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Amyloid-like aggregates formation by blood plasma fibronectin

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ABSTRACT

Fibronectin (FN) is a multifunctional glycoprotein of the extracellular matrix (ECM) playing critical roles in physiological and pathological cell processes like adhesion, migration, growth, and differentiation. These various functions of FN are modulated by its supramolecular state. Indeed, FN can polymerize into different types of assemblies like fibrils and aggregates. However, the mechanism of polymerization and the effects of such assemblies on cell behaviors still remain to be elucidated. Here we show that upon irreversible thermal denaturation, human blood plasma fibronectin forms high molecular weight aggregates. These compact and globular aggregates show amyloid features: they are stabilized by intermolecular b-sheets, they bind Thioflavin T and they are resistant to reducing and denaturing agents. Their characterization by electrospray ionization charge detection mass spectrometry shows that two populations can be distinguished according to the mass and charge density. Despite their amyloid features and the presence of hydrophobic patches on their surface, these aggregates are not toxic for cells. However, their binding abilities to gelatin and RGD are drastically decreased compare to native FN, suggesting possible effects on ECM-cell interactions.

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1. Introduction

Protein aggregation is a key feature that can modulate the physiological role of proteins or lead to deleterious effects when uncontrolled. Protein oligomerization is a simple way to modify the activity of a protein. Hence, self-activation, inhibition, or variation in the selectivity of binding partners can be regulated by the modulation of binding interfaces [1,2]. On the other hand, protein aggregation can escape the regulatory mechanisms of cells and can have disastrous pathological effects [3]. This is the case in a number of neurodegenerative diseases. Indeed, mutation or dysregulation can induce protein polymerization and often fibrillogenesis, resulting into the formation of protein inclusion bodies in cells [4,5].

Fibronectin (FN) is one of the proteins whose roles depend on its oligomerization state. It is a multifunctional glycoprotein that mediates cellular interactions and plays critical roles in cell adhesion, migration, growth and differentiation in the extracellular matrix (ECM) [6]. FN is a dimeric protein composed of disulfide-linked subunits with a molecular weight of 220–250 kDa each [7]. It is a modular protein consisting in a repetition of three different independently folded domains named type I, type II, and type III [8,9]. Depending on their solubility, FN can be subdivided into at least two forms: the soluble blood plasma FN and the less soluble cellular FN [10]. Plasma FN is produced predominantly by hepatocytes [11] and secreted into plasma as a soluble protein, while cellular FN is secreted by fibroblasts and endothelial cells and is mostly bound to the cell surface or deposited as insoluble multimers in the extracellular matrix [12]. FN structural properties could affect its function through a variety of mechanisms. FN conformational changes can either expose or hide binding sites, thus turning them on or off. Modifications in FN quaternary structure can open up binding sites that were sterically blocked, resulting into an alteration of function without change of secondary/tertiary structure. Finally, FN conformational extension can lower avidity for partners that use multiple binding sites without altering the affinity of each binding domain but by separating them. Moreover, FN structural properties can affect ECM porosity and, hence, the storage of growth

Abbreviations: FN, fibronectin; ECM, extracellular matrix; ThT, thioflavin T; ANS, 8-anilinonaphthalene-1-sulfonate; DLS, Dynamic Light Scattering; ESI-CDMS, electrospray ionization charge detection mass spectrometry; RGD, Arginine-Glycine-Lysine.

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factors within the matrix. ECM stiffness can also be modified, with major effects on cell behavior [13].

Insoluble FN can be found under various forms in tissues, i.e. as fibrils but also as high molecular weight aggregates, which have been shown to be involved in different phenomena. Fibrils of FN play important roles in the dynamic of the ECM, in the deposition of other ECM components like collagen, and in cell adhesion and proliferation [14,15]. Besides fibrillogenesis, FN aggregation may be required during initial stages of wound healing and tissue regeneration [16]. However, in pathological context, excessive FN deposition and incorrect remodeling contribute to scarring and fibrosis, and prevent complete tissue regeneration [16]. Inappropriate remodeling, rather than continuous deposition, could promote FN aggregation in multiple sclerosis lesions resulting into remyelination failure [17]. In kidney, hereditary disease glomerulopathy has been linked to mutations in FN forming non-fibrillar aggregates potentially causing defects in matrix assembly [18].

