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The CLU-files: disentanglement of a mystery

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Abstract: The multifaceted protein clusterin (CLU) has been challenging researchers for more than 35 years. The characterization of CLU as a molecular chaperone was one of the major breakthroughs in CLU research. Today, secretory clusterin (sCLU), also known as apolipoprotein J (apoJ), is considered one of the most important extracellular chaperones ever found. It is involved in a broad range of physiological and pathophysiological functions, where it exerts a cytoprotective role. Descriptions of various forms of intracellular CLU have led to further and even contradictory functions. To untangle the current state of knowledge of CLU, this review will combine old views in the field, with new discoveries to highlight the nature and function of this fascinating protein(s). In this review, we further describe the expression and subcellular location of various CLU forms. Moreover, we discuss recent insights into the structure of CLU and assess how structural properties as well as the redox environment determine the chaperone activity of CLU. Eventually, the review connects the biochemistry and molecular cell biology of CLU with medical aspects, to formulate a hypothesis of a CLU function in health and disease.

Keywords: Alzheimer's disease; apolipoprotein J; clusterin; LDL-receptors; molecular chaperones.

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Introduction: clusterin (CLU), its history and the challenge for researchers

In the year 1979, a protein was first discovered, whose complexity, abundance and involvement was initially unknown (1). Later, this sulfated glycoprotein was identified by virtue of its blood cell aggregating ability *in vitro*, and was therefore named clusterin (CLU) (2). Most strikingly, it surfaced in a broad spectrum of tissues and is overexpressed in the face of pathological processes, such as atherosclerosis, cancer and Alzheimer's disease (3–6). Moreover, CLU was found in the bodily fluids of almost all vertebrates from zebra fish to humans (7, 8). In accordance with these findings, an extensive repertoire of names emerged ranging from complement lysis inhibitor (CLI) and testosterone repressed prostate message-2 (TRPM-2) to apolipoprotein J (apoJ). Nevertheless, in 1992 the name CLU was the generally accepted term for all discovered proteins (9).

Thus far, CLU has been demonstrated to be a highly glycosylated glycoprotein of 80 kDa, consisting of two polypeptide chains connected by four to five disulfide bonds (10). The protein is one of the most prominent extracellular chaperones. The chaperone activity of CLU has been intensively studied by Mark Wilson and his colleagues (11–14). In connection with its chaperone activity, CLU is described as a protein that allows for the clearing of cellular debris and misfolded proteins, as well as the clearance of Aβ via the blood-brain barrier (BBB) (15–17). The concerted action of chaperone activity, scavenging- and clearance-function, may be one basis for the cyto- and tissue protective role of the protein (5, 15, 18, 19). Previously, it was further shown that the protein acts as a signaling molecule, inducing cellular prosurvival and proliferatory pathways, which may convey another mechanism of its cytoprotective function (20–22).

This understanding of CLU (as previously described) became complicated when intracellular CLU forms were described in damaged cells, in addition to the predominant secreted form, and attributed to a diverse and even opposing role (23–26). This review will summarize the

biosynthesis of CLU, its structural features and chaperone activity as well as focus on aspects of intracellular CLU forms to assess their cellular and physiological function. Finally, the involvement of CLU in the modulation of various signal transduction pathways, its role in the immune system and its involvement under pathological conditions, such as ischemia and necrosis will be discussed. The goal is to summarize current views about CLU to enable future studies to finally solve the 'CLU mystery'.

Biogenesis of clusterin (CLU)

The biosynthesis of human secretory CLU (sCLU) follows the canonical pathway of secretory proteins (Figure 1). Translation of CLU starts from a startcodon located on exon 2 of the CLU-mRNA, resulting in a pre-proprotein composed of 449 amino acids. The first 22 amino acids represent a signal sequence ensuring co-translational translocation into the endoplasmic reticulum (ER). Subsequently, the signal sequence is cleaved off and four to five disulfide bonds are formed (10). Hereafter, N-glycosylation at six Asn-residues ($Asn_{86,\ 103,\ 145,\ 291,\ 354,\ 374}$) takes place converting the proprotein to a high-mannose ER-precursor (pre-secretory CLU, psCLU) of 60 kDa (27, 28). After translocation to the golgi-apparatus, complex carbohydrate moieties are attached to the maturating psCLU, composed of galactose, fucose, mannose, N-acetylglucosamine and N-acetylneuraminic acid (29–31). The resulting 80 kDa protein is further cleaved by a furin-like proprotein convertase (FC) (amino acid recognition motif: RIVR) between Arg_{227} and Ser_{228} to produce an N-terminal α-chain and a C-terminal β-chain which are interlinked by disulfide bonds (29–31). Finally, mature sCLU is secreted as a heterodimeric complex of two 40-45 kDa subunits (28, 29).

Under cellular stress additional CLU forms emerge by diversion from the canonical secretory pathway (Figure 1). They encompass core-glycosylated forms, presumably derived from retrotranslocated CLU out of the ER (32, 33) or intracellular forms which failed to be segregated into the ER and thus are not carrying any sugar residues or disulfide bonds, respectively (34). In addition, intracellular forms may arise from alternative splicing events or from non-canonical/alternative translation-initiation start sites downstream of the ER-leader peptide (Figure 1) (34). All mentioned intracellular CLU forms are single-chain proteins, since they are not proteolytically processed.

