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HYPOTHESIS

Comparison of molecular functions of lactoferrin and amyloid precursor protein support their functional roles in the innate immune system and links with infection in Alzheimer's disease risk

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Systemic and brain-localised inflammations are hallmark features of ageing that are further elevated in dementia and particularly in Alzheimer's disease (AD). However, although present in other chronic diseases co-associated with AD, the potential role of chronic inflammation as a *causative* risk factor for cognitive decline and AD may have been overlooked. Peptide-derived forms of amyloid precursor protein (APP) present as amyloid beta peptides (Aβ) together with intact and peptide-derived forms of lactoferrin (Lf), are both present and co-localised in amyloid deposits in the eye and in senile plaques in the brain. It is proposed that their co-incidence supports the hypothesis that APP and Lf exert similar and mutually supportive biological roles. There is a strong evidence base for the protective role of Lf in host defence during infection with its very high affinity to ferric iron representing a front line of attack against pathogenic microbes and binding interactions that scavenge virus particles. Lf turn-over involves release of peptides exerting anti-inflammatory effects via multiple pathways, representing a 'self-regulating' biological system. We present compelling evidence that APP exerts a similar functional role to Lf as a signaling molecule of the innate immune system, which can account for its co-expression with Lf in AD. The hypothesis is supported by membrane-localisation of APP, metal and other ligand binding capacities, involvement in chemo-attraction of immune cells to the endothelium and cell binding to the extracellular matrix. Consistent evidence supports that systemic APP expression is correlated with inflammation status in conditions of chronic disease and ageing, and is lowered by treatments that regulate inflammation. While APP over-expression occurs in pro-inflammatory conditions other than infection, it is possible that the co-incidence of APP and Lf is specific for the presence of *infection-mediated* causes of APP upregulation. If APP does participate in the innate immune response, then the relationship between development of chronic inflammation and *onset* of APP over-expression represents a new basis for understanding AD risk. Furthermore, if substantiated, managing longitudinal changes in APP expression and amyloid-mediated AD pathology, by treating infection and chronic inflammation, offer promising targets for AD prevention and potentially therapy.

Keywords: Amyloid precursor protein; inflammation; Alzheimer's disease risk; innate immune system; prevention; lifestyle; diet; intervention

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Molecular and functional characteristics of lactoferrin and amyloid precursor protein

Lactoferrin

Lactoferrin (Lf), is a member of the transferrin family of iron-binding glyco-proteins that are found in exocrine fluids (breast milk) and mucosal secretions (saliva, tears) [1]. Expression of the Lf gene, which is both constitutive and inducible, produces two isoforms of Lf with both types present in most tissues. However, Lf isoform-1 is exclusively expressed in brain, testis and peripheral blood leucocytes whereas Lf isoform delta is exclusively expressed in placenta, liver and ovary [2]. This work is focused on molecular and functional properties of Lf isoform-1 that is released in the apo-form by neutrophils during infection, inflammation, tumor development and iron overload [3, 4] , and thereby influences homeostasis of neural, endocrine and immune systems [5].

Human Lf is a single polypeptide chain of 691 amino acids, folded into 2 distinct ferric Fe-binding N terminal and C terminal lobes [4]. Respective lobes of Lf co-operatively and reversibly bind ferric iron at slightly different binding affinities over a broad pH range [6, 7] electrically and stereochemically stabilized by 2 bicarbonate anions [8]. The co-operative interaction between both N and C lobes 'closing' the binding cavity induced by Fe-binding support that Fe-loading can regulate Lf conformation, with the structure of the N-lobe relatively more sensitive to the status of Fe-saturation [8]. Superior stability (100-fold) to tryptic digestion of human Lf than bovine Lf is attributed to its glycosylation pattern [9] and *in vitro*, to its tendency for self-assembly [10].

