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Amyloidogenesis and Responses to Stress

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Abstract

Amyloidogenesis is a primitive, physiological response that seems to be an ancient process widely distributed in different cell types of evolutionary distant organisms. The amyloid fibril synthesis is part of a more general inflammatory response to stressful conditions all entailing overproduction of reactive oxygen species (ROS). Interestingly, this event has been integrated into additional physiological functions: (i) the formation of a scaffold promoting the activation and packaging of melanin; (ii) the formation of a scaffold to compartmentalize hormones in the cytoplasm; (iii) the ability to reversibly link different types of molecules to drive close to the nonself; (iv) the construction of a framework to close body lesions. Amyloid fibril formation is a cellular response harmonically integrated with the stress response but for a deregulation in assembling/dismantling, dangerous depots, as in a lot of pathologies, can occur.

Keywords: amyloidogenesis, ROS, melanin, invertebrates, vertebrates

1. Introduction

Bacteria, fungi, yeasts, unicellular algae, plants, invertebrates, and vertebrates can synthesize functional amyloids and physiological amyloidogenesis is a key process in response to stress. Even though the aggregation of proteins into amyloid fibrils is associated with human neurodegenerative diseases [1], including Alzheimer's, Parkinson's, and prion disease, it has been evidenced that amyloid is also a fundamental nonpathological protein folding process

that spans from bacteria to humans [2–8]. There are many examples of functional and physiological amyloidogenesis: the bacterial biofilm, the insect silk to form the cocoon, the spider silk for constructing their webs, producing egg sacs, and wrapping in their prey, etc., the scaffold to package melanin in human melanocytes when activated by UV, etc.

The various proteins forming amyloids do not necessary display the same primary structure and/or the same biochemical function and/or similarities in biophysical properties: irrespective of the nature of the polypeptide, the proteins, under defined conditions, share the ability to change folding and adopt cross- β sheet conformation with high resistance to enzymatic cleavage, and denaturant. Amyloids can be easily recognized due to their ability to bind to specific dyes such as Thioflavin T/S and Congo Red (CR) and due to their ultrastructural features.

Once the aggregates, in form of amyloid fibrillar material, are formed and tailor-made for the intra- or extracellular space, they can contribute in numerous ways to cellular/tissue/organism activities.

In functional amyloidogenesis, the synthesis of fibrils, occurring in a controlled folding pathway, is balanced by mechanisms involved in their clearance through enzymatic cleavage. When there is a slow down or a total block in this complex balance between assembling and dismantling, a spatial or temporal accumulation of fibrillar material, responsible of deleterious/pathological state, occurs.

The exposure to any kind of chemical or physical nonlethal stress is responsible of an excessive production of cytoplasmic reactive oxygen species (ROS) that, if overpowering the endogenous antioxidant mechanisms, leads to a progressive cellular damage that, in turn, impairs the survival of the cell. In other words, transient or persistent increase of ROS, as a harmful bioproduct deriving from regular cellular metabolism, is coped in all cellular types with the same sequence of events. Constant basal ROS concentration is strictly maintained with the activation of enzymatic and nonenzymatic defences such as production of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), methionine reductase, ascorbic acid (vitamin C), glutathione (GSH), and vitamin A. Uncontrolled, imbalanced production of ROS, and an inefficient free radical scavenging systems may result in inflammation, hypersensitivity, and in the pathogenesis of numerous human diseases such as neurodegenerative diseases, atherosclerosis, allergy, and cancer [9]. In healthy cells, ROS are produced in a small amount in mitochondria but the dysfunction of these organelles, resulting in increased ROS production, can be caused by rough endoplasmic reticulum (RER) stress. In stressed cells, RER cisternae release Ca^{2+} that in turn is taken up by mitochondria where determine the opening of membrane pores with the consequent enhancement of ROS production and release in cytoplasm. Resulted vicious cycle leads to impaired cellular homeostasis up to cellular death [10].

ROS overexpression, responsible of a change in cytoplasmic pH, can be considered the earliest event and an absolute prerequisite to induce proteins to adopt a β -sheet conformation, thus the amyloid fibril production is strictly linked to this event [2, 4, 5, 11].

1.1. Types of cells and subcellular districts involved in amyloid fibril synthesis

Even though the formation of amyloid or amyloid-like structure under physiological condition is a generic property of polypeptides across all kingdoms of life and can be considered as a response to different types of stimulations, it is of paramount importance to have an overview about which kinds of organisms, which types of cells, and for which purpose amyloid fibrils are synthesized.

