplemented with glutamine, antibiotics, and 10% FCS. Cells were stimulated for 5 min with 20 ng/ml TNF- α in the presence or absence of 20 μ M MG132 (N-CBZ-Leu-Leu-Leu-AL; Sigma-Aldrich). After stimulation, the cells were harvested and lysed in 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, with protease and phosphatases inhibitors. Cell lysates were precleared with protein A-agarose beads (Sigma-Aldrich) for 30 min and supernatants were incubated overnight with 5 μ g/ml rabbit polyclonal Abs anti-CLU (H330, sc8354) or control rabbit IgG Abs and then incubated with protein A-agarose beads for 1 h. Immune complexes were eluted with Laemmli buffer, separated by SDS-PAGE, and revealed by Western blot using anti-IkBa phosphorylated (Cell Signaling Technology).

Quantitative estimation of NF- κ B by ELISA

Quantitative analysis of NF- κ B/p65 and NF- κ B/p50 translocation in nucleus were performed using ELISA. For this purpose, we used commercially available Trans-AM kit (Active Motif) using the manufacturer's protocol. For this assay, the nuclear extract of HeLa cells or synoviocytes was

prepared using the Nuclear Extraction kit (Active Motif) according to the manufacturer's protocol. The assay was done in triplicate and absorbance read at 450 nm with reference taken at 650 nm. Results are expressed in terms of variation of OD of different treatment samples.

Statistical analysis

Determinations were conducted at least in duplicate. Nonparametric Mann-Whitney *U* test was used to evaluate the difference between groups. A *p* value < 0.05 was considered statistically significant.

Results

Evaluation of CLU mRNA expression in synovium

Comparative analysis of RA and OA tissues by cDNA microarrays led us to identify genes differentially expressed in these two diseases. Among those selected genes, one of the most striking findings was the weak expression of CLU mRNA in RA. Furthermore, using various softwares to find genes in a set of DNA chips which best classifies samples, we identified *clu* to belong to a set of 14 genes that have a higher prediction factor (3). To confirm and extend these results, we studied CLU mRNA expression in a larger number of samples (Fig. 1) using the real-time quantitative RT-PCR (QT-PCR) technique.

We found a highly significant decrease of CLU transcripts in RA, as compared with OA (Fig. 1A, left panel). Thus, the ratio RA-OA for CLU expression was very low with either method of quantification (0.11 and 0.19 for microarray and QT-PCR, respectively). Increasing the number of samples (Fig. 1B, middle panel) analyzed by real-time PCR (23 OA and 14 RA) further validated these results because the ratio RA-OA of CLU expression was 0.14 (*p* < 0.0001). Measure of the level of spliced *clu* mRNA showed a significantly lower expression of this mRNA form in RA compared with OA (Fig. 1A, right panel). In addition, Northern blot analysis (Fig. 1B) of four RA and four OA synovial tissues strongly confirmed the weak expression of CLU in RA compared with OA. We compared CLU expression in RA synovium to that in healthy synovium from trauma patients. We found that CLU mRNA was significantly lower in RA synovium (*p* < 0.001) than in healthy tissue (Fig. 1C). Thus, compared with healthy synovial tissue, CLU mRNA is expressed at a higher level in OA and a lower level in RA.