Lf is multifunctional, displaying metal and ligand binding activities and even amyloidogenesis associated with specific domains [11]. As such, functional properties of Lf are dynamic and 'evolve' from its primary iron-scavenging function during proteolytic turnover. Iron-binding induces a structural change that affects its proteolytic stability and consequent peptide release profile [12]. Others have also recognized that Lf is conformationally dynamic and its structure is affected by iron [13] and ligand binding [11]. While a key molecular feature, the iron-binding function of Lf is not considered to be important for iron uptake or homeostasis *per se* [14] but instead, contributes to its primary host defense functions [4]. An understanding of the integrated progression of the functions of Lf has been considered but is not established [11, 15].

Immediate effects of inducible expression of apo-Lf by leucocytes by pathogen infection are to scavenge iron, thereby suppressing growth and regulating defence against a broad range of gram positive and gram negative bacteria, yeasts and parasites [16-22]. A number of N and C terminal peptides released from Lf by phagocytic proteases (lactoferricins [23],) subsequently exhibit strong pathogen growth suppression by bacterial membrane disruption [24] and binding of immune system mediators [25]. Basic N-terminal peptides interact strongly with the glycosaminoglycans of cell surfaces [26]. In contrast with N-terminal peptides, C-terminal peptides are glycosylated and anti-bacterial activities are mediated through glycan-binding and permeabilisation of outer-membrane structures such as lipopolysaccharides (LPS) and porins [16,20,27] and consequent sensitization to lysozyme and antibiotics [28-30]. Likewise, capture by Lf and lactoferricins of virus particles or blocking cellular uptake receptors, can prevent primary infection as demonstrated for herpes simplex virus-1 [31], cytomegalovirus [32], human immunodeficiency virus-1 [33], hepatitis C virus (HCV [34]) and rotavirus [35]. Indeed, it is possible that progressively iron-saturated forms of Lf may acquire enhanced tendency for release of specific anti-bacterial peptides that bind mediators and regulate immune responses.

Amyloid precursor protein

The amyloid precursor protein (APP) gene expresses 8 known protein isoforms with another recently reported in platelets [36, 37]. APP is a single pass transmembrane protein with a large extracellular domain, and encrypts the amyloidogenic suite of Aβ peptides associated with Alzheimer's disease (AD). APP is preferentially expressed in brain, kidney, heart, spleen, cerebral spinal fluid (CSF) and plasma with isoforms APP695, APP770 and APP751 expressed in neuronal, non-neuronal and T-lymphocytes (including microglial cells), respectively [38]. APP695 is the predominant isoform found in the central nervous system, while APP751 and APP770 are ubiquitous in peripheral tissues. Peripheral isoforms of APP contain a Kunitz-type proteinase inhibitor (KPI) domain, which is lacking in the APP695 variant. However, genotypes and APP isoforms associated with familial AD that favour amyloidogenic processing, contain the KPI domain [39].

APP is a cell surface molecule that is involved with Notch and apoptosis signaling. Peptides released by secretase and caspase enzymes from APP are potent drivers of neuronal cell death. APP contains two Cu/Zn/Fe binding sites, one of which is present in amyloidogenic Aβ, that are reduced by APP and lead to radical attack of lipoproteins *in vitro*. Metal-bound versus metal-free forms of APP and Aβ can

either promote or suppress damage by reactive oxygen species (ROS), respectively, reflecting the reducing capacity of the protein/peptide *per se*.

APP is required for neuromuscular synapse assembly and synatpogenesis [40]. APP is thought to be structurally similar to cell surface receptor proteins, with capacity to mediate cell adhesion and to bind components of the extra-cellular matrix [41] inferring a potential signaling role. After emerging from the endoplasmic reticulum and passing through the Golgi, newly translated APP is either transported to the cell surface or directed to an endosomal compartment. At the cell surface, APP is predominantly processed by α- and γ- secretases to release peptides αAPP and p3 into the extracellular space. Alternative pathways of APP processing are operative in AD, presumably reflecting biological responses to over-expression and non-membrane localisation. In AD, APP remains or becomes internalised within endosomes where processing by β- and γ-secretases produce βAPP and the amyloidogenic peptide Aβ. Release of Aβ into the extracellular space by exocytosis permits its self-assembly into oligomeric and fibrillar structures that accumulate as senile plaques. Subsequent apoptotic death of neurons releases aggregated forms of Aβ into the extracellular space and deposition at nearby sites.