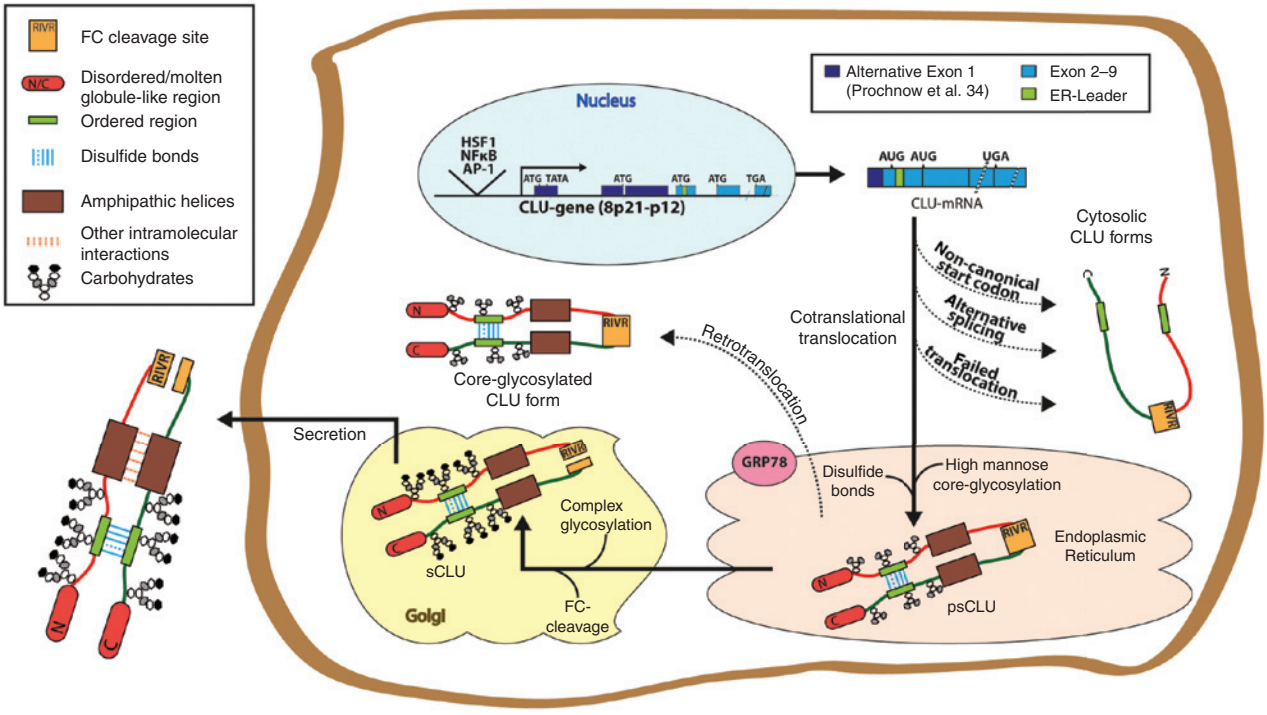

Figure 1: Illustration of CLU biosynthesis.
The canonical pathway of secretory proteins leads to the synthesis of secretory CLU (sCLU). It undergoes proteolytic maturation and carries complex carbohydrate moieties as well as disulfide bonds. Upon cellular stress, non-canonic CLU forms emerge, mainly derived from failed translocation, alternative splicing or translation-initiation events on exon 3, as well as from retrotranslocation. All non-secreted CLU forms are incompletely maturated. For details please see text.

Structure and function of secretory clusterin (sCLU)

Structure

So far, one of the most challenging questions pertains to the structure of sCLU. Over the past three decades numerous studies have been conducted, but the structural properties of sCLU are still not fully understood. One reason for this lack of knowledge lies in the aggregating nature of sCLU. Besides interacting with a plethora of ligands, it aggregates with itself, forming di-, tetra- and even higher oligomers, depending on the pH-value (2, 12–14, 35). In combination with distinct ligands, such as fibrinogen, sCLU can form high molecular weight complexes with a putative molecular mass up to 40 000 kDa and a diameter ranging from 50 to 100 nm (14). These characteristics together with a heterogeneous glycosylation pattern, renders it difficult to obtain X-ray structures or reliable NMR spectra from purified sCLU-samples (13, 36). Additionally, it is hard to obtain suitable protein samples in sufficient quantities (30, 36). Nevertheless, a number of studies have been conducted, which focus on distinct properties of sCLU and shed light on its structural elements (10, 13, 37–39). Beyond doubt, the primary structure of sCLU is highly conserved between different species, with the highest homologies found in the regions of disulfide bonding cysteins and the FC cleavage site pointing to their significance regarding sCLU-function (8, 40). Already after the first successful isolation of sCLU, its amphipathic character was apparent (2). Later on, secondary structural elements were investigated by means of circulardichroism (CD)- and infrared-spectroscopy (14, 30, 35, 38, 41, 42). In all of these a predominant α-helical content of up to 60% was calculated. *In silico* analyses further predicted five amphipathic α-helices (37). On the tertiary structure level, sCLU is believed to belong to the family of intrinsically disordered proteins, meaning that it partially lacks a defined tertiary structure, thus exposing hydrophobic regions, so-called molten globule-like domains, towards the external space (43). This in turn allows for binding to other molecules via hydrophobic interactions (Figure 2A). Remarkably, sCLU shares this