Bacteria are able to produce and utilize amyloid material in adhesion to solid surfaces as a starting point to form the biofilm that is, for the most part of microorganisms, the guarantee to live in a consortium spatially and metabolically organized [12]. Amyloid proteins, contributing with exopolysaccharides to form the biofilm, provide a key component of this protective scaffold [13, 14].

Figure 1. Unstimulated and stimulated *H. virescens* circulating cells. Optical and ultrastructural images (A–H). Scanning electron micrographs (SEMs) (A, B): the surface of several granulocytes shows bowl-shaped depression with exocytosed fibrillar material (white arrowheads). Transmission electron micrographs (TEMs) (C, E, H): the fibrillar material (white arrowhead) is spatially organized close to an electron dense core (E) in dilated RER cisternae surrounding the central nucleus (C). The Thioflavin positivity (green/yellow fluorescence), localized within RER cisternae (D, arrowhead), identify the amyloid fibrils. Melanin is ultrastructurally identified by using specific technique (the periodic acid-silver methenamine staining (PASM). Pigment deposition is localized on exocytosed amyloid fibrils (H, arrowhead). ROS generation is detected by monitoring the increase in fluorescence of the oxidized dye H_2DCFDA (F, G). The increase in ROS level is evident comparing control granulocytes (F) with those from activated hemocytes (G). Immunocytochemical characterizations for ACTH (I, J), α -MSH (K, L), and IL-18 (M, N) show higher positivity in activated granulocytes (J, L, N).

Moreover, many bacterial species, under stress condition, are able to induce not only the biofilm production but also melanin synthesis [15].

As in bacteria, many protozoa are able to secrete huge amounts of melanin and generally, the production of the pigment is strictly linked to the presence of an amyloid scaffold.

In 2006, Fowler et al. [4] demonstrated that, in mammals, nonpathogenic amyloids are involved in melanin synthesis in melanocytes where the glycoprotein Pmel17 polymerizes into amyloid fibrils forming a framework on which pigment assembled.

Our data (based on light microscopy (LM), transmission and scanning ultrastructural analysis paralleled by immunocytochemical ones) (see Section 3) about activated hemocytes in the lepidopteran-moth *Heliothis virescens* [7, 16], underline the relationship between melanin synthesis and the production of amyloid fibrils to template the pigment. Moreover, we have described and characterized the morpho-functional events sustaining the amyloid fibril assembly in relation to melanin production, in immune cells under stress condition, not only in insects such as *H. virescens* and *Spodoptera elicoverpa*, but also in different invertebrates (protostomes and deuterostomes) such as *Hirudo medicinalis* (Annelid), *Helix pomatia* (Mollusc), *Ciona intestinalis*, and *Botryllus schlosseri* (Ascidian). In all these animals, melanin synthesis/amyloid fibril assemblage always starts with ROS overproduction that, in turn, causes a cytoplasmic pH variation [8]. Amyloidogenesis takes place in RER where the large amount of fibrils, spatially organized, dilate them (**Figure 1**, panels **A** and **B**). RER is specialized in folding and maturation of a considerable number of secreted proteins and it is highly sensitive to external (chemical or physical) and internal stimuli (such as change in the redox state) [10].

This amyloid production due to RER deregulation/accumulation can be morphologically evidenced by Thioflavine S staining and by ultrastructural analysis. During this amyloid/pigment productive phase, a cross-talk between immune and endocrine systems, able to mutually influencing, occurs. These intercommunications are mediated by neuromodulators with the activation of stress-sensing circuits to produce and release molecules such as adrenocorticotrophic hormone (ACTH), melanocyte-stimulating hormone (α -MSH), and neutral endopeptidase (NEP) [7, 8, 16] (**Figure 1**, panels **I–N**). These molecules have a direct role in immune defences. ACTH is widely expressed in various tissues (as well demonstrated in immunocytochemical localizations) and it is synthesized not only by cells of nervous system but also by nonneural cells [17–19]. ACTH expression is induced in stress conditions (such as ROS increase, lipopolysaccharide (LPS) administration, presence of bacteria and proinflammatory cytokine administration) to regulate the immune cellular functions influencing proliferation, migration, production of immune mediators, and trafficking of immunocytes.

The temporal extend of ACTH action is restrained by the production of a neutral endopeptidase (NEP) that is concomitantly synthesized. This protease inactivates ACTH and the released cleaved product, α -MSH, is able to inhibit chemotaxis, production of proinflammatory cytokines (nuclear factor kappa-light-chain-enhancer of activated B cells) NFkB stimulation, as well as to stimulate melanin synthesis.

