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Research Article

Nuclear Amyloid Fibrils Detected in Human SH-SY5Y Cells in Presence of $A\beta_{1-42}$ and LPS

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Abstract

Amyloid-β peptide (Aβ) represents the main component of amyloid plaques in Alzheimer's disease (AD); Aβ belongs to the group of antimicrobial peptides (AMPs) small peptides that kill pathogens through their antimicrobial activity and also have affinity for bacterial lipopolysaccharide (LPS). If amyloid is part of the antimicrobial mechanism of Aβ, fibrillar material would also be expected to accumulate as long as the innate immune system, correctly or incorrectly perceives an infection. Repeated reactivations of the chronic latent infection are constantly producing new Aß peptide, this situation lasts for a long time in the decades preceding the manifestation of AD, progressively leading to neurodegeneration and neuroinflammation. Aim of this work was to evaluate the concomitant synergizing action of $A\beta_{1-42}$ and LPS in human SH-SY5Y cells; AMPs and LPS have an amphipatic structure that is able to form heterogeneous micelles, in this way LPS acts as a fibrillogenesis promoter, Furthermore, depending on peptide concentration, the action of Aβ as AMP can be bacteriostatic or bactericidal.

Keywords: Amyloid-beta (Aβ); Bacterial Lipopolysaccharide (LPS); Antimicrobial Peptide (AMP); Blood Brain Barrier (BBB); Alzheimer's Disease (AD)

Introduction

Recent studies suggest that Amyloid-beta (Aß) is an ancient highly conserved effector molecule of the innate immunity, an antimicrobial peptide (AMP) [1-3]. This new etiology model is characterized by Aβ deposition as an innate chronic immune response, which normally protects against microbial infection in brain. Nonetheless, the view that Aß is functionless remains wildely held, despite evidence highlighting that human Aß sequence is 100% conserved across most vertebrate species up to at least 400 millions years [4,5]. Growing of Aß fibrils [6], capture, agglutinate and finally entraps microbes in a resistant network of ß-amyloid, the antimicrobial protection hypothesis reveals how an increased brain microbial burden may directly exacerbate fibrils deposition, inflammation and neurodegenerative disease progression.

The human AMPs are not incidental vestiges of immune evolution; low AMP levels can result in a seriously compromised

immunity. Available data suggest that Aß is an inducible rather than constitutive AMP. If amyloid is part of the antimicrobial mechanism of Aβ, fibrillar material would also be expected to accumulate as long as the innate immune system, correctly or incorrectly perceives an infection [1]. What remains to be determined is if Aß-mediated activation of immune pathways in AD is pathological disregulation or an immune response to a genuine chronic infection. In the decade preceding the full manifestation of AD, there may be repeated reactivations [7,8] of the chronic latent infection constantly producing new Aß peptide in response, progressively leading to neurodegeneration and neuroinflammation [9]. Chronic neuroinflammation, over time, causes marked changes in the brain that lead to neuron degeneration and, in turn, to non-steroidal functional decline. Microglia forms a sort of lattex through the brain and express many immunological receptors, TLR2, TLR4 and TLR6 that act together augmenting the response to Aβ exposure [10], as well as their co-receptors. Activation of caspase-1 [11] itself is controlled by large multimolecular signaling complexes called inflammasome, a cytosolic multiprotein oligomers belonging to the innate immune system the inflammasome sensor NLRP3 [11] is important for mediating neuroinflammation, and it is activated in response to extra- and intracellular danger signals including Aß aggregates. Active caspase-1 is present in the brains of patients with AD as respect to those of age matched control subjects [12]. Blocking the NLRP3 decreases the Aß induced formation of IL-1ß in the brain and improves the clearance of Aß by microglia in APP/PSI mice; NLRPs inflammasome is also an important contributor to normal-age related system inflammatory response [12].

Microglia contributes to the killing of neurons through the induction of neurodegeneration by soluble inflammatory mediators but it can destroy functional Aβ by direct phagocytosis. These mechanisms could exist in parallel but operate in specific disease phases and/or depend on the state of activation of the innate immune system [9,13].