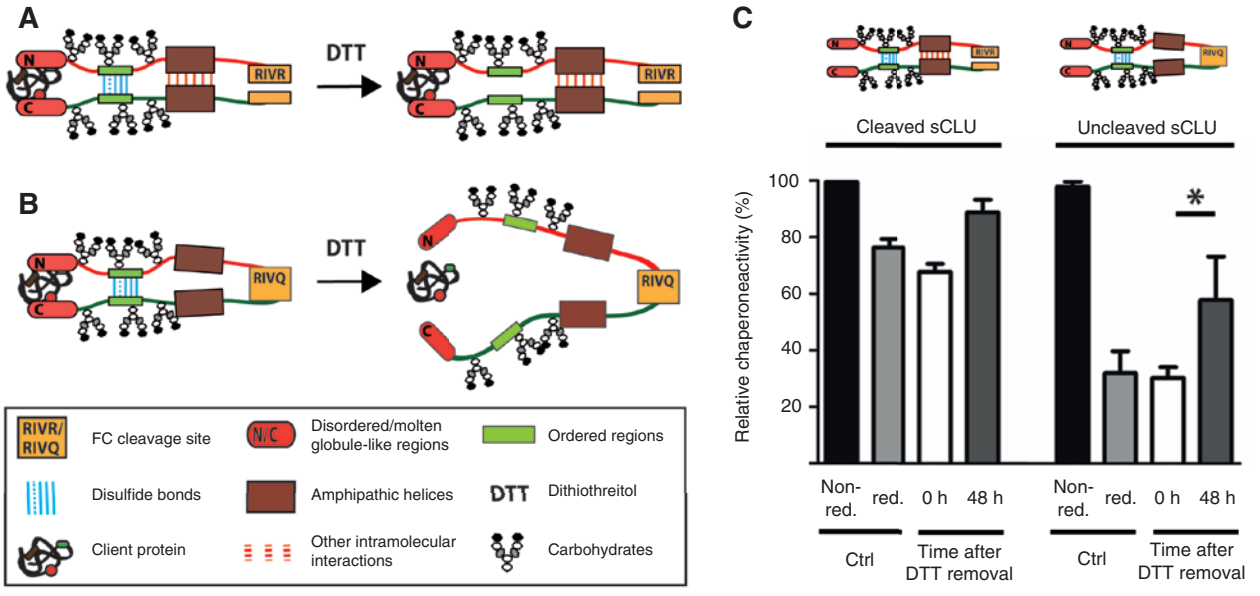

Figure 2: Schematic depiction of sCLU chaperone activity and hypothetical influence of structural elements.
Fully maturated sCLU is stabilized by disulfide bonds and intramolecular interactions, such as hydrophobic interactions in the region of amphipathic helices (A). In contrast, artificial uncleaved sCLU relies on its disulfide bonds and therefore displays a high sensitivity towards reducing conditions (B). Preliminary data show that uncleaved sCLU partly regains its chaperone activity after removal of DTT within 48 h (C). The procedure of sCLU purification, DTT treatment and chaperone activity assay are in accordance with Rohne et al. (30). After 12 h of incubation with or without 40 mM DTT at 37°C upon gentle agitation, sCLU was subsequently used for chaperone activity assays (Ctrl reducing and non-reducing) or DTT was substituted by PBS using a Vivaspin 4 5000 MWCO PES (Sartorius) at 4°C and used for chaperone activity assays subsequently or after 48 h at 4°C. All chaperone activity assays were performed in the presence of 5 mM DTT with Catalase being the client protein. Additionally, BSA was mixed with Catalase and served as a negative control to calculate the relative chaperone activity of sCLU as described by Rohne et al. (30). For evaluation, the data of two to three independent experiments, each with the mean of two to three measurements were used (unpaired *t*-test $*p<0.05$). The error bars correspond to the mean±standard errors.

feature with other intrinsically disordered proteins, most prominently heat shock protein (Hsp) 27 or α-crystalline (11, 44). As pointed out in the following, sCLU indeed possesses a chaperone activity similar to that of Hsp27 and other heat shock proteins.

Chaperone and scavenging function

Chaperones are part of the basic molecular defense mechanism for a cell overcoming stress situations induced by UV light, ionic irradiation, heat, oxidants, heavy metals, hyperoxia or certain drugs. They can be divided into different classes: chaperonins, Hsp100, Hsp90, Hsp70, Hsp60 and small Hsps (sHsps) (45). These intracellular chaperones regulate the correct folding of maturing proteins or prevent the aggregation of denatured proteins thus adopting a cytoprotective function. Some even initiate the refolding of misfolded proteins in an ATP-dependent manner (Hsp70, Hsp90, chaperonins). For chaperones, natively disordered regions are required for binding with a plethora of structurally diverse client proteins (46).

Similar to the above mentioned Hsps, CLU-mRNA is up-regulated upon cellular stress due to a heat shock element-like motif present in the CLU promotor (19, 34, 47, 48). The chaperone function of sCLU was revealed 20 years after the protein's discovery (11). Thus it became the first molecular chaperone known to act outside of the living cell. Similar to sHsps inside the cell, it is assumed that sCLU binds to client proteins through its molten globule-like regions via hydrophobic interactions (13, 14, 37, 49). In particular, proteins that are on the off-folding pathway, e.g. as induced by heat or reducing conditions, serve as clients for sCLU (12, 30). The binding occurs in an ATP-independent manner, leading to formation of soluble high molecular weight complexes with different molar chaperone-client ratio between 1:5 and 1:0.33 (14, 50, 51). As a consequence, sCLU prevents the uncontrolled aggregation of the denaturing proteins. Interestingly, binding of sCLU does not retain potential enzymatic activities of denaturing client proteins. However, in cooperation with ATP-dependent Hsps, such as Hsc70, the enzymatic activity of some proteins can be restored (11, 50).

In cell culture experiments it was observed, that sCLU is able to facilitate the uptake of the bound client complexes into surrounding tissue cells to allow their removal via lysosomal digestion (Figure 3) (15, 52). Receptors responsible for the binding of sCLU and/or sCLU-protein complexes, such as megalin (15, 42, 53), LRP1 (15) and ApoER2, VLDLR (22, 54) are members of the LDL-receptor gene family. In a recent study, sCLU-client complexes were intravenously injected into mice, confirming the sCLU-chaperone/scavenging activity (17). sCLU-ligand complexes were found to be enriched in the mice liver and kidneys, which are involved in degradation and subsequent excretion of cellular toxins. The finding that the enrichment in the liver can be blocked by administration of fucoidan (a potent inhibitor of scavenger receptor class A, E and F) indicates that sCLU is able to interact with scavenger receptors beside those of the LDL-receptor gene family. In addition, a decrease in the removal of glomerular protein deposits was found in CLU-K.O. mice (55). In humans, reduced sCLU secretion is accompanied by a higher risk for Alzheimer's disease (56). These findings are in line with observations that show that sCLU prevents the aggregation and oligomerization of Aβ and transthyretin (57, 58). Conclusively, these data argue for the pronounced role of sCLU in protein homeostasis in the body.

