Chapter 14

Clusterin and Alzheimer's Disease

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Abstract: Clusterin (apolipoprotein J) is a ubiquitous multifunctional glycoprotein with the capability to interact with a broad spectrum of molecules, among them the Alzheimer's Aβ peptide. Due to its co-localization with fibrillar deposits in systemic and cerebral amyloid disorders, clusterin is also considered an amyloid-associated protein. Although no genuine function has been attributed to this protein so far, it has been implicated in a wide variety of physiological and pathological processes, a role that may vary according to the protein maturation, sub-cellular localization, and the presence of certain tissue- or cell-specific factors. This review focuses on the importance of clusterin in health and disease conditions, with particular emphasis in its role in Aβ amyloidosis and other disorders of protein folding.

Key words: apolipoprotein J; amyloid β; cerebral amyloidosis; neuroprotection; neurotoxicity

1. INTRODUCTION

Clusterin[1] (also known as apolipoprotein J, apoJ) is a fascinating highly conserved glycoprotein with a nearly ubiquitous tissue distribution, that is remarkably regulated under certain conditions among them apoptosis, cancer, inflammation, as well as in nearly all neurodegenerative disorders

[1] Alternative names commonly used in the literature: sulfated glycoprotein 2, SGP2; complement-associated protein, SP-40,40; complement lysis inhibitor, CLI; testosterone-repressed prostate message 2, TRPM2.

described to date. Clusterin is a unique molecule both from a structural and a functional perspective and some of its most interesting aspects are often displayed as a dichotomy of opposite effects or a multiplicity of attributes.

The impressive number of ligands reported and the many, and often opposite, functions attributed to clusterin clearly indicate the need for a better understanding of the protein structure-function relationship, as well as the determination of its physiologically relevant functions. Fortunately, recent studies are starting to shed light on its role in certain physiological processes and the actual significance of many of its attributed ligand-binding interactions. The development of clusterin-deficient mouse models, the understanding of the disparity of roles played by secreted and nuclear isoforms, and the appreciation of the involvement of unstructured regions of the molecule in binding to hydrophobic ligands have played a key role for gaining new insights into the genuine function of this molecule.

2. CLUSTERIN STRUCTURE AND EXPRESSION

2.1 Secreted and intracellular forms of clusterin

Clusterin, a 70-80 kDa secreted protein (Kirszbaum *et al.*, 1989), was first described in 1983 as one of the major proteins present in ram rete testis fluid and was named after its ability to elicit clustering of different cell types (Blaschuk *et al.*, 1983; Fritz *et al.*, 1983). In humans, clusterin is coded by a single gene located on chromosome 8p21-p12 (Slawin *et al.*, 1990; Purrello *et al.*, 1991; Tobe *et al.*, 1991) that is organized into nine exons spanning a region of 16,580 bp (Wong *et al.*, 1994). Heat shock and apoptotic signals in rat and human cells are able to induce alternative splicing of the clusterin gene resulting in the generation of a variant mRNA lacking exon V and coding for a protein isoform missing the C-terminal two-thirds of the molecule (Kimura and Yamamoto, 1996; Kimura *et al.*, 1997). Recently, another alternative splicing has been described that omits exon II, which contains the first translation start site (AUG) and codes for the endoplasmic reticulum-targeting peptide. In this variant, a downstream AUG in exon III becomes the first available translation start site originating a shorter mRNA and resulting in a 49-kDa intracellular protein (Leskov *et al.*, 2003) normally present at low levels and which expression presumably increases under cellular stress conditions.

Clusterin gene promoter is highly conserved among vertebrates and contains several potential regulatory elements such as AP-1, AP-2, Sp1 and

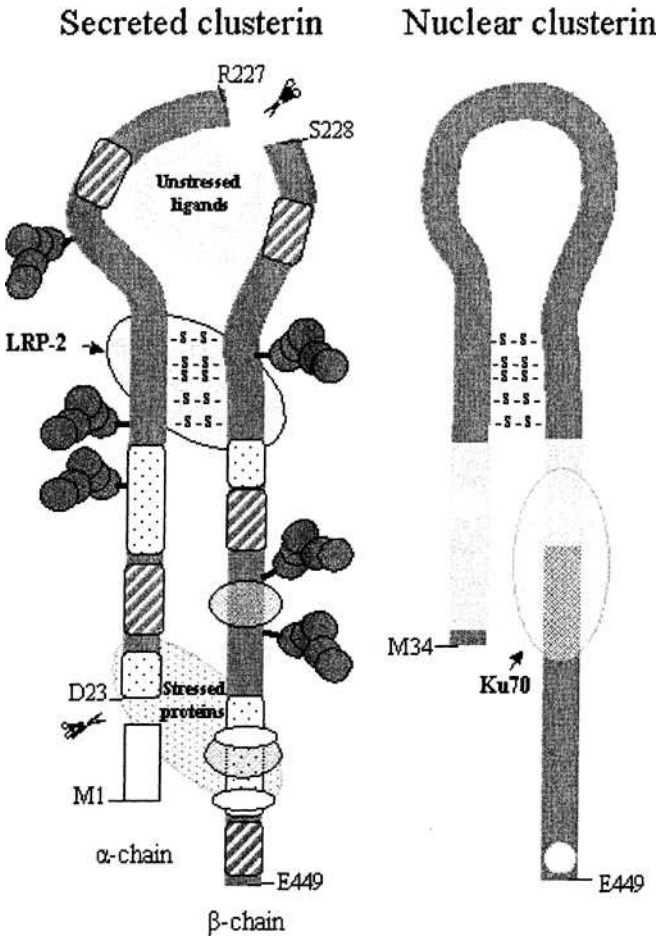

Figure 1: Schematic representation of secreted and nuclear clusterin. The secreted form contains a leader sequence (amino acids 1-22, open box), six N-glycosylation sites (positions 86, 103, 145, 291, 354 and 374), five putative amphipatic helices (hatched boxes), four predicted disordered regions (dotted boxes), two heparin-binding sites (open ovals), and two segments with cell adhesion activity (shadowed ovals). The regions involved in the binding of stressed proteins, LRP-2 and unstressed ligands are indicated. The nuclear form contains two coiled-coil segments (light grey areas) and two nuclear localization signals (open circles). The Ku70 binding site is indicated. Both, secreted and nuclear forms contain five disulphide bonds (-S-S-).