The physiologically produced and circulating Aβ may have such functions [14]; it can be hypothesized that amyloid peptide secreted by certain types of bacteria helps to maintain the pathological bacteria under control [8], The production of Aβ, as an AMP, helps to limit the original pathogen at the beginning of an infection [15].

An high dose of endotoxin (LPS) can induce blood brain barrier (BBB) permeability, allowing plasma components to enter into the brain, thus potentially resulting in neuroinflammation and neurodegeneration [16,17] but also potentially allowing endotoxin to penetrate into the brain. LPS may induce brain inflammation by activating the NF-kB signaling pathway. This implies that response to blood endotoxin requires the presence of TLR4 [18] because the long term effect of blood LPS on the brain may be mediated at least in part by LPS which stimulate the above cells to produce cytokines within the brain [18].

High plasma level of endotoxin can then increase the permeability of BBB allowing toxic plasma components including Aβ and α-synuclein (aS), which form insoluble fibrils in pathological conditions such as Alzheimer's disease (AD) and Parkinson disease (PD) into the brain [19]; this suggests the possibility that LPS synergizes with different aggregable proteins to favour different neurodegenerative diseases [20,21].

In AD patients endotoxin levels are increased threefold in blood, and two or threefold in the brain [14] and endotoxin is also found in AD amyloid plaques [22,23].

On the other end the increased risk of AD is associated with the ε4 variant of the apolipoprotein E gene [24], carriers of the ε4 allele have higher rates of CNS infection for several pathogens [1,9]. If AD is at least in part mediated by endotoxin, it then is possible that gene variants associated with AD interact with endotoxin. APOE isoform is the main genetic risk for AD [25]; humans with APOE4 variant are more sensitive to LPS and show higher Aβ levels in the brain, thus having a higher risk for infections and cardiovascular disease. Sequence variants of TLR4 and the LPS binding receptor TREM2 are also associated with an increased risk of AD, an additional genetic link between AD and the brain endotoxin.

Aim of this work was to evaluate the concomitant synergizing action of $A\beta_{1-42}$ and LPS in human SH-SY5Y cells [26]; Aβ for its positive charge has a high affinity for the LPS present in the outer membrane of Gram negative bacteria. They both have an amphipathic structure that is able to form heterogeneous micelles that can in turn, generate amyloid fibrils. As a function of the peptide concentrations, ionic strength and type of stressor the action of Aβ as AMP can be bacteriostatic or bactericidal [1]. In this work the most significant results were obtained after 72 hrs.

Materials and Methods

The LPS stock suspension from E. coli serotype 0128:BI2 (SIGMA-Aldrich Chemie, Germany), was dissolved in distilled water at the final concentration of 0,05µg/ml.

The $A\beta_{1-42}$ fragment (SIGMA-Aldrich Chemie, Germany) was dissolved in DMSO and then diluted in PBS, to obtain a final concentration of 0,5 µg/ml.

Cell culture

Human SH-SY5Y cells provided by the American Type Culture Collection (ATCC, Manassas, VA) were grown in Eagle's minimum essential medium, supplemented with 10% fetal bovine serum 1% penicillin-streptomycin, L-glutamine (2 mM), with the addition of non-essential amino acids (1 mM), and sodium pyruvate (1 mM) (all from Euroclone, Milan, Italy) at 37°C, in an atmosphere of 5% CO_2 and 95% humidity. SH-SY5Y cells were plated (1.0×10^6) on 100 mm petri dishes; each experiment was performed for 24, 48, and 72 h at 37°C, 5% CO2; $A\beta_{1-42}$ was in the form of monomers at the starting concentration of 10 µg/ml.

Cell conditions

Experiments were performed at 37°C for 24, 48 and 72hrs: SH-SY5Y untreated (control), SH-SY5Y/LPS (0,05µg/ml), SH-SY5Y/ $A\beta_{1-42}$ (0,5µg/ml), SH-SY5Y/LPS (0,05 µg/ml)/$A\beta_{1-42}$ (0,5µg/ml). After each treatment, cells were washed three times with PBS.