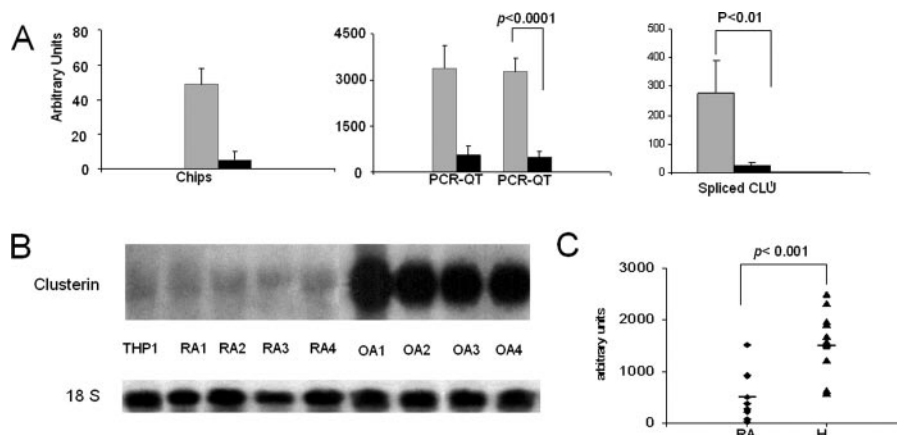


FIGURE 1. A, Down-regulation of CLU in RA tissues. CLU mRNA expression was assessed in RA (■) and OA (▨) synovial tissues. Left panel, *clu* transcription by means of cDNA microarray (chips). Middle panel, quantification of CLU mRNA levels by QT-PCR on the same samples used for cDNA microarray (first two bars) and a greater number of patients (23 OA and 14 RA). Right panel, Quantification of the spliced CLU mRNA levels by QT-PCR in 6 OA and 6 RA patients. Results are presented as mean \pm SD. Statistical significance between RA and OA groups was determined by the Mann-Whitney test. B, Northern Blot analysis of CLU expression in RA and OA tissues. Total RNA from 4 RA, 4 OA, and THP1 cell lines were studied. 18S (R45) labeling is shown as loading control. C, Differential expression of CLU between RA and healthy (H) synovial tissues analyzed by QT-PCR; *p* < 0.001 (Mann-Whitney *U* test).

Next, we wanted to determine whether this differential expression was due to down-regulation of expression in RA or reflected the fact that the RA synovium is infiltrated by large numbers of CLU-negative inflammatory cells, which would dilute the RNA from the synovial component. We evaluated expression of CLU mRNA in OA, RA, and healthy synovium by the in situ hybridization technique.

In all synovial tissues that we studied, synovial lining cells appeared to be the main CLU mRNA-expressing cells. CLU mRNA was expressed in almost all cells of the lining layer of all normal and diseased synovium (Fig. 2). The strongest expression was observed in synovium from OA and the lowest in normal synovium. In situ hybridization showed that CLU mRNA was overexpressed in RA synoviocytes in comparison to healthy synoviocytes. Thus, these results demonstrated that both in OA and RA CLU mRNA expression was induced in synoviocytes although at a different level.

Histological analysis of CLU protein expression in the synovium

To further address the question of the cellular source of CLU in synovium and evaluate CLU protein expression, CLU protein expression was studied by means of immunostaining of RA and OA and healthy synovial tissue. Expression of CLU was performed with a polyclonal anti-CLU Ab. RA tissues were markedly infiltrated by inflammatory cells and exhibited synovial hyperplasia and neovascularization (Fig. 3). Although OA synovial tissue exhibits histological abnormalities as already reported (17), in the great majority of the cases, the histological changes in OA may be easily distinguished from those observed in RA. In contrast, histological abnormalities were absent in healthy synovium. In all cases, the strongest staining with anti-CLU was detected almost

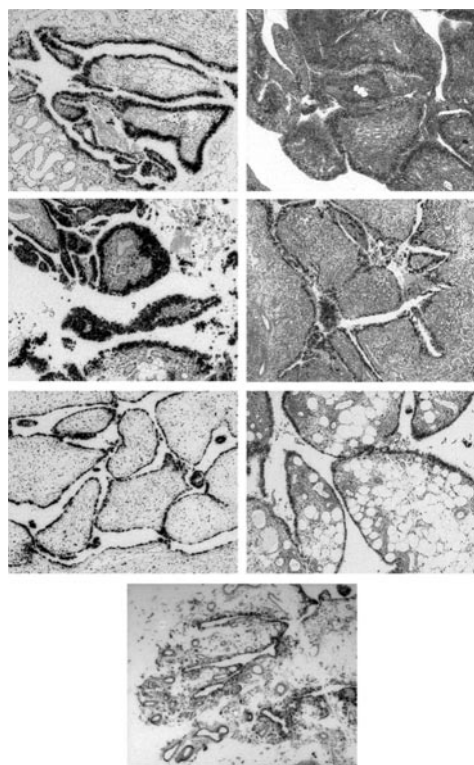


FIGURE 2. Analysis of CLU mRNA production in various specimens of synovial tissue: OA (left panel) compared with RA (right panel) synovium and with normal synovium (bottom). Scale bar, 500 μ m. The figure shows comparison of CLU mRNA expression in three RA, three OA, and one normal specimen.