NF (Herault *et al.*, 1992; Wong *et al.*, 1994; Michel *et al.*, 1995; Michel *et al.*, 1997) that result in differential expression in response to a number of growth factors, cytokines, and stress and apoptosis inducing agents in a cell-type or tissue specific manner (Jin and Howe, 1997; Michel *et al.*, 1997; Gutacker *et al.*, 1999; Trougakos and Gonos, 2002).

In humans, clusterin is mainly a secreted glycoprotein, although intracellular -cytoplasmic and nuclear- isoforms originated by the above described alternative splicing mechanisms have also been described (Jin and Howe, 1997; Leskov *et al.*, 2003). Secreted clusterin (sCLU) results from the translation of the full clusterin mRNA that codes for the 449 residues-long protein and contains a 22-mer leader sequence. After synthesis, the structure of the molecule is stabilized by the formation of five disulfide bonds (Choi-Miura *et al.*, 1992) and the mature form of the protein finally generated by posttranslational cleavage at peptide bond R^{227}-S^{228} in the trans- or post-Golgi, rendering two ~40 kDa antiparallel subunits (named α- and β-chains) (Murphy *et al.*, 1988; Jenne and Tschopp, 1989; Kirszbaum *et al.*, 1989; de Silva *et al.*, 1990a; de Silva *et al.*, 1990b; Kirszbaum *et al.*, 1992) (Figure 1). Clusterin is heavily glycosylated and the carbohydrate molecules attached to the six existing sites (three on each chain) constitute about 30-40% of the molecular mass of the secreted protein (Urban *et al.*, 1987; Kapron *et al.*, 1997). Other reported post-translational modifications include sulfation (Urban *et al.*, 1987), lipid binding (Jenne *et al.*, 1991; Burkey *et al.*, 1992), iodination, mannose-6-phosphorylation (Lemansky *et al.*, 1999), and glycation (Mitsuhashi *et al.*, 1997). In addition, similarity searches to identify protein motifs and potential post-translational modifications performed against the Swiss-Prot and Prosite data bases at the ExPASY (Expert Protein Analysis System) server (http//www.expasy.org) of the Swiss Institute of Bioinformatics indicate putative phosphorylation sites for protein kinase C, casein kinase II and tyrosine kinase.

Cytoplasmic clusterin (cCLU) is normally expressed at very low levels as the translation product of a shorter mRNA produced by alternative splicing, as described above. The translated protein, lacking any signal peptide, is unable to be secreted and, probably as a result of retrograde transport from the Golgi to the ER, accumulates as a 49-kDa cytoplasmic, disulfide-linked, non-glycosylated, and uncleaved isoform. Under cellular stress, cCLU is assumed to be post-translationally modified, presumably phosphorylated, and subsequently translocated into the nucleus most likely via two functional nuclear localization signals. In the nucleus it is found as a 50-53 kDa nuclear clusterin (nCLU) (Leskov *et al.*, 2003; O'Sullivan *et al.*, 2003) that remains uncleaved at the peptide bond R^{227}-S^{228} (Park *et al.*, 1997) (Figure 1). Recently, this nuclear form has been shown to bind Ku70, a DNA damage sensor and key double-strand break repair protein that mediates cell death responses, through its C-terminal domain (Leskov *et al.*, 2003).

2.2 Tissue expression: an ubiquitous protein

Clusterin mRNA is present in almost all mammalian tissues (Collard and Griswold, 1987; de Silva *et al.*, 1990a) and protein expression in nearly all body fluids (de Silva *et al.*, 1990c; de Silva *et al.*, 1990b; Sylvester *et al.*, 1991; Aronow *et al.*, 1993). The normal concentration of clusterin in plasma, where it is primarily distributed within the high-density lipoproteins (de Silva *et al.*, 1990a; de Silva *et al.*, 1990c; de Silva *et al.*, 1990b; Jenne *et al.*, 1991; Stuart *et al.*, 1992), ranges between 35 and 105 μg/ml (0.44 - 1.35 μM) (Murphy *et al.*, 1988). About 30% of the protein seems to be sequestered by the alpha granules of platelets and can be released upon platelet activation (Witte *et al.*, 1993). Clusterin is about four-times more concentrated in seminal fluid than in plasma (O'Bryan *et al.*, 1990), while the CSF values vary between 1.2 and 3.6 μg/ml (15-45 nM) (Choi-Miura *et al.*, 1992; Harr *et al.*, 1996) with clusterin also co-localizing with high-density lipoproteins.

Within a given tissue, clusterin is expressed predominantly in particular cell types. Secreted clusterin is constitutively and developmentally expressed in epithelial cells, mainly at the fluid-tissue interface in the case of biologically active fluids such as gastric, pancreatic, and biliary secretions, urine and CSF (Aronow *et al.*, 1993; French *et al.*, 1993). In this sense it is present in the epithelial lining of the esophagus, biliary ducts, gallbladder, urinary bladder, ureter, kidney distal convoluted tubules, gastric glands, Brunner's glands, choroid plexus, ependyma, ocular ciliary body, endometrium, cervix, vagina, testis, epididymus, and visceral yolk sac (Aronow *et al.*, 1993). Clusterin is also expressed at high levels in several non-epithelial secretory cell types that line fluid compartments, such as synovial lining cells and ovarian granulosa cells (Aronow *et al.*, 1993).