Transmission electron microscopy (TEM)

TEM was performed by standard technique: samples were fixed in glutaraldehyde 2.5% and cacodylate sodium buffer pH 7.4 for 2 hrs at room temperature. Cells were then rinsed in cacodylate sodium buffer (pH 7.4) overnight and post-fixed in 1% aqueous osmium (OsO_4) for 90 minutes at room temperature. Dehydration was performed at increasing ethanol concentrations (50% to 100%) then samples were embedded in epoxy resin, Epon 812. Thin sections were counterstained; observations and micrographs were performed on a Jeol JEM (Tokio, Japan) operating at 100 kV.

Immunogold immunoreaction with post-embedding method

The samples included in Epon 812 resin were cut at 600–800 Å using the ultramicrotome. The ultra-fine sections were placed on nickel grids and left to dry. Later they were incubated with NGS (Normal Goat Serum), diluted 1:20 in 1% PBS-BSA in order to block non-specific immunoreactivity for 15'. Then exposed overnight in primary anti-Aβ (monoclonal antibody produced by Sigma-Aldrich) at the dilution 1: 100 in PSB-BSA. After washing in PBS, the samples were exposed to the colloidal gold-labeled secondary antibody Goat Anti-mouse (GAM) Abcam) at the diluition 1:20 in 1% PBS-BSA, for 1 hour. Finally the washes were performed with PBS-BSA for 1 h, and then in PBS and distilled water. Samples were then counterstaining with 5% uranyl acetate and 1% lead citrate. Observations and micrograft were performed at a Jeol JEM operating at 100 kV.

Results

TEM analysis

SH-SY5Y cells following incubation with $A\beta_{1-42}$ after 72 hrs (Figure 1). In A, human SH-SY5Y cells as control, In B, is detectable a nucleus with a prominent fibril protruding from nuclear membrane (arrow). Another regular helical twist fibril is visible in the same picture in cell cytoplasm (arrowhead). In C, the same details at greater magnification. A great cell with clumped heterochromatin detectable in nucleus, irregular dismorfic mitochondria showing lost of cristae are recognizable in D; the molecular damage induced by fibrils is enhanced by the increased ROS production (data not shown). In cell cytoplasm micelle-like structures are formed, clumped heterochromatin in nucleus is also detectable in E. A spherical particle, possibly containing little fibrils, embedded in nuclear membrane is detectable in F and in G at greater magnification (arrow).

Figure 1: SH-SY5Y cells following incubation with A-$beta_{1-42}$ for 72 hrs. In A) SH-SY5Y cells without A-$beta_{1-42}$ as control, bar = 2 µm; in B) is detectable a nucleus with a prominent fibril protruding from nuclear membrane (arrow), a long twisted fibril is also visible in the same picture (arrowhead), bar = 1 µm. In C) the same picture at greater magnification, bar = 500 µm. In D) a great cell with clumped heterochromatin in nucleus is detectable, irregular dysmorphic mitochondria showing lost of cristae, bar = 2 µm. Structured micelles are detectable in E) (arrow), bar = 1 µm. In F) a spherical particle embedded in nuclear membrane (arrow), bar = 1 µm, the same picture at greater magnification in G), bar = 2 µm. H) grainy material with the characteristics of thickened chromatin with an intense staining and condensation that are typical of transcriptionally inactive chromatin; spherical structures are also detectable, bar = 1 µm. Micellar particles near nuclear membrane (arrow)in I), bar = 1 µm.

Filamentous grainy material with the characteristics of thickened chromatin with an intense staining and condensation, characteristic of transcriptionally inactive chromatin in H. Globular structures are also detectable in the same picture. Further, micellar particles and fibrils are visible near nuclear membrane in figure 1 (arrow).