Disulfide bonds & proteolytic maturation: Crucial components or dispensable features?

One of sCLU's prominent features is its maturation into an α- and a β-chain occurring within the golgi apparatus of vertebrate cells. The two subunits are connected by 4–5 highly symmetrical disulfide bonds. As suggested by Bon-Hong Min's group, the formation of disulfide bonds is a prerequisite for sCLU maturation/synthesis (59).

Interestingly, a reduction of these disulfide bonds in mature sCLU does not inhibit its activity in chaperone activity assays (11, 12, 50). This tolerance of sCLU for reducing conditions raises the question whether the α- and β-chain of sCLU act independently as chaperones or whether the disulfide bonds are extraneous for their intramolecular association. It must be noted that the proper maturation of sCLU is dependent on appropriate disulfide bond formation. Treatment of cells with dithiothreitol (DTT), which prevents disulfide bond formation in the ER, abolishes the secretion of sCLU (60). More recently it was demonstrated that mutations in the cys-rich region of the CLU protein are leading to reduced secretion of sCLU in patients with Alzheimer's disease (56).

Additionally, it was shown that the inhibition of the proteolytic maturation by *in vitro* mutagenesis of the furin-like proprotein convertase (FC) cleavage site, which generates uncleaved sCLU, does not interfere with sCLU-maturation and its chaperone activity but rather renders it highly sensitive to reducing conditions (30). The lack of proteolytic maturation may therefore cause

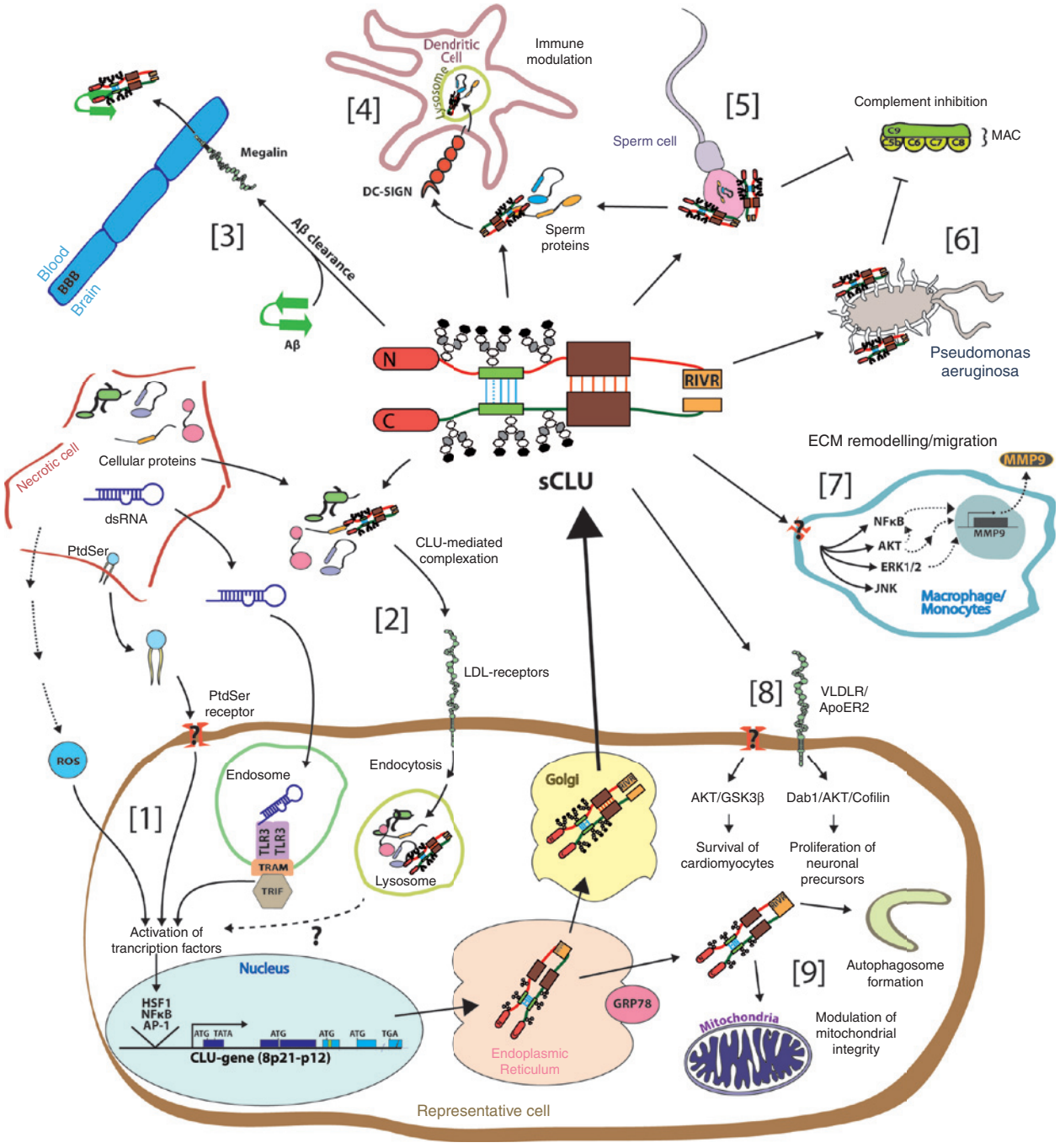

Figure 3: Role of CLU in cell biology, pathology, immunobiology and beyond.
For description please see text.