The anatomical and cellular distribution of clusterin within the CNS has been studied in particular detail. In the adult rat brain the mRNA shows regional differences with the highest levels in the ependymal lining of the ventricles followed by various gray matter areas, mainly the hypothalamic and brainstem nuclei, the habenular complex, and the motorneurons of the ventral horn of the spinal cord (Danik *et al.*, 1993). In situ hybridization combined with immunocytochemistry studies (Pasinetti *et al.*, 1994) demonstrated clusterin mRNA in astrocytes throughout the normal adult brain as well as in specific neurons, but not in microglial cells. Among neuronal areas, clusterin was prevalent in pontine nuclei and in the red nucleus of the midbrain tegmentum whereas it was expressed in only a subset of substantia nigra dopaminergic neurons or locus ceruleus noradrenergic neurons (tyrosine hydroxylase immunopositive). Clusterin immunopositive cells were also observed in the Purkinje cell layer of the cerebellum, medial and interposed cerebellar nuclei, trigeminal motor and

red nucleus, but not in the striatum (Pasinetti *et al.*, 1994). Interestingly, clusterin expression in the adult rat CNS changes dramatically following brain injury from a constitutive restricted neuronal distribution to a widespread inducible astrocytic expression (Danik *et al.*, 1993; Pasinetti *et al.*, 1994).

3. A CONSERVED STRUCTURE FOR A MYRIAD OF LIGANDS

Clusterin is a highly conserved protein amongst mammals, showing 70-80% sequence identity at the amino acid level. Secondary structure analysis by circular dichroism shows a majority of α-helical domains, predicted to be forming three amphipathic α -helices and two coiled-coil regions (de Silva *et al.*, 1990a; de Silva *et al.*, 1990b) (Figure 1). It has recently been proposed that clusterin likely includes three extended regions of natively disordered or molten globule-like structures, flexible and thereby highly sensitive to trypsin digestion, that contain the putative amphipathic α-helices. It has been suggested that these natively disordered regions may confer to clusterin the ability to bind a variety of molecules (Bailey *et al.*, 2001).

A short review of the literature reveals that clusterin binds to an impressive array of different ligands including apolipoproteins, lipids, immune system related molecules, cellular receptors, amyloid- forming proteins and peptides, enzymes, bacterial proteins and heparin. More recently, it has been demonstrated that clusterin is also able to bind to a number of stressed, partially folded proteins (Poon *et al.*, 2000; Lakins *et al.*, 2002; Trougakos and Gonos, 2002). Except for the amyloid-forming peptides that will be further analyzed below, all described ligands of clusterin are summarized in Table 1 and will not be discussed in detail.

According to Lankins and collaborators (2002), secreted clusterin has at least three distinct sites by which it binds three different types of ligands, namely: i) unstressed ligands such as the $A\beta_{1-40}$ peptide, Complement Components C7, C8, C9, and IgG, ii) stressed proteins, and iii) cell-surface receptors. Experimental data and sequence analysis predictions indicate that these sites are located at different parts of the molecule (Figure 1). The binding site for unstressed ligands may involve the amphipathic helices located at the C-terminal of the β-chain and the N-terminal of the α-chain. The binding to stressed proteins – seemingly of high affinity and low specificity- is apparently mediated by natively disordered or molten globule-

Table 1. Clusterin ligands

Ligands	References
Apolipoproteins & Lipids	
ApoAI	Tschopp J, 1993; Jenne DE, 1991; Ehnholm C, 1991
ApoCII	Hatters DM, 2002
Paraoxonase	Kelso GJ, 1994
Lipids	Jenne DE, 1991; Yokoyama M, 1988; Burkey BF, 1992
Bacterial proteins	
Streptococcus pyogenes (Prot SIC)	Akesson P, 1996
Streptococcus sanguis	Hogg SD and Embery G, 1979
Staphylococcus aureus	Partridge SR, 1996
Staphylococcus epidermidis	Li DQ and Ljungh A, 2001
Bacteriorhodopsin	Bailey RW, 2001
Immune system related molecules	
Complement components	Murphy BF, 1988; Kirszbaum L, 1989; Tschopp J, 1993; Tschopp J and French LE, 1994
Immunoglobulins	Wilson MR, 1991; Wilson MR and Easterbrook-Smith SB, 1992
Intracellular ligands	
Ku proteins (Ku70, Ku86)	Leskov KS, 2001; Yang CR, 1999; Yang CR, 2000; Trougakos IP and Gonos ES, 2002
Glycoproteins H, J, K	Palmer DJ and Christie DL, 1992
Cellular receptors	
TGFβ receptors	Golabek A, 1995
Megalin/LRP-2/gp330	Zlokovic BV, 1996; Kounas MZ, 1995
Amyloid forming proteins	
Aβ peptide	McGeer PL, 1992; Oda T, 1995; Matsubara E., 1996; Calero M, 2000, De Mattos RB, 2002
PrP	Sasaki K, 2002; McHattie S and Edington N, 1999; Calero M, 2000
Corneal amyloid GDCD and LCD-I	Nishida K, 1999
α-synuclein	Sasaki K, 2002
ABri	Ghiso J, 1995; Ghiso J, 2002
ADan	Ghiso J, 2002
Stressed ligands	
Catalase, β-lactalbumin, BSA	Humphreys DT, 1999
Denatured IgG, ovotransferrin, lysozyme	Hochgrebe T, 2000
Other molecules	
Glutatione-S-transferase	Humphreys D, 1997
α1-microglubulin	Calero M, unpublished observation
Heparin	de Silva HU, 1990; Pankhurst GS, 1998
Leptin	Bajari TM, 2003
MT-6 MMP	Matsuda A., 2003