However, certain aspects of APP processing and their relative physiological significance remain controversial. For example, in addition to the general framework outlined above, there is evidence for both α- and β-secretase activity in the trans-Golgi network and for β-secretase activity in the Golgi apparatus and at the cell surface [42-44]. Furthermore, it also seems possible that amyloid-β can be produced by the sequential cleavage of tri- or tetra-peptide fragments from longer amyloid-β forms involving γ-secretase [45]. Intact APP has a short half-life [46] and is only present in small quantities on the plasma membrane at any given time. APP may also be processed by pathways that do not involve the secretases, such as in lysozymes and through caspase activity [44].

Although structurally dissimilar, Lf and APP display some common functional features, specifically, their metal binding and ROS-regulating capacities and importantly, the dynamic functionalisation of peptides released during turnover. In contrast, amyloidogenic forms of APP possess (serine) protease inhibitor activity whereas Lf is itself a serine protease [47] suggesting complimentarity of these functions. These protease inhibitor and protease activities may implicate complimentary roles for APP and Lf in regulation of the complement system.

Roles of lactoferrin and amyloid precursor protein in infection

Lactoferrin

Lf is a major protein of the secondary granules of polymorphonuclear neutrophils which is released during infection and pro-inflammatory cycles [29, 48, 49] for the purpose of host defense. Lf is expressed at sites of infection in the apo- form and depletes microbes of essential Fe. Apo-Lf initially promotes immune-stimulatory effects as a weak serine protease that can activate complement [47], promote neutrophil and lymphocyte proliferation and antibody synthesis. However, peptide products released during turnover of Lf exert counter-balancing anti-inflammatory effects also via multiple pathways. It appears that dynamic changes in Fe-saturation may represent the central lever regulating the biological efficacy of Lf as it progresses from pro-inflammatory to anti-inflammatory functional stages.

Lf displays 10^{10}-fold higher affinity to ferric versus ferrous iron [50] which can drive the oxidation of ferrous iron thereby providing anti-oxidant benefits. Apo-Lf was more efficient than Fe-saturated Lf in quenching Fenton-mediated free radical production [51] confirming the stability and lack of reactivity towards oxygen of Lf-bound ferric iron [52]. Similarly, Lf can stabilize oxidized forms of Cu, Zn, Pt or Au, and suppress their capacity for Fenton chemistry-mediated ROS damage [53].

During infection, soluble complexes of microbial LPS are potent activators of the innate immune system [54, 55] stimulating production of the inflammatory cytokines: tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6) [56,57]. Following immune-stimulatory effects of apo-Lf, proteolytic release of Lf-derived peptides subsequently exert down-regulation of the inflammation cycle through LPS binding by the basic C-terminal lobe of Lf [56, 58] yielding protective outcomes [55, 48, 59]. Lf elicits anti-inflammatory efficacy via multiple pathways of the innate and adaptive immune systems [49, 60]. In addition to binding soluble complexes of LPS, lactoferricins down-regulate inflammatory cytokine expression through agonism of receptors on monocytes, lymphocytes, macrophages and enterocytes [61-63]. Furthermore, progressive Fe-binding inhibits the serine protease and complement-activating capacity of apo-Lf while its turnover and release of N terminal peptides inhibit the complement enzyme C3 convertase [64].

Thus, anti-oxidant activities of Lf are mediated through both direct and indirect pathways. Apo-Lf appears to scavenge free iron and regulate its participation in harmful Fenton-type chemistries. In addition, both apo-Lf and Fe-saturated Lf can indirectly exert anti-oxidant activities, as

related to the complexation of LPS, inhibition of complement activation and consequent capacity to down regulate immune-mediated oxidative inflammation [65].