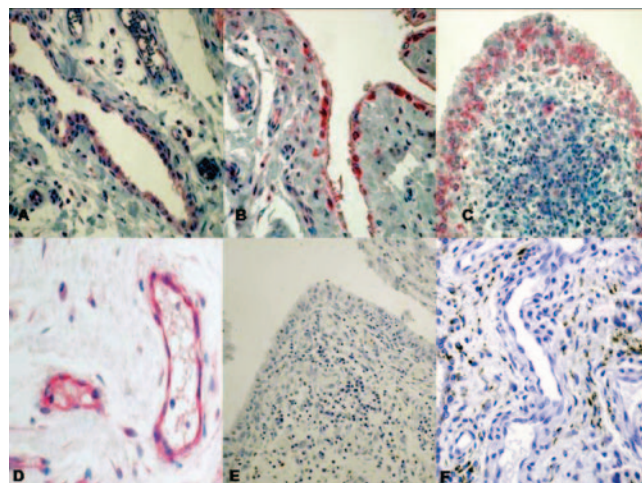


FIGURE 3. CLU protein is expressed by synoviocytes in synovial tissue. Immunohistochemical detection of CLU in synovial tissues. A, Healthy synovial tissue: synovial cells with positive CLU immunostaining. B, Synovial tissue in OA: single layer of flattened synovial lining cells staining positively for CLU. C, Synovial tissue in RA: hyperplastic synovial lining cells with positive CLU immunostaining. D, Expression of CLU by endothelial cells in a RA synovial tissue. E, Negative control without primary Abs. F, Negative control with irrelevant polyclonal rabbit Abs. Results are representative of three independent experiments.

exclusively in the synovial lining cells showing that these cells are the main CLU producers in synovium and that the differential CLU mRNA expression was also observed at the protein level.

Expression of CLU in synovial fluids

The fact that the major source of CLU in the joint appeared to be the synoviocytes prompted us to test whether the differential expression of CLU mRNA in RA and OA had a consequence in CLU protein expression in SF. CLU was detected by ELISA in all the SF tested. However, there was no quantitative difference between RA and OA levels (Fig. 4A). This result was further confirmed by Western blot analysis (data not shown). Thus, despite a quantitative difference in *clu* gene transcripts, the protein appeared to be equally present in the SF of both patients.

Breakdown of intracellular CLU protein expression in RA synovium compared with OA synovium

Because CLU protein is known to be synthesized as different isoforms (extracellular, intracellular, and nuclear) that can be distinguished by their respective molecular mass, we conducted Western blot analyses in OA and RA tissues. The levels of the 40- to 50-kDa forms, which are the major intracellular forms of CLU, were dramatically reduced in RA tissues compared with OA (Fig. 4B). We did not observe differences in the mature form 70–80 kDa of the protein.

Next, we investigated whether the differential CLU protein expression observed in synovium could be observed in cultured fibroblast-like synoviocytes (FLS). Western blot analysis of CLU expression was performed in two RA and two OA third-passage FLS (Fig. 4C). Several different isoforms of CLU were detected. The 40–60 kDa isoforms were found in both OA patients but were very low or absent in RA.

These results indicate that a qualitative and quantitative difference exists regarding CLU protein expression between OA and RA synovium as well as between RA and OA FLS, a result which could have a pathological meaning.

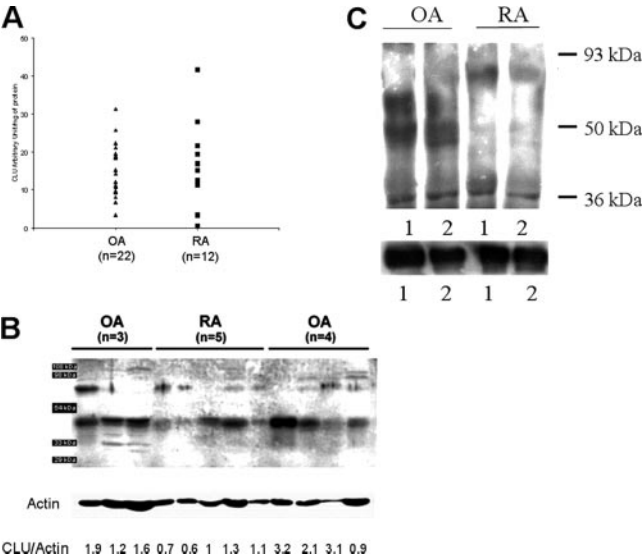


FIGURE 4. A, Clusterin concentration in SF from patients with RA ($n = 12$) and OA ($n = 22$). The bars indicate median value. The concentration of CLU was determined in arbitrary units per milligram of protein of synovial fluid (see *Materials and Methods*). B, Intracellular forms of CLU are absent in RA tissues. Western blot analysis of CLU expression was performed in five RA and seven OA synovial tissues. Several different forms of CLU were detected. The 40–50 kDa isoforms are found in all OA patients but are low or absent in RA. C, Intracellular forms of CLU are absent in cultured RA FLS. Western blot analysis of CLU expression was performed in two RA and two OA third-passage FLS. Several different forms of CLU were detected. The 40–60 kDa isoforms are found in both OA patients but are very low in RA.