GDCD: gelatinous drop-like corneal dystrophy

LCD-I: lattice corneal dystrophy type I

TGFβ: transforming growth factor beta

MT-6 MMP: membrane-type 6 matrix metalloproteinase

like regions at the N-terminal of the β-chain and the C-terminal of the α-chain containing the putative amphipathic α-helices. On the contrary, the highly conserved and well structured disulphide- linked central region of the molecule may be involved in the highly specific, and probably fundamental for its function, binding to the LRP-2 cell receptor (Bailey *et al.*, 2001;

Lakins *et al.*, 2002). More recently, studies with the intracellular form of clusterin have indicated a fourth type of binding site, probably located within the C-terminal coiled-coil region of the protein, involved in cell death responses through the interaction with Ku70 (Leskov *et al.*, 2003) (see Figure 1 and Table 1).

Interestingly, at mildly acidic pH clusterin shows increased binding for some of its ligands -heparin (Pankhurst *et al.*, 1998), IgG, C9, apoAI and GST (Hochgrebe *et al.*, 2000)- most likely due to modifications in its aggregation state than to conformational changes resulting from the change in pH. In this sense it should be noted that at physiological pH clusterin exists in an array of multimeric forms, whereas mildly acidic pH favors the formation of clusterin monomers more easily available for binding interactions. This enhancement has potential physiological relevance as clusterin is locally produced at sites of tissue damage and inflammation, where local acidosis also occurs (Hochgrebe *et al.*, 2000).

4. CLUSTERIN AS A MULTIFUNCTIONAL PROTEIN

4.1 The many professions of clusterin

Mostly as a result of the interaction with the many ligands described above, clusterin has been implicated in a number of diverse biological processes including cell-cell interactions (Fritz *et al.*, 1983; Silkensen *et al.*, 1995), sperm maturation (Collard and Griswold, 1987; Sylvester *et al.*, 1991), apoptosis (Buttyan *et al.*, 1989), complement inhibition (Murphy *et al.*, 1988), lipid transport (de Silva *et al.*, 1990c; Jenne *et al.*, 1991), tissue remodeling, membrane recycling (Danik *et al.*, 1991; Palmer and Christie, 1992), and clearance of cellular debris (Bartl *et al.*, 2001), as well as degradation of the extracellular matrix through the interaction with the membrane-type 6 matrix metalloproteinase (Matsuda *et al.*, 2003).

The structural nature of the different binding sites described above may explain, at least in part, many of clusterin's putative functions that result from its competence for binding such a diverse array of molecules. For instance, the presence of the amphipathic α-helices, a type of secondary structure that mediates interactions with hydrophobic molecules (de Silva *et al.*, 1990a; de Silva *et al.*, 1990b) may originate broad and non-specific interactions with some of the ligands shown in Table 1 that, in turn, may not be physiologically significant. In this sense, recent data suggest that clusterin may not be a relevant regulator of complement activation at physiological concentrations (Hochgrebe *et al.*, 1999) in spite of its ability to bind

components of the membrane attack complex (Blaschuk *et al.*, 1983). In contrast, the interaction with LRP-2, the only known cellular receptor for clusterin, is highly specific and it most likely plays a crucial role in clusterin function by either triggering signal transduction pathways or by directing internalization and lysosomal degradation of different ligands through receptor-mediated endocytosis. Other relevant biological function of clusterin may relate to its capacity to participate in signal transduction pathways such as regulation of the transcription factor NF-κB signaling (Santilli *et al.*, 2003).

Although some results are controversial, a number of evidences support the idea that clusterin is directly involved in the response to cellular stress and mediates in apoptotic signaling. Clusterin mRNA level is increased in regressing tissues (Viard *et al.*, 1999) and endothelial cells undergoing apoptosis show transcriptome changes that include not only down regulation of survival signals, and alteration of cell cycle elements and apoptosis regulators, but a significant increment in clusterin expression (Johnson *et al.*, 2004). Controversially, a number of findings point out to a cytoprotective/anti-apoptotic role. Clusterin over-expression is able to protect cells in culture from the cytotoxic effect of TNF-α (Humphreys *et al.*, 1997), and blocking clusterin biosynthesis by anti-sense oligonucleotides or by silencing gene expression with small interfering RNAs induces apoptosis, reduces growth ability and enhances susceptibility for oxidative stress (Viard *et al.*, 1999; Trougakos *et al.*, 2004). Mouse models also provide contradictory information evidencing once more that the role of clusterin may vary according to particular circumstances, the final maturation and localization of the protein, as well as tissue or cell specific factors. Whereas studies performed on clusterin-deficient mice have demonstrated that, under certain conditions, the protein may have a protective effect against apoptosis (Bailey *et al.*, 2002), mice suffering hypoxic-ischemic brain injuries, a model of cerebral palsy, show clusterin accumulation in dying neurons contributing to caspase-3-independent brain injury (Han *et al.*, 2001)

In order to fully comprehend clusterin's diverse and many times contradictory functions, the importance of protein maturation and sub-cellular localization, as well as the potentially differential roles of the secreted and intracellular isoforms should be taken into consideration. Although secreted clusterin seems to act as an inherent pro-survival and anti-apoptotic protein under stress, intracellular clusterin becomes highly toxic and strongly accumulates in ubiquitinated form in juxtanuclear aggregates leading to profound alterations of the mitochondrial network (Debure *et al.*, 2003) presumably triggering cell death signals through Ku70/Ku80 binding (Yang *et al.*, 2000). Additionally, different stress conditions including

ionizing radiation or apoptosis provoked by treatment with TNF-α or antiestrogens, induce nuclear translocation of intracellular clusterin.