The pigment is accumulated in specific organelles where an amyloid scaffold sustains its packaging. Moreover, NEP can be also involved in the degradation of amyloid fibrils [20, 21].

Furthermore, the activation of amyloidogenesis is accompanied by the overexpression of a furin-like proprotein convertase, a specific enzyme that is activated in melanosomal biogenesis to liberate a fibrillogenic fragment [22] and by the overexpression of proinflammatory cytokines such as interleukin-18 (IL-18) [4, 8, 23, 24]. In particular, this is an important molecule that cooperates both in the activation of innate immune system and in amyloidogenic processes in Alzheimer disease (AD) [23, 24].

It is important to emphasize that in immune cells from phylogenetically distant metazoans (viz. molluscs, annelids, insects, ascidians, and vertebrates) [8, 16], the amyloid scaffold is produced to package melanin and to convey the pigment in close contact to the invader with a twofold advantage: to isolate the nonself from host tissues and to focus the toxic bio-product [7, 8, 16], thus minimizing the diffusion of highly reactive, toxic melanin precursors. This association may occur in intra- or extracellular compartments. In several invertebrates, stimulated granulocytes (granular circulating cells) produce an amyloid lattice into the RER, that is exocytosed in the body cavity where it works as a template for melanin resulting from humoral prophenoloxidase (proPO) system reactions [25, 26]. proPOs synthesized primarily by hemocytes are present after their lysis in the hemolymph. The proPO activation cascade leads to the formation of quinones that undergo additional reactions leading to synthesis of melanin. This pigment is deposited on the parasite surface, on nodules made by hemocytes, and in wound sites.

In other invertebrates and in vertebrates, melanin and amyloid fibril synthesis are not disjointed and the molecules are produced and assembled in the same cell (melanocytes) [4]. In any case, amyloid fibrils, synthesized by circulating immunocytes (we use this generic term to identify the cells with the same morpho-functional characteristic that, in invertebrates, have been called, phagocytes, amebocytes, hemocytes), are a shuttle of melanin made to trap and isolate the nonself in capsules where the killing of pathogens is facilitated.

So far, we have described the formation of amyloid fibrils as an event absolutely necessary to activate, support, and store pigments, but this is also true, always as response to stress and due to its intrinsic adhesiveness [27], for the assembly and the compartmentalization of hormones such as ACTH in the cytoplasm [28].

The activation of vertebrates neutrophils, due to the presence of bacteria or fungi, culminates with the production of the neutrophil extracellular trap (NET). NETs, as we have immunocytochemically demonstrated, are characterized by a fibrillar backbone for addressing cytotoxic proteins and DNA against the nonself, that shows amyloid birefringence upon Congo red staining, Thioflavin S positivity, and harbors a basic mammalian protein, Pmel17 [4], as validated by the colocalization of signals [29] (**Figure 2**, panels **D–J**). In stimulated neutrophils, there is a correlation among amyloidogenesis and ROS generation, change in cytoplasmic pH, enhanced expression of the ACTH/ α -MSH loop, synthesis of specific cytokine, and also autophagy and exosome release (**Figure 2**, panel **C**). These closely related

partners appear to play a key role in the management of the amyloid fibrils that constitute the scaffold of NET [29].

Figure 2. Unstimulated and stimulated human neutrophils. Thin and semithin sections of resting (A) and activated neutrophils (B, C). The unstimulated cells are roundish but after stimulation with LPS (B, C) they lose their globoid shape acquiring migratory phenotype (B, C). (D–F) Identification of amyloid fibrils with Thioflavin S (ThS): unstimulated neutrophils (D) in comparison with stimulated cells (E, F) from LPS administration. Within cells (E) and extracellularly (F), amyloid fibrils are localized by ThS brightly fluorescence. Nuclei and DNA material in NETs are marked in brilliant blue with DAPI staining. The blue (DAPI) and green (ThS) in NETs, released on stimulation, co-localize (white arrowhead). (G–J) Immunolocalization with a specific antibody directed against Pmel17 (a mammalian protein involved in amyloidogenesis): immunofluorescence staining show the increased Pmel17 expression that is present in the cytoplasm of stimulated neutrophils (H) and in the NETs (H, I) (arrowheads). DAPI (blue), staining for DNA, is localized in nuclei, as well as extracellularly in NETs. (J) Double labeling of neutrophils with ThS (green) and antibody against Pmel17 (red). Note the colocalization of two signals (merge in yellow). Nuclei (blue) are stained with DAPI.