an impaired 'flexibility'of sCLU (Figure 2B). Studies of Bailey et al. revealed that the N- and the C-terminus of sCLU are regions with disordered/molten globule-like domains which are likely involved in client binding (37). Therefore, the only regions left for stabilizing the sCLU protein are probably the disulfide bonds and the putative amphipathic helices next to the FC cleavage site. As mentioned above, mature sCLU is still sufficiently active even after long-term reduction (11, 12, 30, 50). However, the activity of uncleaved sCLU after long-term reduction is tremendously impaired (Figure 2) (30). Thus, the amphipathic domains or other neighboring regions may be responsible for sCLU protein stabilization. Intriguingly, preliminary data from our lab indicate that uncleaved reduced sCLU partly regains its chaperone activity when DTT is removed (Figure 2C). In conclusion, we propose

that the disulfide bonds are on one hand important for correct folding and maturation but on the other hand are not essential for the chaperone function of mature sCLU. However, we cannot exclude the possibility, that they are involved in other processes. The latter is supported by the notion that sCLU is able to restore the function of glutamine synthetase by acting as a thiol specific-antioxidant. However, this activity is impaired when the disulfide bonds of sCLU are alkylated or when the electron donor is not thiol-based (59).

Carbohydrates: negligible or essential?

Another prominent feature of sCLU is its high level of glycosylation resulting in carbohydrates, which comprise about 30% of the molecular weight (61). The glycosylation pattern of human serum sCLU was elucidated in 1997. Six N-linked carbohydrate attachment sites are found within the molecule, with three on the α- and three on the β-chain in humans (29). The carbohydrate composition is highly diverse and depends on the expressing tissue (4, 31). Serum sCLU mainly contains carbohydrate moieties with sialic acid in a mono- and biantennary fashion (29, 31). Similar glycosylation patterns were found with recombinant human sCLU (30). In contrast, semen sCLU carries mainly fucose-rich glycans, enabling semen sCLU to bind to the C-type lectin receptor DC-SIGN found on dendritic cells. This binding was not observed with serum sCLU (31, 62). Therefore, the glycan moieties either change the spatial structure of sCLU so that its receptor affinity is altered, or the receptors and other interacting molecules have varying affinities to distinct glycan moieties.

Interestingly, it was observed that the glycosylation of sCLU is crucial for correct polar secretion in epithelial cells (28), but not for chaperone function (41). sCLU treated with the endoglycosidase PNGase F only showed little decrease in chaperone activity (41). Indeed, it was recently shown that the removal of the terminal sugars by exoglycosidases does neither alter the chaperone activity nor the secondary structure of sCLU. However, it became obvious that plain PNGase F-digestion results in residual sugars still being attached to the protein-core. Fully deglycosylated recombinant human sCLU was obtained by application of a more elaborated protocol and was shown to possess a tremendously decreased chaperone activity. CD-spectroscopy further revealed that upon full deglycosylation the amount of α-helices lowered from 60% to 40%, whereas the amount of disordered regions increased significantly from about 20% up to 30%. After removal of all carbohydrates the rearrangement of the secondary structure indicates that the folding of sCLU is correlated with its glycosylation (30). In summary, the core-glycosylation is a prerequisite for the folding and chaperone activity of sCLU and terminal sugars might be instrumental for other mechanisms, such as receptor binding and signal transduction (21, 31, 62).

Intracellular clusterin: significance vs. occurrence

Nuclear CLU (nCLU)

Since its discovery in the early 1980s until the mid-1990s, CLU was regarded solely as a secreted protein (sCLU). It was not until 1995 that researchers observed an association of CLU with the nucleus after induction of cell death with anti-estrogens (26) or TGF-β (63). They speculated that if translation would start at a start codon located on exon 3 of the CLU-mRNA, an otherwise cryptic nuclear localization sequence (NLS) could be active. Thus, Reddy et al. postulated internal translation initiation as one possible mechanism leading to N-terminally truncated forms of CLU. These truncated forms are lacking the signal sequence for segregation into the ER, exposing an NLS and therefore localizing CLU in the nuclear compartment (Figure 1). This hypothesis, however, is still lacking supporting data.

Around the turn of the millennium, the group of David Boothman reported the expression of a nuclear form of CLU (nCLU) in MCF-7 cells after treatment with ionizing radiation. They proposed that this nCLU form would act as a pro-death factor by interacting with the DNA-repair-associated protein Ku70. While it was initially assumed that nCLU derives from an ER-borne psCLU form (64), the authors stated in later publications that ionizing radiation favors internal translation initiation at a start codon on exon 3, resulting in the expression of nCLU (Figure 1) (24, 65). Finally, in 2003, the same group reported that an exon-skipping event, resulting in a transcript lacking exon 2 with its signal sequence coding region (hereafter termed variant 1 [Δex2]), precedes the expression of nCLU (25). Surprisingly, overexpression-experiments with variant 1 [Δex2] resulted in strong cytosolic localization of the corresponding protein as determined by confocal microscopy. Nuclear associations, however, could only be detected by overexpression of artificial constructs lacking distinct portions of variant 1 [Δex2]. Despite this

controversial observation, the authors hypothesized that the protein translated from the exon 2-lacking mRNA is the pre-nuclear form of nCLU. It would then be transformed into mature nCLU of unknown post-translational modifications induced by ionizing radiation. However, experimental proof of this hypothesis is still missing.

Throughout the first decade of the 2nd millennium many research groups claimed a nuclear localization of CLU in stressed cells, including cellular stress induced by various agents such as Etoposide (66–68), Interleukin-6, Somatostatin (69), heat (70), Ca^{2+}-depletion (67, 71), serum-starvation (72), TNF-α-treatment (73), indocyanine-treatment with green tea extracts (74), proteasome inhibition (75), treatment with 5′-Fluorouracil and Fas receptor-binding antibodies (76) or vanadium-treatment (77). Furthermore, nuclear localization of CLU has been observed upon overexpression of c-fos (77), Interleukin-24 (78) or pVHL (79), as well as spontaneously in untreated cells (69, 76, 80–82). Additional to the mechanisms discussed above, it was argued that inhibition of sCLU secretion could lead to intracellular accumulation and subsequently nuclear localization (83). However, diminished secretion and therefore intracellular accumulation of CLU, as described by Bettens et al. (56), did not provide any evidence for nuclear localization of CLU.