4.2 Clusterin as an extracellular chaperone

Clusterin has been described as the first identified extracellular mammalian chaperone which binds to a wide variety of partly unfolded stressed proteins, inhibiting their stress-induced precipitation, via an ATP-independent mechanism (Poon *et al.*, 2000; Wilson and Easterbrook-Smith, 2000). Although the ultimate biological function of clusterin is still not well defined, its role as an extracellular chaperone is consistent with its involvement in tissue remodeling and protective responses against cellular stress. Chaperone activity may arise from the presence of the amphipathic helices in combination with natively disordered stretches in the protein structure that allows the formation of a putative, dynamic, molten globule-like binding site with ability to transiently bind to a variety of molecules (Bailey *et al.*, 2001), preferentially partly folded protein intermediates that are slowly aggregating as a result of stress. Through this interaction, clusterin contributes to solubilize high molecular weight complexes and inhibits rapid and irreversible protein precipitation producing a pool of inactive but stabilized molecules from which in turn, other intracellular refolding chaperones, such as heat shock protein 70 (HSP70) can subsequently rescue functional proteins (Poon *et al.*, 2000; Poon *et al.*, 2002a). Interestingly, clusterin is also the first chaperone molecule shown to be activated by mildly acidic pH and not by increased temperature. This unique mode of activation appears to result from an increased solvent-exposed hydrophobicity of the molecule with acidic pH, independent of any major changes in the secondary or tertiary structures (Hochgrebe *et al.*, 2000; Poon *et al.*, 2002b).

5. AMYKLOID β AMYLOIDOSIS: A DISORDER OF PROTEIN FOLDING

Protein misfolding and aggregation associate with a variety of human disorders. Among them, particular interest has been devoted to a large group of chronic and progressive neurodegenerative conditions characterized by the selective loss of neurons associated either with cognitive, motor or sensory systems as well as the intra- or extra-cellular deposition of protein aggregates and/or fibrils in different regions of the CNS (Table 2).

Table 2. Disorders of protein folding, characteristic neuropathology and deposited proteins

Disease	Neuropathology	Protein
Alzheimer's disease, Down's syndrome, normal aging, Sporadic cerebral amyloid angiopathy, Familial AD	Neuritic plaques, pre-amyloid lesions neurofibrillary tangles, cerebrovascular amyloid deposits.	Aβ
Familial British dementia	Cerebrovascular amyloid deposits, neurofibrillary tangles, neuritic plaques, pre-amyloid lesions.	ABri
Familial Danish dementia	Cerebrovascular amyloid deposits, pre-amyloid lesions, neurofibrillary tangles	ADan
Creutzfeldt-Jakob disease, Gerstmann-Straussler-Schinker disease, fatal familial insomnia, Kuru	Spongiform degeneration , gliosis, neuronal loss	PrP^{Sc}
Fronto-temporal dementia, Pick's disease	Loss and athophy of neurons, Pick's inclusion bodies	tau
Diffuse Lewy body disease	Diffuse Lewy bodies	α-synuclein
Parkinson's disease	Neuronal loss, Lewy bodies in pigmented neurons	α-synuclein, parkin
Huntington's disease	Neuronal loss	Huntingtin
Spinocerebellar ataxia Type 1	Neuronal loss	Ataxin 1
Spinocerebellar ataxia Type 2	Neuronal loss	Ataxin 2
Spinocerebellar ataxia Type 3	Neuronal loss	Ataxin 3
Spinocerebellar ataxia Type 6	Neuronal loss	α_{1A}V-Ca C
Spinocerebellar ataxia Type 7	Neuronal loss	Ataxin 7
Spinocerebellar ataxia Type 8	Neuronal loss	untranslated
Multiple-system atrophy	Neuronal and glial inclusions	α-synuclein
Friedereich's ataxia	Neuronal loss in sensory and motor systems	Frataxin
Dentatorubropallidolusyan atrophy	Neuronal loss	Atrophin I
Amyotropic lateral sclerosis	Upper and lower motor neurons loss	SOD1
Familial Spastic paraparesis	Upper motor neurons loss	Paraplegin
Spinal muscular atrophy	Lower motor neurons loss	SMN
Spinal and bulbar muscular atrophy	Lower motor neurons loss	Androgen receptor

A subset of these diseases, collectively known as cerebral amyloidosis, is characterized by the deposition of poorly soluble, long, twisted amyloid filaments composed of low molecular weight proteins that are normally soluble under physiologic conditions. In cases in which the amyloid lesions are primarily restricted to the cerebral vessel walls, the common clinical manifestation is stroke; contrastingly, a widespread distribution throughout selected parenchymal areas, particularly the limbic structures, is associated with dementia. Alzheimer's disease (AD) is the most common form of cerebral amyloidosis in humans and the major cause of dementia. In hereditary as well as in sporadic AD, extracellular amyloid deposits in the

form of amyloid plaques and cerebral amyloid angiopathy co-exist with intraneuronal neurofibrillary tangles in the brain parenchyma. The major component of the amyloid lesions, Aβ, is a 40-42 residues internal proteolytic fragment of a larger type I transmembrane precursor molecule APP codified by a single multi-exonic gene located on chromosome 21. Although a soluble form of Aβ (sAβ, predominantly 40 residues in length) has been identified in biological fluids, systemic deposits of Aβ cannot be demonstrated in AD patients (Ghiso and Frangione, 2002). Whether the circulating soluble forms of Aβ represent immediate precursors of the deposited amyloid, reflect brain clearance, or both, are not solved issues. Certainly, the blood-brain barrier has the capability to modulate this equilibrium.