In the absence of infection, oral Lf therapy (bovine) can provide immune-stimulatory functions that suppress cancerous cell growth [66] involving: stimulating natural killer (NK) cells [67], modulating G1 proteins [68], inhibiting vascular endothelial growth factor (VEGF)-mediated angiogenesis [69] and promoting apoptosis [70]. Most of the anti-cancer mechanisms of bioactivity of Lf were not related to the iron-binding capacity of Lf [21] but specifically linked with the N-terminal of bovine Lf (amino acids 14-31) [71] although growth regulation of sub-populations of mononuclear phagocytes with Lf receptors are favoured by iron-saturated forms of Lf [72].

Amyloid precursor protein

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a ubiquitous transcription factor, present in most mammalian cell types and an important regulator of the innate immune system orchestrating cellular, cytokine and growth factor signaling responses. NF-κB activation status reflects inflammation status. The promoter region of the APP gene has a NF-κB transcription factor binding site which stimulates the expression of APP [73]. Similarly, a NF-κB binding site is present on the promoter region of β-secretase and expression of β-secretase in brain neuronal and microglial cells is stimulated by pro-inflammatory conditions and vice versa [74]. Immune and prostaglandin E_2 (PGE_2)-mediated pathways of inflammation not only stimulate APP expression, but also promote amyloidogenic processing of APP. Therefore, NF-κB and APP expression are both expected to be correlated with inflammation status and furthermore, elevated inflammation appears to promote amyloidogenic processing of APP and risk of AD.

A primary role for inflammation in AD is supported by genetic profiles of early onset AD subjects (ie, highest risk) being comparatively enriched in pro-inflammatory alleles and independent of APOE alleles whereas lowest AD risk genetic profiles were devoid of both APOE and pro-inflammatory alleles [75]. Thus, lifestyle factors, infections and physiological changes in ageing all contribute to inflammation status and may contribute to AD incidence.

Chronic inflammation prevails in AD brains to which the progressive loss of neuron viability is largely attributed [76]. Hypothetical *initiation* of APP upregulation can be accounted for by infection [77] and other origins of acute and chronic inflammation (i.e., *primary* inflammation, either systemic or in the brain) associated with disease and ageing, termed 'inflammageing' [78]. Ageing *per se* is associated with a 2-4-fold increase in plasma levels of cytokines and acute phase proteins [79] and loss of integrity of the blood brain barrier (BBB) that can permit T cell recruitment and inflammation status signaling from the periphery to the brain [78]. Systemic infection can produce similar effects to inflammation associated with chronic disease and ageing [80] and has been attributed to generalised symptoms known as 'sickness' behavior [81]. Systemic inflammation also exacerbates neurodegeneration [82].

The membrane localization of APP in neuronal and other cells and its role in inflammation signaling by chemo-attraction at the endothelium can explain the dependence of its expression on inflammation status, which is further exacerbated by amyloidogenic processing and plaque deposition. Pro-inflammatory mediator signaling drive proliferation of microglial cells and astrocytes seeking to clear aggregated forms of Aβ [83]. However, chronic activation of microglia in the ageing brain [84] compromises their neuroprotective role [78]. Mouse knock-out studies indicate that APP has important functions in both the brain and periphery [40], affecting the phenotype of immune and endothelial cells [41]. APP can be linked with mediating cell binding to extracellular matrix, cell-cell adhesion, acting as or interacting with neurotrophic factors, mediating axon pruning and participation in intracellular signaling including the apoptotic pathway. It is not clear whether APP acts as a receptor or ligand (or both) at the cell surface, if the influence on cell signaling is direct or driven through gene regulation, or how full length APP and numerous potential intracellular binding partners are coordinated [85].

Expression of APP is specifically sensitive to iron via a type II iron response element (IRE) at its 5′-untranslated promoter region [86]. Like Lf, APP preferentially complexes and stabilises the ferrous form of iron. The oxidation of ferrous to ferric iron increases the expression of APP [87] while chelation of ferric iron by deferoxamine reduced APP expression in AD transgenic mice [88]. Indeed, compelling epidemiological evidence for a role of infection as a possible cause of AD [77] is also supported by the biological role of APP in managing iron supply associated with pathogen invasion.