Amyloid fibrils are also synthesized in inflammatory response induced by the presence of abiotic material such as nanotubes that, as waste, can be found in the air, in the soil, and into the water having a harmful effect to the health of many animals, including humans [30, 31]. Leeches, as animal model, exposed to multiwall carbon nanotubes (MWCNTs) powder dispersed in water, reflect the situation of aquatic animals subjected to uncontrolled carbon nanotube (CNT) exposition. MWCNTs, showing an average 9.5 nm external diameter and 1.5 μm mean length, are able to across the epithelial superficial barrier promoting inflammatory responses. MWCNTs in the leech body wall provoke nonspecific responses characterized by proliferation and migration of macrophages toward the stimulated area. Girardello et al. [32] show that these immune cells, recruited in the area of inflammation, produce amyloid material, as demonstrated by Thioflavin-T staining. In addition, the authors correlated this synthesis with a concomitant overexpression of IL-18, confirming the importance of this cytokine in the

process. Extracellular amyloid fibrils entrap and isolate the nanotube aggregation but, forming a kind of spongy coating, can have an additional function in concentrating the cytokines produced by neighbor activated cells thus attracting and driving the macrophages engaged in immune responses [32] (Figure 3, panels A–E).

Figure 3. Effects of carbon nanotube exposure and wound healing in leeches. Optical (A, C, D, E) and ultrastructural (B) images. Leech body wall sectioned in wound healing area (A, C, D, E). The specific Thioflavin-T colorimetric method evidences the presence of amyloid fibrils close to the aggregates of nanotubes (arrowheads). Ultrastructural (B) image of carbon nanotubes (CNTs) crude powder (used as control). The fibrillar material, Thioflavin-T positive, produced by migrating cells (E) contribute to form a plug (D) in the area of lesion (arrowheads).

Amyloids are also synthesized by the cells that are recruited in the lesioned body wall of diverse invertebrates to work as a “collision mat,” the strategic solution patterned to close a boat leak, by forming a clot. The fibrillar material, Thioflavin-T positive, deposited in correspondence of damaged area, contributes to block the ingress of water/pathogens/external materials and prevent the loss of cells and hemolymph/blood from the body (unpublished data).

We have shown (combining light and electron microscopy, staining reactions, and immunocytochemical characterization—see paragraph about methodologies) that different types of cells characterized by different lineages (i.e., from mesoderm and ectoderm) and from diverse organisms, such as larval hemocytes, IPLB-LdFB and *Drosophila* Schneider’s S2 cells from insects; NIH3T3 embryonic fibroblasts from mouse; human umbilical vein endothelial cells (HUVEC), and mesenchymal stem cells from human, under stress condition (for instance, LPS stimulation) are characterized by an increase of cytoplasmic ROS [33].

After stimulation, these cells are characterized by a morphological change and in the cytoplasm, empty vacuoles or RER cisternae filled with material showing staining properties typical of amyloid fibrils are visible (Figure 4, panels A–L). Moreover, the activation of amyloidogenesis is also linked with an extensive production of ACTH and α -MSH in all cultured cell types. These data suggest that amyloidogenesis could be a common, physiological cellular response to weak ROS, starting when other antistress cellular systems failed to restore homeostasis. Then the morphological evidence and/or functional characterization of synthesized amyloid fibrils could be an early indicator of oxidative stress that may lead to a general inflammatory process.

Figure 4. Effects of LPS stimulation on different cell types. Optical (A–C, E–G, I–K) and ultrastructural (D, H, L) images of S2 cells from *Drosophila melanogaster* (A–D), IPLB cells from insect (E–H) and NIH3T3 murine fibroblast (I–L). Amyloid fibril presence is detected with Thioflavin T. The yellow-green brightly fluorescence is more evident in stimulated cells (C, G, K) as compared to controls (B, F, J). Nuclei are stained with DAPI and marked in brilliant blue. TEM

analysis: thin sections of LPS activated cells (D, H, L) show the presence of dilated reticulum cisternae filled with fibrillar material (arrowheads).

2. Conclusions

It is intriguing to underline that amyloid fibril synthesis is a cellular dynamic and versatile system evolutionary conserved. Recent evidence strengthen the idea that:

- Amyloidogenesis, shared by invertebrate and vertebrate cells, occurs as a protective response.
- Amyloid fibril synthesis that can be considered, in healthy cells, as a functional event, starts always, independently from their utilization, with an increase of ROS, real key player in defining this cellular response.
- The amyloid fibrillar material has unique features: it is versatile, extremely durable, can be assembled from any type of protein, it is able to paste, reversibly, different molecules due its residual charges, it can be disassembled only using specific enzymes such as NEP.
- The amyloid fibrils are utilized in different ways: they can be part of a more general response to stressful condition all entailing overproduction of ROS; they can form a scaffold to package pigment or hormones within the cell; can convey melanin or cytotoxic molecules; they can form a resistant structure to physically isolate the nonself, thus favoring the killing of pathogens, also conveying and concentrate toxic products; they can build a part of framework to close body lesions.