SH-SY5Y cells following co-incubation with $A\beta_{1-42}$ and LPS after 72 hrs (Figure 2). Long, irregular, flexous fibrils are evident all around the nucleus in A, B, C (arrow); LPS seems to act as a fibrillogenesis promoter since the fibrils visible in these pictures are longer than those observed in figure 1 B, C where cells were incubated only with A-$beta_{1-42}$. Micelles are also detectable in D (arrows); this is in agreement with the amphipathic nature of both Aβ and LPS. Further Aβ and LPS also show affinity for lipid bilayers such as cell membrane. In E long fibrils and swollen mitochondria (arrow). Very long twisted flexous smooth fibrils, not observed in SH-SY5Y incubated only with $A\beta_{1-42,}$ are detectable in F. Strange spore-like structures only formed by LPS in G, while in H the same structure formed by $A\beta_{1-42}$ and LPS**.**

Figure 2: SH-SY5Y cells following incubation with $A\beta_{1-42}$ and LPS for 72 hrs. Potentiation of Aβ fibrillogenesis has occurred, as shown in A-C); long irregular flexous smooth paired fibrils are visible situated all around the nucleus and in cytoplasm (arrows), LPS acts as a fibrillogenesis promoter, bar = 1 μm for A-C). Micelles (arrow) are present in D), showing the amphipathic nature of Aβ and LPS, bar = 500 nm. Swollen mitochondria have partially lost their cristae, E) bar = 1 μm. In F) are depicted long flexuous helical twist fibrils of the smooth Aβ. In G) strange spore-like structures only formed by LPS, bar = 1 μm; in H) the same structure formed by the concomitant action of Aβ and LPS, bar = 1 μm.

Gold labelling, specific for Anti Aβ, of SH-SY5Y cells following incubation with $A\beta_{1-42}$ and LPS (Figure 3). In A untreated control, in B it's possible to appreciate a nucleus with electron-dense particles of 10-nm gold labelling specific for anti A-$beta_{1-42}$ (arrows) without LPS. Some micelles formed by concomitant action of Aβ and LPS are detectable in C and inset, recognizable by the presence of gold particles. In. D is recognizable a nucleus showing apoptotic phenomena; this was evident especially in samples treated with both $A\beta_{1-42}$ and LPS. Scattered fibrils are also present (arrow). Clumped heterochromatin is always detectable in nucleus in E. In F a nucleus with gold particle undergoing an anomalous division.

Figure 3: SH-SY5Y cells co-incubated with $A\beta_{1-42}$ and LPS in presence of gold labelling specific for anti A-$beta_{1-42.}$ A) control, without Aβ and LPS, in presence of gold labelling specific for anti A-$beta_{1-42}$, bar = 2 μm. In B) human SH-SY5Y cells only incubated with $A\beta_{1-42}$; electron-dense particles showing 10-nm gold labelling specific for anti A-$beta_{1-42}$ are detectable in the nucleus (arrows), bar = 2 μm. C-F) SH-SY5Y cells co-incubated with $A\beta_{1-42}$ and LPS in presence of gold labelling specific for anti A-$beta_{1-42}$. Micelles formed by concomitant action of Aβ and LPs, showing 10-nm electron-dense particles for anti-A-$beta_{1-42}$, in C) (arrow). In the inset are detectable micelles with electron-dense particles, bar = 1 μm. In D), a nucleus undergoing apoptotic phenomena is also visible, bar = 2 μm. A clumped heterochromatin is always detectable in nucleus E), bar = 2 μm. In F) a nucleus undergoing an anomalous division, bar = 2 μm.

Discussion

Lipids, fatty acids and detergents have been found to accelerate fibrilization [27-29].

When the ratio of proteins to lipids is low, low concentrations of lipids or detergents result in aggregation, while high concentrations of lipids or detergents tend to prevent their aggregation [30,31]. One possible mechanism by which lipids or lipid-like molecules such as LPS, may facilitate aggregation is by bounding the protein to a small vesicle or micelle (Figure 1 E, Figure 2 D), increasing in this way the effective concentration and driving aggregation through mass action [30].

The incorporation of LPS into Aβ micelles suppressed their ability to generate nuclei of fibrils, to the point that heterogeneous nucleation on non-Aβ seeds dominates the nucleation process; the resulting fibrils are indistinguishable from those nucleated through micelles [32]. AMPs, small molecule peptides, are able to kill pathogens through their antimicrobial activity but on the contrary for the positive charge have also a high affinity for LPS or membrane receptors [33].