The brain uptake of free sAβ entails the participation of two different receptors: RAGE (the receptor for advanced glycation end-products) (Yan *et al.*, 1996) and SR-A (scavenger receptor type A) (Christie *et al.*, 1996; El Khoury *et al.*, 1996). RAGE is present in different cell types, including vascular endothelial and smooth-muscle cells, choroid plexus epithelium and phagocytes (Brett *et al.*, 1993) whereas SR is expressed on macrophages, microglia and vascular endothelial cells (Lucarelli *et al.*, 1997). RAGE mediates patho-physiological responses in the vasculature when occupied with glycated ligands or Aβ (Brett *et al.*, 1993; Yan *et al.*, 1996; Mackic *et al.*, 1998), whereas SR-A promotes endocytosis and degradation of oxidized LDL and glycated ligands (Christie *et al.*, 1996). Both RAGE and SR-A modulate brain endothelial endocytosis and transcytosis of Aβ from the luminal side of the blood-brain barrier (Mackic *et al.*, 1998; Deane *et al.*, 2003). *In vitro*, Aβ binding to brain microvascular endothelial cell monolayers is time-dependent, polarized to the apical side, saturable, and susceptible to inhibition by anti-RAGE antibodies (~63%) and by acetylated LDL (~33%) (Mackic *et al.*, 1998). Consistent with these data, both RAGE-transfected cells and macrophage SR-A displayed binding and internalization of radiolabeled Aβ (Mackic *et al.*, 1998) and systemic administration of soluble RAGE reduced accumulation of Aβ in brain parenchyma of transgenic APP mice (Deane *et al.*, 2003).

The brain clearance mechanism of the Aβ peptide appears to be in part regulated by the LDL receptor-related protein 1 (LRP-1), a promiscuous receptor highly expressed in the CNS. More than twenty ligands have been identified for LRP-1 (Hussain *et al.*, 1999), including apoE-containing lipoproteins, chylomicron remnants, activated α-2 macroglobulin and Receptor Associated Protein (RAP). When injected into the brain, radiolabeled Aβ is rapidly removed across the blood-brain barrier with a $t_{1/2}$ ~25 minutes being its clearance significantly inhibited by RAP and by antibodies against LRP-1 and α2 –macroglobulin. In *in vitro* experiments,

LRP-1 has also been implicated in Aβ clearance by smooth-muscle cells, neurons and fibroblasts (Narita *et al.*, 1997; Urmoneit *et al.*, 1997; Jordan *et al.*, 1998; Qiu *et al.*, 1999). Interestingly, LRP-1 is abundant in brain microvessels of young mice but is down-regulated in older animals (Shibata *et al.*, 2000), suggesting that a failure in the clearance mechanism leads to the accumulation of Aβ in the brain with the consequent amyloid formation.

6. CLUSTERIN AND ITS RELATION TO Aβ

6.1 Clusterin as an Aβ-carrier molecule

Soluble Aβ co-localizes with high-density lipoprotein (HDL) particles in serum (Koudinov *et al.*, 1994) and CSF (Koudinov *et al.*, 1996). Under normal circumstances, about ninety percent of the circulating sAβ is associated with plasma HDL fractions (Matsubara *et al.*, 1999), largely complexed to the carrier clusterin (Ghiso *et al.*, 1993).

The Aβ-clusterin complex is transported from the blood to the brain across the blood-brain barrier through the only known clusterin receptor, the low-density lipoprotein receptor related protein 2 (LRP-2; also known as megalin or gp330) (Zlokovic *et al.*, 1996), broadly expressed in vascular CNS tissues including the choroid plexus, the blood-brain barrier endothelium and the ependyma (reviewed in (Calero *et al.*, 2000). LRP-2 interacts with clusterin with high affinity, mediates clusterin endocytosis and its subsequent lysosomal degradation (Kounnas *et al.*, 1995; Hammad *et al.*, 1997) or transcytosis (Zlokovic *et al.*, 1996). In the case of sAβ40-clusterin complexes, the transport mechanism is identical to that of free clusterin, being specifically abolished by native clusterin as well as by anti-LRP-2 and RAP. Free sAβ peptides which, as described above, are internalized by a completely different mechanism do not exert any inhibitory effect.

In *in vitro* binding experiments, clusterin is specifically retrieved from plasma and CSF by immobilized Aβ species. Clusterin-Aβ interaction is saturable and specific, with Kd values in the low nanomolar range (2-4 nM), and is not affected by the degree of lipidation of the apolipoprotein (Matsubara *et al.*, 1995; Matsubara *et al.*, 1996; Calero *et al.*, 1999). As a result of the complex formation, *in vitro* peptide aggregation, polymerization and amyloid fibril assembly is significantly prevented (Oda *et al.*, 1995; Matsubara, *et al.*, 1996, Calero *et al.*, 2000), as it is the Aβ neurotoxicity (Oda *et al.*, 1995; Boggs *et al.*, 1996; Lambert *et al.*, 1998). These protective properties of clusterin, consistent with its extracellular chaperone activity described above, are not restricted to the Aβ peptide. Clusterin was shown to prevent *in vitro*, in a dose-dependent manner, the spontaneous aggregation

of a synthetic peptide homologous to residues 106-126 of human prion protein (McHattie and Edington, 1999). Sub-stoichiometric levels of clusterin inhibited apoC-II amyloid formation, although they did not promote fibril dissociation (Hatters *et al.*, 2002). Clusterin binds to slowly aggregating proteins, such as some forms of stressed α-lactalbumin, α-crystallin and lysozyme preventing their aggregation (Poon *et al.*, 2002a). Moreover, as supported by data obtained from a clusterin knock-out mouse model, the expression of clusterin participate in the prevention of progressive glomerulopathy in aging mice, which is characterized by the deposition of immune complexes consisting of IgG, IgM and IgA together with complement components (Rosenberg *et al.*, 2002).