3. Methodologies

3.1. Light microscopy (LM) and transmission electron microscopy (TEM)

LM and TEM analysis give important information about morphology of cell and tissues.

Collected samples were fixed in 4% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2) and washed in 0.1 M Na-cacodylate buffer at pH 7.2. The initial fixation is followed by a postfixation in 1% osmic acid in cacodylate buffer (pH 7.2) for 20 min. After standard dehydration in an ethanol series, samples were embedded in an Epon-Araldite 812 mixture and sectioned with a Reichert Ultracut S ultratome (Leica, Nussloch, Germany). Semithin sections were stained by conventional methods utilizing histological dyes such as crystal violet and basic fuchsin and then observed with a light microscope (Eclipse Nikon, Amsterdam, Netherlands). Thin sections were stained by uranyl acetate and lead citrate and observed with a Jeol 1010 electron microscope (Jeol, Tokyo, Japan).

3.2. Intracellular reactive oxygen species evaluation

Oxidative stress can induce proteins to adopt an insoluble β -pleated sheet conformation, and according to numerous authors, oxidative damage appears to be the earliest events preced-

ing amyloid fibril formation. Thus, it is important to evaluate the overproduction of ROS in relation to LPS activation responsible for amyloid fibril production. ROS production and its derivation was validated by use of 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA, Molecular Probes, Eugene, OR, USA), a fluorogenic probe commonly used to detect the overall degree of intracellular level of ROS. H₂DCFDA is a nonfluorescent compound that readily crosses cell membranes. It is hydrolyzed to 2',7'-dichlorofluorescein (DCF) within cells and becomes fluorescent when it is oxidized by ROS. Oxidation can be detected by monitoring the increase in fluorescence. Fluorescence was determined by excitation at 488 nm and emission at wavelength of 525 nm; fluorescent images visualized on a fluorescence Eclipse Nikon microscope, were acquired with a DS-5 M-L1 Nikon digital camera system.

3.3. Amyloid fibril characterization

Amyloid structures were identified by staining cells/tissues with Thioflavin S and visualizing the amyloid-specific green/yellow fluorescence with an Eclipse Nikon microscope. Images were acquired with a DS-5 M-L1 Nikon digital camera system. Amyloid fibrils were also characterized with Congo red staining, according to published methods and observed under cross-polarized light with an Axioskop 2 microscope (Carl Zeiss, Jena, Germany), equipped with a MC 80 DX camera (Carl Zeiss).

Amyloid fibrils (see the paragraph below) were also localized immunocytochemically using an antibody directed against Pmel17, protein that has amyloid characteristics and contributes to form fibrillar structures in mammals.

3.4. Immunocytochemistry for ACTH, α -MSH, NEP, interleukin 18, and Pmel17 localization

The presence of amyloid fibrils was confirmed by using the primary antibody antihuman Pmel17 (H-300) polyclonal antibody (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The presence of ACTH and its cleavage product (due to degrading enzyme NEP), α -MSH, and of the proinflammatory interleukin 18 (IL-18) were assessed by using the following primary antibodies: antihuman ACTH polyclonal antibody (1:100 dilution, Abcam, Cambridge, UK); antihuman α -MSH polyclonal antibody (1:100 dilution, Abcam); antihuman IL-18 polyclonal antibody (1:100 dilution, Abnova, Taipei City, Taiwan); antihuman CD10/CALLA (NEP) monoclonal antibody (1:100 dilution, GeneTex, Hsinchu City, Taiwan). Incubations with suitable secondary antibodies conjugated with Cyanine5 (Cy5; 1:50 dilution, Abcam) were performed for 1 h in a dark humid chamber at room temperature. Nuclei were stained with 4',6'-diamino-2-phenylindole (DAPI, Sigma-Aldrich). The PBS buffer used for washing steps and antibodies dilutions contained 2% bovine serum albumin (BSA, Sigma-Aldrich) and 0.1% Tween20.

In colocalization experiments, the cells were first stained with Thioflavin S (as described above) and then incubated with anti-Pmel17 antibody (as described above).

In control samples, primary antibodies were omitted, and samples were treated with BSA/Tween20-containing PBS. Coverslips were mounted in citifluor (citifluor Ltd., London, UK). Slides were observed under an Eclipse Nikon microscope.

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