The haemopoietic stem cell environment is also compromised in ageing. There are decreased circulating $CD34^+$ (progenitor) cells in early AD [89]. Furthermore, plasma levels of granulocyte colony stimulating factor (G-CSF) which promotes mobilisation and differentiation of haemopoeitic progenitor cells in response to inflammatory status, are inversely correlated with age and specifically in AD, with conversion from mild cognitive impairment (MCI)

to AD inversely correlated with plasma G-CSF levels [90]. Low G-CSF also compromises the immune cell regulation of pro-inflammatory cytokines (IL-1, TNF-α, IFN-γ).

There are many studies that demonstrate the sensitivity of APP expression to inflammation status. Exposure of human endothelial cells to the inflammatory cytokine IL-1 stimulated APP mRNA [38]. In rat microglia, PGE_2 and the PGE_2 receptor agonist butaprost, both stimulated APP expression [91], which was reversed by the PGE_2 receptor antagonist AH0609 [91]. Increased expression of APP and levels of amyloidogenic peptides (Aβ40 and Aβ42) were found in aged APPswe transgenic mice exposed to injections of lipolysaccaride (LPS) for 12 weeks [92]. Similar effects were reported in staggerer mutant mice, a model of chronic inflammation in the cerebellum [93]. Compared with wild type mice, the staggerer mutant mice displayed upregulation of pro-inflammatory IL-1β and IL-6 and changes in the ratio of KPI containing-APP to non-KPI APP695 protein [93]. In addition, challenge with LPS, further increased the expression ratio of KPI-APP to APP695 in the cerebellum of both wild type and staggerer mutant mice, an effect that was more pronounced in the mutant mice [93]. These results suggested that the KPI domain and its protease inhibitor function may have been an important feature of the host defence response involving APP. Pro-inflammatory effects of a high-fat diet (male wild type mice fed a 21.2% w/w fat diet for 22 weeks) were demonstrated by elevated levels of inflammatory cytokines (TNF-α, the astrocyte marker: glial fibrillary protein and prostaglandins) and APP expression detected in brain (hippocampus) and adipose tissues [94].

Apart from direct inflammatory stimuli, many other factors are known to modulate status of inflammation and therefore potentially influence APP expression. For example, levels of pro-inflammatory advanced glycation end products (AGEs) are increased in metabolic disorders such as obesity and Type 2 diabetes. When compared with bovine serum albumin, intravenous administration of a preparation of AGEs to wild type mice, led to a significant increase in brain APP [95]. In morbidly obese patients, gastric bypass surgery lowered both inflammation status and APP expression measured in blood mononuclear cells [96]. Likewise, vitamin D, a hormone regulating over 900 genes, is important in regulating inflammation. Vitamin D deficiency is associated with increased chronic inflammation [97] while lower expression of Vitamin D receptor confers higher risk of AD [98]. Supplementation with Vitamin D decreased APP promoter activity in neuronal cells [98]. Pro-inflammatory effects of sleep deprivation [99] and its positive association with dementia risk [100] may reflect elevation of APP expression, as supported by higher amyloid plaque loads detected in sleep-deprived APPswe mice compared with sleep-deprived wild type mice [101].

Does the co-localisation of lactoferrin and amyloid precursor protein in AD biology support infection as a causative and treatable factor?

Amyloid precursor protein and Alzheimer's disease

The significance of APP overexpression in the brain and its amyloidogenic processing into Aβ peptides and deposition as senile plaques, is very well established. The culmination of 10 years prior research was presented as the 'amyloid hypothesis' of Alzheimer's disease by Hardy and Higgins in 2002 [102]. The hypothesis suggests that Aβ peptides are the main component of amyloid plaques and the causative agent of AD. Furthermore, the hypothesis also suggests that neurofibrillay tangles, cell loss, vascular damage and dementia follow as a direct result of the plaque deposition. The basis of this hypothesis comes from the identification of an autosomal dominant mutation in the amyloid precursor protein (717Val→Ile) which led to the disease [103]. Other mutations in APP and the presenilins (catalytic component of the γ secretase complex) have been identified with carriers usually having an early onset AD (30-40 years old). These mutations are not present in Late Onset AD (LOAD/sporadic AD) however genome-wide association studies have found that there is genetic predisposition associated with cholesterol metabolism and the complement cascade [104, 105, 106]. The latter suggests that following over-production of APP and Aβ products, individual responses to Aβ clearance versus accumulation can greatly affect the risk of AD.