Another point of intense debate is the potential exon 2-skipping of CLU-mRNA (Figure 1). The resulting variant 1 [Δex2], was commonly considered to explain the occurrence of nCLU in many studies, although rigorous mRNA-analyses have not been performed. This led to controversies, as some groups could not validate the existence of variant 1 [Δex2] (84–86). It was speculated that its expression might be limited to MCF-7 cells or even an experimental artifact (75, 84–87). However, Prochnow et al. confirmed the existence of variant 1 [Δex2] in several human cell lines using validated, variant-specific primer sets for RT-PCR (34). Yet, mRNA-quantification indicated that variant 1 [Δex2] accounts for <0.13% of total CLU-mRNA, even in stressed cells. The exon 2-containing variant 1, in contrast, represents the pre-dominant CLU-mRNA (>99% of total CLU-mRNA). In this study it was also shown that the protein encoded by variant 1 [Δex2] localizes solely in the cytoplasm of unstressed and stressed cells. In fact, very recent studies support this observation, challenging the theory of a nuclear localization of CLU (32, 79, 80, 88–91). Only by incorporating an artificial nuclear localization sequence (NLS) at the 5′-end of variant 1 [Δex2], a nuclear localization of the translated protein could be achieved (92). This in turn renders the existence of a functional NLS hidden in the CLU pre-proprotein unlikely.

CLU associated with mitochondria

Over the course of the last few years, an association of CLU with mitochondria has frequently been reported and interactions with intrinsic apoptose-related proteins Bax and Bcl-xL have been discussed (32, 93–96). Thus, it was speculated, that either psCLU or sCLU might act anti-apoptotic by sequestering Bax in its inactive state in the cytosol (95, 96). However, in none of these studies it was investigated, how extracellular sCLU or ER/golgi-resident psCLU can reach the cytosol in order to bind Bax (Figure 1). Li et al. (32) recently showed that a hypoglycosylated form of sCLU can escape the secretory pathway with the aid of the chaperone GRP78 (BiP) and stabilize the mitochondrial membrane to avoid paclitaxel-mediated apoptosis (Figures 1 and 3). An interaction with Bax was, however, not investigated in this study. Other reports suggest that CLU can act pro-apoptotic by preventing Bcl-xL from binding to Bax (88, 97). The authors assumed that non-secreted CLU forms similar to nCLU, are responsible for this effect. This was attributed to a potential BH3-domain found *in silico* within the nCLU-sequence (97). However, upon overexpression of distinct intracellular CLU forms, no pro-apoptotic properties could be assigned to any case (34). Thus, the significance of CLU association with mitochondria still needs to be challenged.

How significant are distinct intracellular CLU forms?

The diverse results and proposals put forward in the studies on intracellular CLU over the past 20 years are inconclusive and even contradictory. One reason for this may be that the described effects are restricted to stressed/damaged cells and that the abundance of intracellular CLU forms is negligible (34). Most importantly, to our knowledge, no distinct intracellular CLU forms have been described in other organisms besides humans, yet. Additionally, CLU forms lacking carbohydrates and/or proteolytic maturation are not capable to maintain a chaperone activity in the cell (30). Furthermore, Prochnow et al. found no evidence for an apoptosis-modulating effect of unglycosylated cytosolic CLU forms. Therefore, we suggest that intracellular CLU forms may accidently evolve in compromised cells and might have no beneficial function for the cell. One exception may be hypoglycosylated psCLU forms retrotranslocated from ER/golgi. These forms have been described in the course of modulating mitochondria integrity or autophagy to promote cell survival (Figure 3) (32, 91).

Cellular challenges: from necrosis to immunobiology and beyond

sCLU and necrosis

The expression of sCLU is highly correlated to tissue degeneration, necrosis and redox imbalances (98, 99). The stress-associated upregulation in response to UV light, heat-shock, oxidants, heavy metals and in particular proteotoxic stress is mediated by the *clu* promotor which contains HSF1, NFκB and AP-1 binding sites (100). Cytotoxic stress can also be induced by necrosis in neighboring cells (Figure 3) (101). As a consequence, misfolded proteins, free radicals, lipids and other components accumulate in the extracellular space causing an activation of the immune system and affect neighboring cells (101, 102). Remarkably, it was observed that CLU upregulation also occurs in cultured cells upon exposure to necrotic cell debris (103). This upregulation is driven by components of the necrotic cells, such as membranous phosphatidylserine (PtdSer) (103) or by RNA released from the cytosol (Figure 3) (104). PtdSer, under vital conditions, is restricted to the cytosolic side of the plasma membrane and is exposed to the extracellular environment upon damage or apoptosis (105). Extracellular RNA is endocytosed by surrounding cells and upregulates CLU via the TLR3-signaling pathway (104). Once sCLU is secreted into the extracellular space, it mediates the binding and endocytic uptake of cellular debris by receptors of the LDL-receptor gene family, such as megalin or LRP1 thus reestablishing the extracellular proteostasis (Figure 3) (15, 30).

sCLU and immunobiology

Quite recently, data suggest that semen sCLU can bind stress-damaged proteins, facilitating the uptake by dendritic cells via DC-SIGN thus making a priming of immune cells feasible (Figure 3) (31, 62). As a further consequence, sCLU has been attributed to mediate tolerance of the female genital tract towards male antigens (Figure 3) (62). This suggests an immune regulatory role of sCLU, which is evident from multiple *in vivo* and *in vitro* studies. Initially, sCLU was found to be co-localized with complement SC5b-9/MAC (membrane attack complex) in glomerulonephrititis but not in healthy individuals (106). Subsequently, in association with the SC5b-9 complex sCLU was characterized as an inhibitor of the complement system (Figure 3) (38, 49, 107). The complement system causes cell damage under renal pathologies. Thus, sCLU may alleviate renal injuries by preventing an uncontrolled MAC activity (108). However, in 1999 Hochgrebe et al. (109) showed that complement inhibition by sCLU is not possible at physiological concentrations. Despite these facts, the most recent data suggest that *Pseudomonas aeruginosa* can protect itself from complement attack by recruiting sCLU (110). Similar suggestions were made for sperm cells in the female genital tract (Figure 3) (49). Thus, an increase in the local sCLU concentration may achieve a complement inhibiting effect.