6.2 Clusterin as an amyloid associated protein

Paradoxically to its chaperone activity, clusterin has been found co-deposited with all amyloid molecules tested so far, not only in cerebral forms but also in systemic types of amyloidosis. There is extensive immunohistochemical data indicating that besides clusterin, a variety of unrelated proteins -serum amyloid P-component, α_1-antichymotrypsin, apolipoprotein E, complement components, vitronectin, cytokines, glycosaminoglycans and extracellular matrix proteins, among many others- co-deposit in the lesions composed of different amyloid molecules, including Aβ in senile plaques and vascular deposits. These components, collectively known as amyloid associated proteins, co-localize with the lesions but are not a structural part of the final fibril. It is still not clear whether these elements are innocent bystanders or if their presence is related to the mechanism of amyloidogenesis / fibrillogenesis. Several lines of investigation favor the latter notion, at least for some of these proteins, since various studies suggest that they can modulate the formation of amyloid-like fibrils *in vitro* (Ghiso and Frangione, 2002).

As illustrated in Figure 2, anti-clusterin antibodies effectively highlight parenchymal and vascular lesions in Aβ-related disorders [AD (panel A), Down syndrome (panel B), FAD cases associated to the Iowa and Dutch mutations (panels C and D)] as well as in non-Aβ cerebral amyloidosis [CJD panel E), HCHWA-I (panel F), familial British dementia (panel G) and familial Danish dementia (panel H)]. In all these cases, clusterin is present (in both, fibrillar (amyloid) and non-fibrillar (pre-amyloid) deposits.

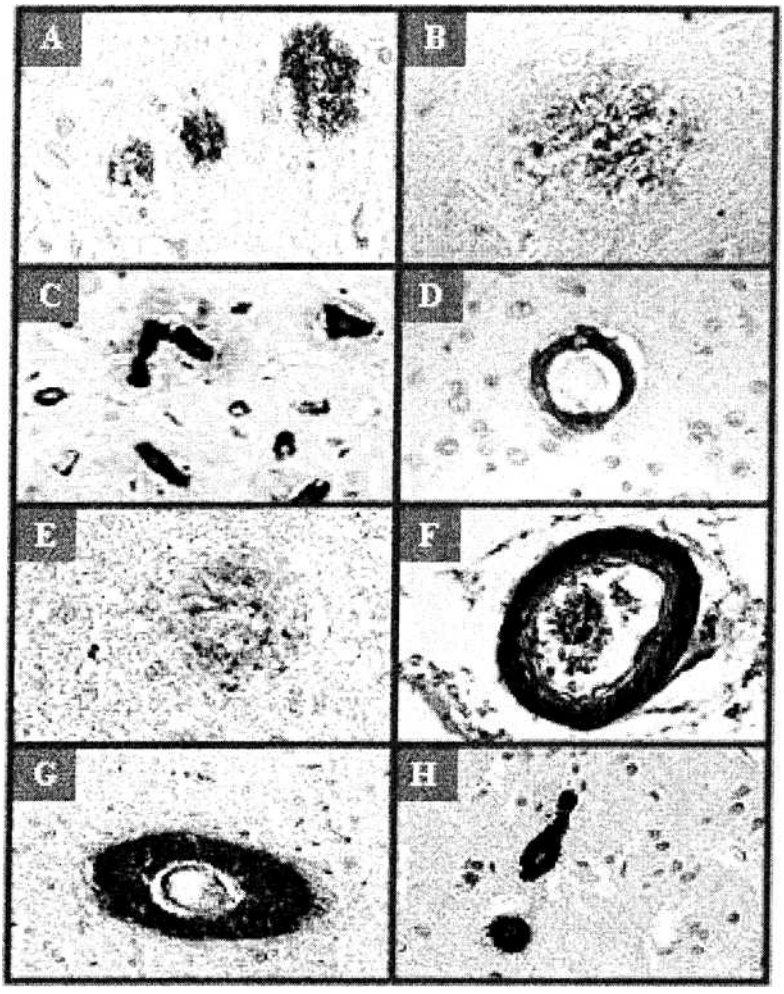

Figure 2: Clusterin co-localizes with parenchymal and cerebrovascular amyloid deposits. Clusterin in **A:** AD, Aβ plaques; **B:** Down syndrome, primitive Aβ plaques; **C:** familial AD (Iowa kindred), cerebrovascular AβN23 deposits; **D:** familial AD (Dutch kindred), cerebrovascular AβQ22 deposits; **E:** Creutzfeldt-Jakob disease, PrP plaques; **F:** hereditary cerebral hemorrhage with amyloidosis - Icelandic type, leptomeningeal Cystatin C Q68 deposits; **G:** familial British dementia, perivascular ABri plaques; **H:** familial Danish dementia, cerebrovascular ADan deposits. Magnification X880.

In addition, clusterin co-localizes with plaque-type deposits but not with punctuate-type prion protein (PrP) lesions in human and animal TSEs (Manuelidis *et al.*, 1997; McHattie *et al.*, 1999; Sasaki *et al.*, 2002b; Sasaki *et al.*, 2002c). It is associated with α-synuclein aggregates in cortical Lewy bodies and glial cytoplasmic inclusions in cases with multiple system atrophy, Parkinson's disease, and dementia with Lewy bodies (Sasaki *et al.*, 2002a). Furthermore, clusterin also co-localizes with amyloid in gelatinous drop-like and lattice type I corneal dystrophies (Nishida *et al.*, 1999). Abnormal staining for clusterin has been described in dystrophic neurites, some neurofibrillary tangles in the Parkinson's dementia of Guam and in ischemic Purkinje cells (Yasuhara *et al.*, 1994). Clusterin is also differentially expressed in the retinas of patients with retinitis pigmentosa (Jones *et al.*, 1992), and elevated levels were described in human cerebrospinal fluid in a number of acute neuropathies (Polihronis *et al.*, 1993; Schreiber *et al.*, 1993). Complete cerebral ischemia also leads to the accumulation of clusterin in neurons and in multiple extracellular deposits located close to microvessels (Kida *et al.*, 1995).