Further support of the amyloid hypothesis is a new mathematical model based on the retention of the amyloid ligand, ^{11}C-Pittsburgh compound B, in the brain. This positron emission tomography imaging evidence shows that amyloid deposition occurs 17-23 years before AD is symptomatic [107]. Hence, recent failures of amyloid-centric drug trials may reflect that timings of interventions in relation to stage of AD development were not optimal and might have been effective in early symptomatic or pre-symptomatic stages, perhaps as identified by a defined load of Aβ in the brain. Clearly there are research challenges with validating efficacy of interventions with a 20 year trajectory of disease development.

In the brain, Aβ is generated mostly by neurons from the transmembrane protein, APP. Under physiological conditions, the extracellular part of APP is sequentially cleaved at the cell membrane by α-secretase and a complex of γ-secretases to generate a form of extracellular soluble Aβ which does not aggregate. Conversely, cleavage by β-secretase instead of α-secretase invokes the

"amyloidogenic pathway" whereby APP releases extracellular Aβ peptides which have propensity to form amyloid fibrils. Conditions which are conducive to the amyloidogenic pathway include metabolic stress associated with diabetes, obesity, cardiovascular disease and other chronic conditions, all of which are accompanied by a strong pro-inflammatory phenotype.

With disease progression, increased Aβ in brain frontal cortex of sporadic AD patients is accompanied by increases in β-secretase expression in the AD-affected temporal lobe [108, 109]. Whilst the amount of Aβ in the brain accumulates, the level of $A\beta_{1\text{-}42}$ is reduced in cerebrospinal fluid (CSF) of AD patients compared with controls [110]. Hence, the accumulation of Aβ peptides in the brain arise through increased production of APP and its amyloidogenic processing and/or by reduced clearance from the brain.

The brain, compared to other tissues in the body, is particularly vulnerable to oxidative stress due to the low levels of antioxidant enzymes, its high metabolic rate and reliance on oxidative phosphorylation [111]. The $A\beta_{1\text{-}42}$ peptide is shown to complex with transition metals (copper, zinc and iron) which promote rate of aggregation [112]. Copper and zinc binding to Aβ is also associated with production of neurotoxic hydrogen peroxide (H_2O_2) [112]. Aggregated Aβ not only places further demand on the already compromised efficiency of monoctye/macrophage phagocytosis [113] but increases the expression and activity of β-secretase [114] leading to further amyloid accumulation.

Further complexity to understanding AD is that APP and APP products including amyloidogenic Aβ can be found inside the cell suggesting that after cleavage there is re-uptake of APP and/or its fragments by the cell [115]. Re-uptake supports cellular regulation of extra-cellular signaling by APP as suggested earlier. APP which is made at the endoplasmic reticulum may also undergo intracellular degradation [115]. Exosomes which are cell membrane-derived particles are released extracellularly and can contain APP, APP fragments including Aβ [116], β-secretase and some components of the γ-secretase complex [117]. It has been suggested that exosomes may have a role in cell to cell transmission of Aβ and APP fragments [118]. The phenomenon of transmission is also known as seeding nucleation and may occur not only with misfolded Aβ but with other misfolded proteins such as tau and α-synuclein [119].

Lactoferrin and Alzheimer's disease

Unlike for APP, the detection of Lf in senile plaques and its significance is not fully understood. Dynamic molecular functions of Lf indicating capacity for self-regulation from pro-inflammatory to anti-inflammatory phenotype during normal processing was described above. It is not known if these properties of Lf turnover in the periphery are operative in the brain but like APP, Lf (selected genetic variants) and Lf-derived peptides display propensity for amyloidogenic folding. An N-terminal peptide of Lf (lactoferricin-B) was reported to adopt a non-native β sheet structure with amyloid fibril characteristics under controlled conditions *in vitro* [120]. In addition, mixtures of amyloidogenic peptides derived from Lf with intact Lf also formed fibrils *in vitro* [121].