Differential expression of CLU mRNA between OA and RA is an intrinsic feature of synoviocytes

Because FLS appeared to be the main *clu*-expressing cells in the synovium, we measured the expression of CLU mRNA by real-time PCR in cultured third-passage FLS. Substantial amounts of *clu* transcripts were detected in all the OA-cultured FLS tested (Fig. 5). Interestingly, as far as RA-cultured FLS were concerned, a consistently lower CLU expression was noted. Accordingly, the level of CLU expression was significantly lower in RA-FLS compared with OA-FLS ($p < 0.001$) with an average magnitude of 11-fold. Thus, regarding *clu* mRNA the differential regulation observed in the ex vivo-collected synovial tissues was still evident after in vitro culture of synovial cells.

CLU overexpression in RA synoviocytes results in higher cell death

Because intracellular CLU has been described to have proapoptotic functions, we evaluated the effect of CLU transient overexpression in synoviocytes. We transduced RA synoviocytes with plasmid coding for CLU and viable cells were counted 24h and 48 h after transfection. As reported in Table I, CLU expression induced a significant cell death in synoviocytes compared with untransfected, sham-transfected, or GFP-transfected cells. We found that at least a part of the cells died through apoptosis as attested by DNA laddering (not shown). Thus, induced expression of CLU resulted in higher RA synoviocyte death.

*Efficient silencing of *clu* gene expression by using siRNA increases baseline IL-6 and IL-8 production in synoviocytes.*

We evaluated the ability of CLU to regulate expression of cytokine genes involved in inflammation. We analyzed the effects of CLU

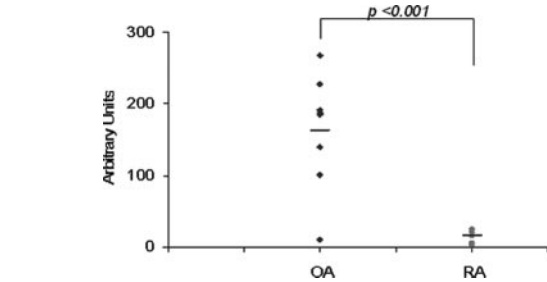


FIGURE 5. Differential expression of CLU in third-passage synovial cells from RA and OA patients. Quantification of gene expression by QT-PCR in FLS. *, Mann-Whitney test, statistically significant ($p < 0.001$).

siRNA transfection on *clu* expression. Treatment of five different FLS cultures with either CLU siRNA induced significant knock-down of the cellular CLU mRNA as measured by quantitative PCR (Fig. 6A) and CLU protein (Fig. 6B). No silencing of *clu* was seen in the presence of the control scrambled siRNA and in the absence of RNA duplexes from the transfection medium. Because NF- κ B is a key transcriptional regulator of IL-6 and IL-8 synthesis, we evaluated the effects of CLU siRNA on IL-6 and IL-8 production. Silencing of *clu* gene expression by CLU siRNA induced a significant and reproducible increase of the baseline production of IL-6 and IL-8 by FLS ($p < 0.05$ and $p < 0.002$, respectively) whereas we observed no significant difference between parental cells or cells transduced with the scrambled siRNA (Fig. 6C). These findings indicate that CLU exerts a negative regulatory role in NF- κ B-regulated cytokine production synoviocyte.

CLU interacts with phosphorylated I κ B

In cells, degradation of I κ B α occurs only after stimulation of the NF- κ B pathway, which leads to I κ B α phosphorylation on serine 32 and 36. Suspecting that CLU would also interact with phosphorylated I κ B α , we therefore stimulated HeLa cells with TNF- α and submitted cell lysates to immunoprecipitation using Abs recognizing endogenous CLU (Fig. 7A). Pretreatment of cells with proteasome inhibitor greatly enhanced phosphorylated I κ B α expression (Fig. 7B, lanes 1 and 2 compared with lanes 3 and 4). Phosphorylated I κ B α was detectable in the anti-CLU immunoprecipitate using Abs that recognize specifically Ser³²-Ser³⁶ phosphorylated I κ B α (Fig. 7A, lanes 2 and 3). As a control, phosphorylated I κ B α was not pulled down when immunoprecipitation was performed with irrelevant Abs (Fig. 7A, lanes 1 and 4). No interaction was detected between nonphosphorylated I κ B α and CLU (Fig. 7A, lane 2, lower panel). Similar results were obtained with third-passage FLS cultures showing that like in HeLa cells, CLU

Table 1. Transient transfection of synoviocytes with CLU-induced cell death^a

	% of Dead Cells	
	Day 1	Day 2
Untransfected	10 \pm 2.9	12 \pm 1.9
Sham transfection	20 \pm 8.2	22 \pm 6.7
GFP transfection	15 \pm 7.1	19 \pm 5.7
CLU transfection	57.5 \pm 12.6	60.4 \pm 13.2

^aResults are mean \pm SD of four different experiments. Synoviocytes were either untransfected or transfected with various plasmid constructs using the Amaxa Nucleofector technology according to the manufacturer's instructions. Apoptosis was ascertained by evaluating DNA fragmentation in two experiments.