In AD tissue, antibodies to clusterin strongly stain senile plaques, dystrophic neurites and neuropil threads, while cellular processes around Aβ are not highlighted. Clusterin antibody also shows punctuate staining of some normally appearing AD pyramidal neurons, and very scattered reactivity with intracellular neurofibrillary tangles (Choi-Miura *et al.*, 1992; McGeer *et al.*, 1992), although it was rarely observed in NFT-containing neurons (Giannakipoulos *et al.*, 1998). In cortex and hippocampus extracts clusterin is about 40% higher in AD than in control individuals (Oda *et al.*, 1994), while normal clusterin levels are present in cerebellum (Lidstrom *et al.*, 1998). Within the AD group, there is a significant negative correlation between clusterin levels in hippocampus (but not in the frontal cortex) and severity of dementia. No significant correlations are found between clusterin levels and the number of senile plaques or neurofibrillary tangles (Lidstrom *et al.*, 1998). Interestingly, there are no changes in CSF-clusterin levels from patients with different neurological disorders such as AD, vascular dementia, Parkinson's disease or acute stroke, when compared to controls (Harr *et al.*, 1996; Lidstrom *et al.*, 2001).

6.3 The role of clusterin tested in a transgenic mouse model of AD

The protective properties of clusterin, preventing aggregation and polymerization of Aβ *in vitro*, drastically contrast with its co-localization with amyloid and pre-amyloid deposits. An interesting model to test the role of clusterin in amyloid formation emerged from the crossing of the PDAPP mice, homozygous for the APPV717F transgene (Games *et al.*, 1995; Bales

et al., 1997) with a clusterin knock-out mouse (McLaughlin *et al.*, 2000; Han *et al.*, 2001). If clusterin prevented fibril formation as indicated by *in vitro* experiments, an increase in the amyloid load in the off-springs would be expected. However, at 12 months of age, PDAPP-clusterin $^{-/-}$ animals had a similar degree of Aβ deposition compared with PDAPP-clusterin $^{+/+}$ counterparts although the deposits were largely thioflavin negative, indicating that fewer fibrillar Aβ (amyloid) deposits were formed (DeMattos *et al.*, 2002). Although electronmicroscopic evaluation of the lesions was not performed, these results suggest that, opposite to what was observed *in vitro*, clusterin may have a role in facilitating the conversion of soluble Aβ into forms with high β-sheet content *in vivo*. Interestingly, these results were similar to those previously obtained for PDAPP/apoE$^{-/-}$ mice (Bales *et al.*, 1997; Bales *et al.*, 1999).

Surprisingly, breeding PDAPP-apoE$^{-/-}$ with PDAPP-clusterin$^{-/-}$ mice resulted in an unexpected phenotype. Opposite to both single knock-out models, the double knock-out PDAPP-apoE$^{-/-}$-clusterin$^{-/-}$ mice revealed earlier onset and greater levels of Aβ deposition with a parallel increase in both Aβ40 and Aβ42 in interstitial fluid and CSF but not in plasma (DeMattos *et al.*, 2004). Contrary to previous conclusions achieved through the single knockout models, these results suggest that apoE and clusterin cooperatively suppress Aβ deposition. Whether the discrepancies observed between the single and the double knock-out animals are the consequence of significant interactions between both molecules that modulate their responses or a phenomenon self-related to the particular transgenic used in the experiments remains to be elucidated. Certainly, a comparison of the biochemical properties of the Aβ deposits in all these models would help with the interpretation.

7. CONCLUDING REMARKS

Current data indicate divergent and controversial functions for clusterin, ranging from cytoprotection to cytotoxicity. As a result, it is still difficult to assign a definitive role for clusterin in the molecular mechanism(s) of Aβ amyloidogenesis. The double knockout data suggest that clusterin, likely in cooperation with apoE, may normally function as a neuroprotective and anti-amyloidogenic molecule. Consistent with its extracellular chaperone function, clusterin transports the Aβ peptide in biological fluids, maintains its solubility, modulates its uptake by the brain across the blood-brain barrier and contributes to its clearance. In addition, clusterin has the capability to inhibit the formation of the membrane-attack complex during the complement activation cascade and to bind to partly unfolded stressed structures, both contributing elements to the inflammatory conditions seen in

AD. As an amyloid associated protein, however, it co-localizes with Aβ amyloid and pre-amyloid lesions without being a structural part of the final fibrils, possibly allowing the conversion of soluble Aβ into forms of high β-sheet content. Under these circumstances, clusterin may also promote neurotoxicity by triggering uncontrolled cell death signals. Whether these opposite effects reflect structural differences (i.e. full-length vs. truncated forms), specific sub-cellular distribution (i.e. secreted vs. nuclear) or still undefined regulatory interactions with some of its multiple ligands and/or unknown partners remains unknown. Certainly the use of DNA microarrays and comprehensive functional proteomic analysis will provide a large-scale gene expression assessment and a panoramic view of the relevant pathways and binding molecules associated with clusterin regulation in AD. These pathways / physiologic ligands may, in turn, become targets for therapeutic interventions.

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