Amyloidogenesis of Lf also occurs *in vivo,* specifically in the eye and brain. Particular mutant variations of Lf but not wild type Lf were able to form amyloid deposits in human (non-AD) cases of trichiasis (abnormal eye-lash contact with cornea), which produces local inflammation [122]. Amyloid deposits of Aβ peptides have been visualized in the eye using retinal imaging methods and offer potential for early diagnosis of AD [123]. However, while co-localisation of Lf and Aβ peptides has been reported in brain tissues from both AD patients [124] and AD transgenic mice [125], no researcher has yet sought to concurrently detect fibril deposits of *both* APP-derived peptides and Lf (and possibly Lf-derived peptides) in the eye. Considering their co-operative roles in innate immune system regulation and co-expression in brain under pro-inflammatory conditions such as AD, it is hypothesized that fibrillar deposits in the eye are also likely to contain both Lf and APP-derived peptides.

Lf has been detected in senile plaques in brain tissue of AD patients along with a number of proteins apart from Aβ peptides, including: apolipoproten E receptor, a2-macroglobulin receptor, low-density lipoprotein-related protein and others [124, 126]. Although, it is not clear if co-localisation of these proteins reflects their abnormal upregulation associated with AD pathology or inadvertent non-specific 'capture' by sticky plaques, only APP-derived peptides, Lf and Lf-derived peptides display similar tendencies for fibrilogenesis during turnover that has not been shown of the other proteins.

While all of the proteins detected in AD plaques have known functions (Rebeck *et al*, 1995), only precursor proteins Lf and APP cannot be explained without invoking their hypothetical roles in innate immune system signaling. These specific biological roles of Lf and APP in regulation of innate immunity in general and infection in particular, may provide mutually supportive evidence for implicating infection as a trigger for AD pathology rather than simply being *associated* with AD pathology. Many pro-inflammatory triggers arising from infection, chronic disease and ageing can stimulate expression of APP however

the primary iron-scavenging function of Lf signals infection as a significant factor requiring attention by the innate immune system. Monitoring sustained increases in APP and Lf expression of individuals may therefore be considered useful biomarkers for diagnosis and therapeutic efficacy in AD prevention. Indeed, control of infection-dependent and independent types of inflammation by therapies including antibiotics, diet, lifestyle and pharmacological approaches are well established.

Infection and anti-biotic therapy in Alzheimer's disease

The role of unmanaged chronic inflammation in mid-life as a risk factor for AD in later life has been described [127]. Furthermore, lowering of chronic inflammation by therapeutic intervention or approaches that address its origin in mid life, confer protection against AD in later life [127]. However, the use of anti-inflammatory therapies have not been effective in modifying progression or symptom relief in established AD presumably reflecting irreversible brain damage caused by the runaway pathologies of AD including pro-inflammatory aspects.

We have proposed that the co-expression of APP products and Lf in amyloid plaques in eyes and brain of AD patients may be indicative of infection as a specific cause of the pro-inflammatory status of the host. Current research does not permit the specific requirement for peripheral versus brain infection to be confirmed. However, it is known that the inflammation status of the periphery can 'infect' that of the brain [128] and that progressive dysfunction of the blood-brain barrier in advancing AD might permit pathogen transport and cross-infection between the central and systemic circulations, eventually invoking upregulation of Lf in the brain when localized infection is detected. Below we review the evidence for relationships between (a) infection and AD risk and (b) antibiotic therapy and AD treatment or prevention.