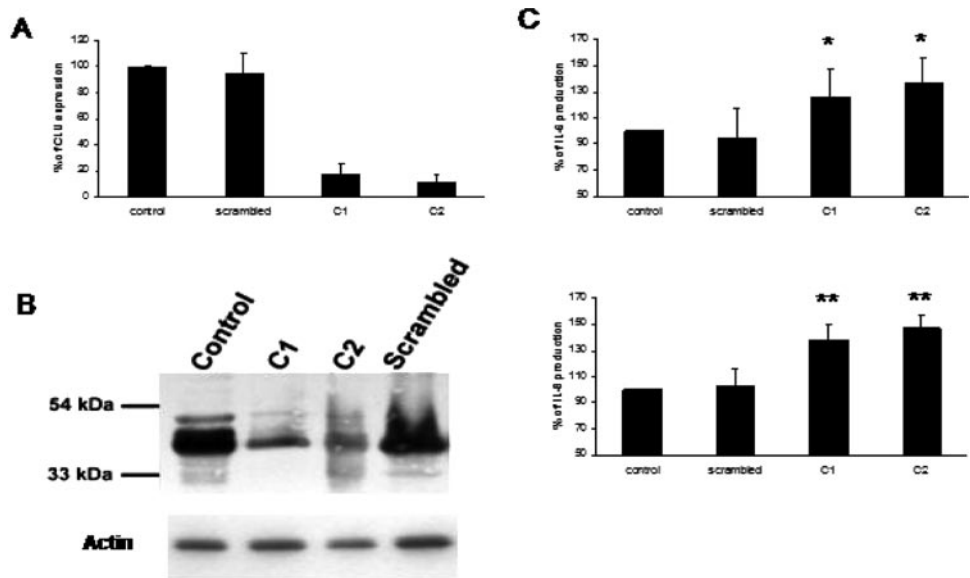


FIGURE 6. Effect of CLU silencing on IL-6 and IL-8 production. FLS were transfected or not with various small interfering RNA (siRNA) duplexes as indicated in *Materials and Methods*. **A**, CLU siRNA for 48 h, CLU mRNA was measured by QT-PCR. CLU expression by cells transfected in the absence of RNA duplexes from the transfection medium was set at 100%. No silencing of *clu* was seen in the presence of the control scrambled siRNA. **B**, Effective silencing of the CLU protein expression in FLS after *clu*-specific siRNA treatment for 48 h as revealed by whole cell lysate immunoblotting analysis (reducing conditions). C1 or C2 siRNA-treated cells show minimal levels of protein expression. **C**, Supernatants were collected at 48 h posttransfection and assayed for IL-6 and IL-8 by ELISA. IL-6 basal production range from 776 to 1332 pg/ml and IL-8 basal production range from 666 to 2262 pg/ml. Results are mean of five different experiments. Five different FLS (three RA and two OA) cultures were used in these experiments. Percentage of increase of IL-6 and IL-8 productions in C1-treated RA-FLS at 24 h were 113 ± 5.8 and 132 ± 13.3 , respectively, compared with 147 ± 16.1 and 146 ± 3.9 in C1-treated OA-FLS. * and **, Differences with respective controls at $p < 0.05$ and $p < 0.002$, respectively.

interacted with the NF- κ B in synoviocytes (Fig. 7, *C* and *D*). Altogether, our results demonstrate that endogenous Ser³²-Ser³⁶ phosphorylated I κ B α and CLU do interact in cells.