Additionally, other data support a role of sCLU in immune modulation: It was shown that sCLU synergize with IL-2 to enhance the proliferation of natural killer cells (111). Moreover, sCLU is described as a transcriptional target of the lymphotoxin-β receptor in germinal centers during an immune response (112, 113). It was further demonstrated that sCLU improved the viability of B lymphocytes (113). In addition, it is important to note that CLU was described as a necessary factor for curation of autoimmune myocarditis, which is accompanied by an increased level of necrosis and inflammation (102, 114). These conclusions were further supported by observations after intravenously administration of sCLU in rats during myocardial infarction. After treatment with sCLU, the infarct size was significantly reduced and led to macrophage invasion (115). This finding is supported by an elevated chemotactic migration of macrophages due to increased matrix metalloproteinase-9 (MMP9) expression induced by sCLU via the ERK1/2-, PI3K/AKT- and NFκB-signaling pathway (Figure 3) (21, 116). The ability of sCLU to modulate macrophage function was further confirmed by an additional study which demonstrated that the migratory effect is related to TNF-α secretion induced by sCLU (116). However, van Dijk et al. (20) have found no evidence of a difference in wound healing after sCLU application. Rather, sCLU protects cardiomyocytes from apoptosis by inducing the PI3K/AKT-signaling pathway (Figure 3).

It is important to note that neither the protective effect of sCLU on cardiomyocytes, the reduction of infarct size nor the interaction with dendritic cells are related to receptors of the LDL-receptor gene family (20, 62, 115). It is therefore important to emphasize that the characterization of other sCLU binding receptors is essential for the unraveling of sCLU function.

sCLU and tissue repair

The significance of sCLU in tissue protection/remodeling was found to be important because of its potential to

prevent organs from damage under ischemic conditions, e.g. ischemia-reperfusion injury (IRI) after transplantation (117). The ischemia-reperfusion damage occurs when the bloodstream is reestablished after periods of limited nutrient and oxygen supply. The consequence of this mechanism leads to initial necrosis, followed by massive apoptosis of the surrounding tissue (102). The surrounding apoptosis is believed to be due to the release of free radicals, generating lipid radicals in the cellular membrane and therefore causing tremendous cellular damage (Figure 3) (101). In fact, sCLU is upregulated after oxidative stress caused by H_2O_2 or cigarette smoke, protecting the cells and maintaining their viability (19, 20, 59, 118). In the case of transplant medicine, it was shown that supplement sCLU in University of Wisconsin-solution (UW-solution) is feasible to prolong the handling-time before an organ can be transplanted (119). Similarly to this finding, it was observed that a genetic inactivation of the CLU gene leads to an increased organ rejection as well as impaired cell viability. These protective actions are probably due to increased membrane fluidity in the presence of sCLU, minimizing cold-induced stiffening and shear stress (117). Furthermore, sCLU is a candidate biomarker for damage in the proximal tubule of the kidney (120). Additionally, it was shown that over time, the absence of sCLU leads to an increased accumulation of components from the immune system within the glomeruli (55). Several *in vitro* studies suggest a cytoprotective function of sCLU after gentamicin- or H_2O_2-induced cytotoxicity in kidney cells (121, 122). When inducing IRI by clamping the renal pedicles of mice, sCLU is an essential factor for renal regeneration. In contrast to macrophages, however, sCLU has no promigratory effect on renal tubular epithelial cells (TECs) but promotes proliferation and cell viability of TECs. In contrast to wild type mice, CLU K.O.-animals exhibit a remarkably decreased survival rate of about 90% to 30%, respectively (123). Previously it was shown that after unilateral ureteral obstruction (UUO) the presence of sCLU was a prerequisite to suppress TGF-β mediated upregulation of plasminogen activator inhibitor-1 (PAI-1). PAI-1 prevents rearrangement of the extracellular matrix and thus attenuates plasmin activation leading to fibrosis (124). However, in contrast to Nguan et al. (123) no influence of sCLU on proliferation or apoptosis on tubular epithelial cells was observed. Finally, it is important to mention that although Girton et al. (121) have excluded an involvement of megalin, the participating receptors mediating the cytoprotective effect of sCLU (directly or indirectly) are still unknown.

sCLU and the nervous system

Although it is well established that sCLU is cytoprotective in the kidney and in the heart, the role of sCLU in the nervous system is still challenging. As mentioned initially, sCLU was also described as an apolipoprotein (apoJ) which is present in serum (free and bound to high density lipoprotein (HDL) particles). sCLU(apoJ)-containing HDLs account for approximately 2% of plasma HDLs (125) with about 20% of the circulating sCLU being actually bound to HDLs (126). In contrast to the brain, sCLU(apoJ)-containing HDL particles in plasma are more lipid-rich (38, 127). In the mammalian brain sCLU (apoJ) and apoE are the two most abundant apolipoproteins [reviewed by Wang and Eckel (128)].

Intriguingly, both apolipoproteins are involved in similar mechanisms, such as Alzheimer's disease or cognitive and memory processes (18, 128–130). It is important to note that even though sCLU is a constituent of HDLs, which are the only lipoprotein subclass crossing the BBB, only LRP1 and SR-B1 are involved in lipid homeostasis of the brain (128). The remaining lipoprotein receptors are involved in a plethora of other functions (128, 129).