The conformational changes induced in the protein by its interactions with the membrane can favour the intermolecular interactions leading to aggregation. Also detergents appear to induce intermediate conformations of Aβ [30] and alpha synuclein (aS) if present at a concentrations that favour aggregation [31]. Unfavorable interactions with the membrane surface eventually affect membrane integrity, leading to its disruption accompanied by micelles formation [34].

Moreover, Aβ may induce intracellular calcium deregulation, that leads to apoptosis by direct interaction with isolated mitochondria (Figure 1 D, Figure 2 E) or by indirect association with the neuronal membrane, figure 1 A, and B at greater magnification.

The above results suggest that infections are important cofactors in promoting the progression of AD [35]. The incorporation of LPS acts in elongation of the amyloid fibril and occurs at an early stage of Aβ aggregation acting as a seed; it acts in elongation of the amyloid fibril [31,36]. Aβ as AMP counteracts the action of LPS only in a concentration-dependent manner. Spherical amyloid intermediates (Figure 1 E-I; Figure 2D; Figure 3 C and inset) represent a common state of assembly and aggregation for many different amyloids.

Furthermore, in human only one cathelicidin (hCAP-18), namely LL-37 is expressed [37]; LL-37 and its precursor were found in different cells, tissue, lymphocytes and body fluids; this human cathelicidin is produced or synthesized in response to the presence of bacteria or their products and it is an important effector molecule within the innate immunity mechanism [38,39]. Furthermore LL-37 is an AMP and exhibits two common motifs: cationic charge and amphiphilic form; of interest LL-37 binds more strongly to Aβ oligomers than to Aβ fibrils, for this reason its presence tends to prevent the formation of Aβ fibrils [40]. It is interesting to note that if patients express low levels of LL-37 have an high risk to develop serious infections, while high levels of LL-37 are associated with pathology including atheromathous plaques [41,42].

Several images reported in this work show the presence of heterochromatin tightly packed nuclei (Figure 1 B-I; Figure 2 A-C, E); these regions have no or very few replication sites, since the DNA within the heterochromatin is in a transcriptionally-repressed state.

Heterochromatin is a conserved feature of eukaryotic chromosomes that serves to repress the transcription of certain genes and to confer genome stability within repetitive regions of the genome [43]. The two forms of chromatin, heterochromatin (highly condensed) and euchromatin (less condensed), are functionally and structurally distinct regions of the genome, The difference is in large part due to the presence of methyl groups on DNA, indeed DNA methylation prevents transcriptional activity; this process is called transcriptional silencing [44]. Heterochromatin is now known able to give rise to small RNA interference (RNAi) that direct the modification of proteins and DNA in heterochromatic repeats [45]. The classical view is that 'silent' heterochromatin is not transcribed into RNA, on the contrary RNAi has been found to have a central role in heterochromatic gene silencing. Heterochromatic silencing depends on the processing of repeat RNA transcripts into short interfering RNAs (siRNAs), which then direct chromatin modification. The first biological function attributed to RNAi was defence against transposons and viruses; we might speculate that this function could also plays a role in the experimental conditions performed on cells incubated with Aβ and LPS. This RNAi-dependent gene silencing mechanism will surely have implications that are still unknown for the biological role of heterochromatin [45].

Conclusion

Gut microbiota-derived amyloid may enhance inflammation in response to cerebral $A\beta_{42}$ by acting upon the innate immune system [46,47]. "Inflammaging" increases gut and BBB permeability which in turn influences the production of Aβ in the brain. After activation by LPS, monocytes, microglia and astrocytes increase the expression of amyloid precursor protein and release Aβ peptides. In agreement with this concept, in a mouse model it has been observed that LPS induces amyloid precursor protein expression and subsequent Aβ plaque deposition. These finding suggests that the secretion of the Aβ peptide is to function as an AMPs, as part of the innate immune defense within the CNS [48].

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