CLU inhibits TNF-induced activation of NF- κ B

We investigated the regulation of NF- κ B activation by CLU at the transcription level by determining the activation of the NF- κ B transcription factor in HeLa cells. Our study demonstrated that

there was a significant increase in the activation of NF- κ B in the nucleus when cells were stimulated either with TNF- α . The TransAM ELISA-like assay was used to gain a quantitative assessment of NF- κ B p65 DNA-binding activity in nuclear extracts. The assay uses immobilized dsDNA corresponding to κ B DNA elements to capture NF- κ B complexes and anti-RelA/p65 or anti-p50 Abs to detect bound transcription factors. Immobilized mutant DNA elements and excess dsDNA-containing wild-type elements

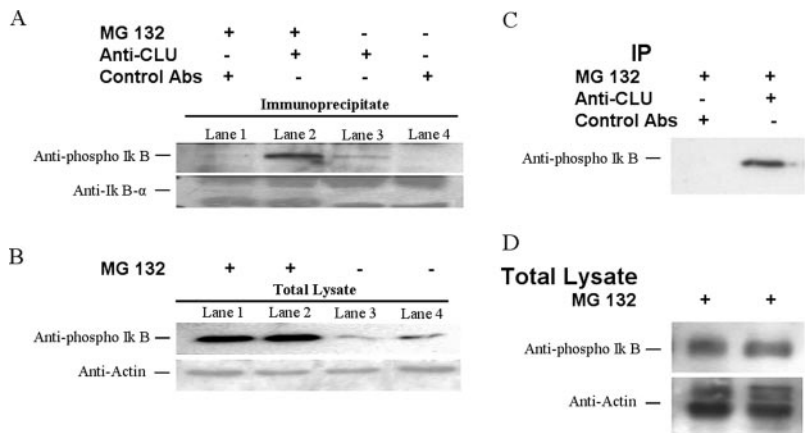


FIGURE 7. Clusterin interacts with phosphorylated I κ B α . **A**, HeLa cells were treated with 20 ng/ml TNF- α for 5 min in presence or not of MG132, lysed and immunoprecipitated with anti-CLU (lanes 2 and 3) or control Abs (lanes 1 and 4). Immunoprecipitates were probed either with anti-phosphorylated (upper panel) or anti-nonphosphorylated (lower panel) I κ B α Abs. Proteasome inhibitor (MG132) allows higher expression of phosphorylated I κ B α . **B**, Same amount of proteins were used for immunoprecipitation studies. Total lysate of HeLa cells used for immunoprecipitation experiments were probed either with anti-phosphorylated I κ B α Ab (upper panel) or with anti- β Actin Abs (lower panel). **C**, Ten million third-passage synovial cells were treated with 20 ng/ml TNF- α for 10 min following 4 h pretreatment in presence of MG132, lysed and immunoprecipitated with anti-CLU or control Abs. Immunoprecipitates were probed either with anti-phosphorylated I κ B α Abs. **D**, Same amount of proteins were used for immunoprecipitation studies. Total lysate of cells used for immunoprecipitation experiments were probed either with anti-phosphorylated I κ B α Ab (upper panel) or with anti- β -actin Abs (lower panel). Results are representative of two independent experiments made with two different FLS cultures.

are used to control for nonspecific factor binding. When HeLa cells (Fig. 8A) or synoviocytes (Fig. 8B) were transfected by CLU construct and induced by TNF- α a dose-dependent inhibition in NF- κ B activation was observed, which was determined by the quantitative analysis of NF- κ B/p50 and NF- κ B/p50 activation in nuclear fraction as shown in Fig. 8. We performed these experiments with Amaxa Nucleofector technology allowing transfection efficiency of >40%. Transfection of CLU to HeLa cells resulted in the inhibition of TNF- α -induced activation of NF- κ B p50 by 25% ($p < 0.05$), and NF- κ B p65 by 30% ($p < 0.01$), respectively, at the dose of 1 ng/ml at 10 min after treatment. Transfection of CLU to synoviocytes resulted in the inhibition of TNF- α induced activation of NF- κ B p50 by 33% ($p < 0.02$), and NF- κ B p65 by 41% ($p < 0.005$), respectively, at the dose of 5 ng/ml at 10 min after treatment. In contrast, the inhibition was absent or barely detectable when a very high concentration of TNF- α was used (20 ng/ml) (data not shown).

These observations further support the fact that CLU is an inhibitor of NF- κ B activation at least at suboptimal stimulation with TNF- α and that under these conditions the inhibition of cytokine-induced activation of NF- κ B by CLU resulted in a reduction in NF- κ B nuclear translocation.

Discussion

Among the different genes identified as a molecular signature of RA vs OA during our previous microarray study (3), *clu* was a good candidate because it was one of the genes that differentiate the most RA from OA tissues and also because it has multiple activities closely related to the pathological process involved in RA. Indeed, numerous functions have been assigned to CLU such as complement inhibition, antiproliferating factor, apoptosis, neg-

ative regulator of matrix metalloproteinase, chaperone, and cell-cell or cell-substratum interactions (18–23).