Inside the brain, CLU is primarily expressed and secreted by astrocytes but also by other types of cells, e.g. neurons (127, 131, 132). Upon neuronal death, ischemia and with advancing age, however, a significant upregulation of CLU is observed (7, 131, 133, 134). Previously, it was not clear whether an elevated level of CLU in the brain was helpful or harmful. In 2001, two groups performed brain-ischemia experiments and reported contradictory results (133, 134). Whereas Han et al. (133) found that the absence of CLU reduces cell death in neonatal hypoxia-ischemia, Wehrli et al. (134) observed a neuroprotective effect of CLU in permanent focal cerebral ischemia. It is believed that these different findings are due to different experimental settings; more beneficial effects of CLU *in vivo* and *in vitro* were observed elsewhere. For instance, brain ischemia induced by permanent middle cerebral artery occlusion shows heavily impaired tissue remodeling in CLU K.O.-animals. In wild type animals, however, a significant CLU upregulation by astrocytes and increased microglia/macrophage invasion was detected (135). Interestingly, another study has shown that microglia activation by sCLU leads to chronic inflammation and thus neurotoxicity due to observing an increased release of nitrite oxide and TNF-α (136). Moreover, sCLU in astrocyte-conditioned media from the hippocampus and midbrain had a contributing effect on cell survival and differentiation but not on proliferation of neural precursor cells (137). However,

Leeb et al. recently showed that sCLU promotes ApoER2/VLDLR-mediated proliferation by inducing the Dab1/AKT-signaling pathway in neuronal precursors, suggesting a role of sCLU in neurogenesis (Figure 3) (22). In contrast, conducting oxygen-glucose deprivation experiments increased propidium iodide (PI) uptake and impaired excitatory postsynaptic potential (EPSP) in hippocampal neurons of wild type animals as compared to CLU K.O. mice were observed (138).

Further, even though being an established genetic risk factor for late onset Alzheimer's disease (56, 130), the mode of sCLU action in Alzheimer's disease is still unclear. In fact, it is not fully understood if sCLU protects or promotes Aβ-induced cell death, respectively. At the one hand it was shown that sCLU binds Aβ, preventing its oligomerization/fibril formation and finally allowing the megalin-mediated removal across the BBB (Figure 3) (16, 42, 52). In other reports, however, a contribution to complex formation and therefore neuronal death was reported (139, 140). Intriguingly, it was further suggested that smaller oligomers would be more harmful to the cell by inducing oxidative stress after interaction with the plasma membrane. In contrast, higher oligomers generated by chaperone-client interactions have a reduced cytotoxic potential (18, 141). In line with this, other reports indicating that sCLU stabilizes Aβ-oligomers preventing their further aggregation or disaggregation (58). Moreover, it was demonstrated that a lower sCLU to client ratio promotes, whereas a higher ratio prevents cytotoxicity (51). Therefore, the role of sCLU may depend on its concentration as suggested by Bettens et al. (56).

In conclusion, the role of CLU in the brain is still unresolved. However, it is thinkable that sCLU acts in a combined manner as a receptor-mediated signal transducer, a molecular chaperone which facilitates the removal of misfolded proteins or debris after injury and in pathologies, as well as a lipid carrier to reestablish damaged nerve tissue. These processes, however, are fine-tuned and susceptible to disturbances that may lead to opposing results.

Perspectives

The enigmatic protein clusterin (CLU) has challenged researchers for almost four decades. Its versatility has brought numerous insights into different fields of biomolecular science but has also led to even more questions. In this review, we provided a comprehensive understanding of the basics of CLU and gave a wider view concerning future research possibilities. Thus far, the biosynthesis of CLU, its chaperone/scavenging activity and its occurrence in diseases was intensively investigated. It can be concluded that sCLU is a cytoprotective factor that is particularly involved in situations of cell/tissue damage.

An overview of CLU's role in health and disease is depicted in Figure 3. In situations, such as tissue damage, CLU upregulation is induced by components of necrotic cells and by oxidative stress via stress inducible promotor elements [1]. Subsequently, sCLU is secreted and allows the binding and removal of proteins released from necrotic cells [2]. Moreover, sCLU stimulates the clearance of misfolded protein such as Aβ via the BBB [3]. The removal of proteins and Aβ is mediated by receptors of the LDL-receptor gene family. Moreover, in the female genital tract sCLU-associated with sperm cells, allowing the uptake of male antigens (sperm proteins) into female dendritic cells via DC-SIGN and, thus, preventing rejection by the female immune system [4]. Further, sperm cells protect themselves from the membrane attack complex (MAC) by binding of sCLU [5]. On the other hand opportunistic bacteria such as *P. aeruginosa* escape MAC-induced cell lysis after binding sCLU via dihydrolipoamide dehydrogenase [6]. Additionally, sCLU attracts macrophages by inducing various signal transduction pathways which lead to upregulation of pro-migratory proteins [7]. In some cell lines, such as cardiomyocytes and neurons, sCLU induces the PI3K/AKT-signaling pathway leading to survival or proliferation [8]. Other cytoprotective effects of CLU are executed by the retrotranslocated psCLU form (mediated by the ER chaperone GRP78), which is involved in the modulation of autophagy and apoptosis [9].

It must be noted that under healthy conditions, there is no difference in vitality between wild type and CLU-deficient mice (114). CLU seems to be primarily important under pathological conditions where it protects the organism and helps to reestablish proteostasis. On the downside, by being beneficial to organs, sCLU can be devastating as in the case of cancer. Based on this dual role, phase III clinical trials with the CLU antisense oligonucleotide OGX-011 (Curtisen) in cancer research are underway which may highlight this fact in the upcoming months or years (6). The results of these trials will not only shed light on treatment of cancer, but will also allow new insights in the biology of CLU and, thus, the understanding of many other physiological mechanisms and diseases.

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