Infection and AD risk

The significance of a causative relationship between bacterial and viral infection and AD has been recently reviewed [with extensive evidence for a positive association reported but limited by lack of proof of any specific infection causing AD [77]. Furthermore, pathogen DNA has been detected in brain tissues of both AD and normal subjects. In support of an association, the prevalence of accelerated cognitive impairment that accompanies AIDS infection is undisputed [129]. More recently, infections originating from oral [130] and fungal sources [131] have also been linked with AD risk. It is clear that infection does not necessarily dictate progression to AD as reflecting multiple opportunities for biological defence, with the robustness of the innate immune system of particular importance. It is possible that inadequate resolution of treated or untreated infections that subsequently reach the brain permit APP-mediated pathologies to become dysregulated. This is the first time that a hypothesis centered on APP and Lf as biomarkers of infection status has been proposed.

Antibiotic and anti-viral therapy and AD treatment or prevention.

Recognition that infection represents a potential risk factor for AD leads to the logical question of the use of antibiotic or anti-viral therapies for prevention and treatment [132]. A related, alternative approach to targeting the pathogen *per se* has been to elicit immunosuppression and cognitive improvement by brain-targeted counter infection, which was successfully demonstrated using *Toxoplasma gondii* in Tg2576 mice [133]. A number of early phase trials are currently underway with various antibiotic therapies including: doxycycline, rifampicin, minocycline and moxifloxacin (clinicaltrials.gov). Results posted for the minocycline trial (NCT01463384 involving AD, MCI, healthy control (HC) subjects treated with 50 mg of minocycline twice daily for 6 months), revealed trends (statistical analysis not provided) towards (1) improvement of neuropsychological status for HC but decline for AD and MCI; (2) increased hippocampal volume for MCI but no change for AD or HC and (3) trend towards 'normal' range of ratio of N-acetylaspartate/myo-inositol for AD and MCI but no change in HC groups. These results might be considered most promising for HC and MCI groups supporting a beneficial role for antibiotic therapy in pre- and early symptomatic stages of disease. However, the trial participants were not screened for baseline infection status and probably included those with both infection-dependent and independent APP/amyloid pathology (ie, non-infection related chronic inflammation) with low power for influencing infection-specific effects. There do not appear to be any trials for anti-viral therapies that specifically target the virus and not related symptoms (clinicaltrials.gov). Thus, it appears that recognition of APP and Lf as biomarkers of the innate immune system in AD offer new opportunities for diagnosis and intervention studies.

Conclusions

The molecular and functional properties of Lf and APP were compared and interpreted to inform previously unrecognized and co-operative roles of respective proteins. Lf and APP display many common and some complimentary biological properties. Functional properties of these proteins each evolve during turnover as reflecting specific properties

and localizations of encrypted peptides. Peptides derived from APP and Lf each display propensity for self-assembly into amyloid fibrils both *in vitro* and *in vivo*. By virtue of the co-expression and co-localisation of Lf and APP-derived peptides in amyloid deposits in the eye and brains of AD patients, the functional properties of APP were proposed to capitulate those of Lf and infer a previously unrecognized role of APP as a signaling molecule of the innate immune system. APP expression is consistently found to reflect the inflammation status of the host but appears to be responsive to many types of inflammatory stimuli associated with ageing and chronic disease. Loss of regulation of APP expression in the pro-inflammatory state of the brain in AD leads to APP-dependent pathologies including deposition of Aβ in senile plaques. However, the co-expression of Lf and APP can infer a supportive role for APP in the innate immune defense specifically mounted against infection. It is suggested that the presence of Lf and APP together reflect infection-specific inflammation and can account for the substantial but inconclusive association of AD with infection. It is proposed that APP and Lf expression represent novel and important biomarkers for future clinical studies of AD risk and treatment, with significant implications for identifying when anti-biotic, anti-viral or general anti-inflammatory therapies are needed. Longitudinal studies of APP and Lf expression may provide a 'window' to the inflammation status of the brain. Finally, supplement forms of bovine Lf may also offer a therapeutic tool assuming exogenous Lf is absorbed and subjected to turnover and release of peptides that stimulate anti-inflammatory immunomodulation, as observed for endogenous Lf. These hypotheses require further substantiation.

Abbreviations

Aβ: amyloid beta peptide; AD: Alzheimer's Disease; APP: amyloid precursor protein; TNF-α: tumour necrosis factor alpha.

Conflicting interests

The authors have declared that no competing interests exist.

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