Using several complementary approaches, we found a highly statistically significant ($p < 0.0001$) lower expression of CLU mRNA in RA than in OA tissues with a magnitude close to 10-fold. Altogether, we evaluated the levels of CLU mRNA in 23 OA and 14 RA tissues and found almost no overlap between the two groups, which confirms that CLU gene expression may serve as a marker to differentiate OA from RA tissues. Moreover, CLU mRNA was also significantly underexpressed in RA as compared with healthy synovium. In contrast, CLU is overexpressed in OA as compared with healthy synovium, extending from chondrocytes to synoviocytes the results of Connor et al. (24).

Interestingly, cultured synovial fibroblasts exhibited the same pattern of CLU expression as ex vivo tissue with an 11-fold reduction in RA synoviocytes as compared with OA FLS. It is not certain that the genes expressed by a cell in the tissue are the same as those expressed in culture after a short- or long-term period. For instance, in a work using the microarray approach to study inflammatory diseases, Heller et al. (25) have already addressed such issue for synoviocytes and demonstrated that the genes and their respective levels of expression vary soon after culture, even when whole synovial tissue is cultured. Altogether, these results emphasized that the difference between OA and RA tissues was not due to different stages of diseases or to in situ production of cytokines and raised the hypothesis that an intrinsic mechanism may account for differences in CLU expression between OA and RA FLS.

Applying immunohistochemistry and in situ hybridization to synovial tissues, we provided evidence that CLU was present almost exclusively in synovial lining cells though it was also expressed in vascular endothelium.

These findings point to intriguing questions about the role of *clu* overexpression in this localization. Different mechanisms of action could be postulated to explain the cytoprotective role of CLU during cellular stress in the synovium: its function as an antiapoptotic signal, its protection against oxidative stress, its inhibition of the membrane attack complex of locally activated complement protein, or its binding to stressed protein thus avoiding aggregation in a chaperone-like manner. The defectiveness or loss of any of these mechanisms could increase the synovium destruction in RA. It is possible that CLU serves to maintain cell integrity, thereby being protective against inflammation in synovial tissue. This is further substantiated by a report by McLaughlin et al. (26) that provides insight into the in vivo function of *clu* in autoimmune myocarditis which has pathophysiological similarities with RA. CLU-deficient mice exhibited a more severe disease than wild type. Although the onset of the disease was concomitant with the induction of T cell-mediated responses, the extent of inflammation was significantly more severe and broad in *clu*^{-/-} mice, suggesting a cytoprotective role of *clu* in the progression of autoimmune diseases. Another study (27) found a reduced serum CLU expression in patients with active systemic lupus erythematosus compared with patients with RA, OA, or healthy donors, which corroborates the role of *clu* in autoimmunity. Measuring the levels of sCLU in the synovial fluid, we did not find any difference between patients affected from OA or RA both by ELISA (Fig. 4A) and Western blot (data not shown). This result is in accordance with previous reports (18, 27), and could be the consequence of either CLU production by cells other than synovial cells (28) or high stability of the extracellular form of the protein. In contrast, the lower *clu* mRNA levels observed in RA synovium could be related to the difference in expression of other forms of CLU protein. In agreement with this hypothesis, we found a striking difference in intracellular forms of CLU between

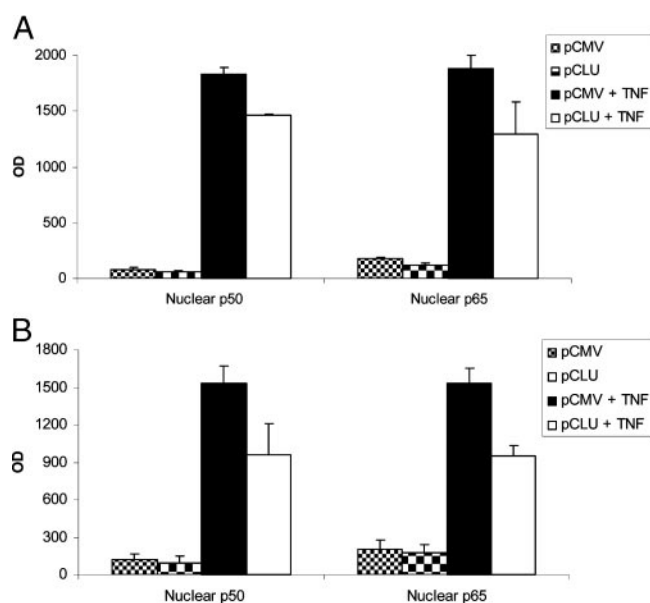


FIGURE 8. Clusterin inhibits NF- κ B translocation. **A**, HeLa cells were transfected with pCLU or pCMV and treated or not with 1 ng/ml TNF- α for 10 min. **B**, Same as in **A** following transfection of third-passage synovial cells treated or not with 5 ng/ml TNF- α for 10 min. Nuclear extracts were prepared and used in a TransAM (Active Motif) ELISA-like assay to quantitate the NF- κ B p50 and p65 DNA-binding activity. Data were processed as described by the manufacturer, with correction for nonspecific binding of the transcription factor. Data represent the difference of the ODs at the measurement (450 nm) and reference (650 nm) wavelengths after color development. Error bars represent the means and SDs of duplicate (HeLa) and triplicate (synoviocytes) experiments.

OA and RA samples with low or no expression in RA compared with OA, whereas the levels of the extracellular form were similar.

Determination of the exact role of *clu* in cell death has been the focus of numerous studies and has been largely debated (19). For instance, CLU is a stress-associated cytoprotective protein up-regulated by various apoptotic triggers in many cancers and confers treatment resistance when overexpressed whereas suppression of CLU expression resulted in increased cell death by drug treatment (15). In contrast, Trougakos et al. (16, 29) reported that in osteosarcoma cells either *clu* overexpression or *clu* knockdown could result in apoptosis of these cells. Furthermore, it recently became evident that numbers of *clu* functions were related to intracellular forms of the protein, in particular for apoptosis (30). Lastly, it has been demonstrated that overexpression of intracellular CLU caused clonogenic toxicity in PC-3 androgen-independent prostate cancer cells (31). In the context of RA, the low expression of *clu* suggests that *clu* might be a new antiproliferative agent in the synovium.

In contrast, a recent report showed that the intracellular level of CLU could be essential for regulation of NF- κ B activity (12). It is well-known that NF- κ B-induced gene expression contributes significantly to the pathogenesis of inflammatory diseases such as arthritis (32–37). Moreover, I κ B kinase 2 has been showed to be a key convergence pathway for cytokine-induced NF- κ B activation in synoviocytes (38–40). Thus, the intracellular defect of CLU in RA FLS opens new insights on the still unknown molecular mechanism of NF- κ B activation. Because NF- κ B is a key transcriptional regulator of IL-6 and IL-8 synthesis, we evaluated the ability of down-regulated *clu* to regulate expression of these two main cytokines involved in inflammation. Interestingly, knockdown of CLU, by means of siRNA, induced increase baseline production of both cytokines by FLS. These results cannot be explained by the induction of FLS death following CLU knockdown because IL-6 and IL-8 expression have been assessed at 24 and 48 h posttransduction whereas FLS death occurred only after 72–96 h following CLU knockdown.

Intracellular levels of CLU could be essential for regulation of NF- κ B activity via its effect on the modulation of I κ B expression (12). We demonstrated herein that CLU interacts with phosphorylated I κ B, either directly or indirectly, which results in an inhibition of NF- κ B translocation. These results were observed both in HeLa cells and third-passage FLS cultures showing that CLU interacted with the NF- κ B pathway in synoviocytes. We propose that CLU induces I κ B α stabilization by inhibiting E3 ubiquitin ligase binding to phosphorylated I κ B α . Consequently, low expression of CLU in RA would result in enhanced I κ B α degradation.

Our results demonstrate that the expression of CLU is differentially regulated in RA and OA synovium. At the protein level, this differential expression results in a low expression of intracellular form of the protein in RA synoviocytes both in synovium and in cultured FLS showing that it is an intrinsic property of these synoviocytes. To our knowledge, our findings are the first description of the involvement of intracellular forms of the protein in human inflammatory diseases. Considering the emerging role of CLU in apoptosis and NF- κ B signaling, these results highlight the interest of such glycoprotein in articular pathological processes and suggest that CLU is a potentially interesting target not only for RA but also for OA therapy. Altogether these data emphasize the potential critical importance of CLU expression and its regulation both in OA and RA. Clearly, understanding why this multifunctional protein is strongly differentially expressed in the course of these two diseases and defining the exact role(s) of the various isoforms of the protein are playing in either pathology is a real and crucial challenge.

Acknowledgments

We thank Olivier Nègre for his help on CLU cloning and Mariana Casas for her help on Northern blot. Special thanks to Profs. Saverio Bettuzzi and Maurizio Scaltriti for providing CLU constructions.

Disclosures

The authors have no financial conflict of interest.

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