Hence, supramolecular states of FN seem to have important roles in different physio-pathological processes. Still, the mechanisms of FN oligomerisation remain to be elucidated. Concerning FN fibrillogenesis, it has been shown that the stretching of FN by cells is a crucial step for FN polymerization [19]. Fibril formation is initiated by the binding of integrins resulting in a conformational change in the FN molecule and an exposition of self-association sites. Mechanical stress unfolds the FNIII domains and may expose some of the internal β-strands in a conformation relevant for hydrogen bonding with a similarly unfolded domain, thus forming stable polymeric structures [20–23]. Receptors clustering and increased local concentration of FN allow fibril formation, propagation and ultimately, the generation of detergent-insoluble FN matrix. The latest is composed of high-molecular weight FN multimers [24,25]. FN structure in such self-assemblies, as defined by deoxycholate (DOC)-insolubility, is likely the result of strong, noncovalent protein-protein interactions [26,27].

It has been reported that purified plasma FN could form disulfide-bonded aggregates in the presence of a low concentration of reducing agent such as glutathione, i.e. under physiologically relevant conditions [28]. Disulfide-bonded aggregates can also be formed upon denaturation either in guanidine [29] and urea [30] or by temperature [31]. A fibrillar form of FN not stabilized by covalent bonds can be formed using SDS and sonication [32]. These studies have provided some cues about the different conformations of full length FN, and suggest that this protein can self-assemble into different types of supramolecular assemblies, other than fibrils. However, the mechanisms of FN self-assembly still remain to be elucidated.

Hence, in this work, we studied full-length human blood plasma FN self-assembly by monitoring spectroscopically the kinetics of aggregation and the associated conformational changes. Thermal unfolding was used to trigger FN multimerization. Morphological information on FN assemblies has been obtained by atomic force microscopy (AFM) and molecular weight of intact FN aggregates has been measured using an innovative charge-detection mass spectrometry method. Using Fourier transformed infrared spectroscopy (FTIR), 8-anilinonaphthalene-1-sulfonate (ANS) binding assays and reducing SDS-PAGE, we investigated the conformational changes occurring during the process and analyzed the molecular contacts stabilizing the aggregated state. With kinetics analysis followed by thioflavin T (ThT) fluorescence, we elucidated steps of FN self-assembly. Affinity chromatography and enzyme-linked immunosorbent assay (ELISA) have been used to study the accessibility of FN binding sites, like RGD (Arginine-Glycine-Lysine) and gelatin binding sites, within the aggregated state. Since some functionalities of FN were decreased in multimers, we assessed the cytotoxicity of aggregated FN. As the exact roles of different aggregated FN detected *in vivo* still remained to debate, we discussed the putative link between FN aggregates obtained in our study and extracellular matrix organization, thus cell behavior.

2. Materials and methods

2.1. Purification of plasma fibronectin

Fibronectin has been purified from human plasma as described by Poulouin et al. [33]. Briefly, a gelatin-sepharose column (gelatin-sepharose 4B, GE-Healthcare, Velizy-Villacoublay, France) was equilibrated with Tris-HCl 50 mM, EDTA 1 mM, NaCl 500 mM at pH 7.4. Plasma was then loaded onto the column and the bound fibronectin was eluted using Tris-HCl 50 mM, EDTA 1 mM, NaCl 250 mM, urea 3 M. Fibronectin solution was then loaded onto a heparin-sepharose column (Heparin-sepharose™ 6 fast flow, GE Healthcare, Velizy-Villacoublay, France) previously equilibrated with Tris-HCl 50 mM, EDTA 1 mM, NaCl 75 mM at pH 7.4. After the elution from the heparin column with Tris-HCl 50 mM, EDTA 1 mM, NaCl 500 mM, fibronectin was loaded and eluted from a new gelatin-sepharose column as described above. Fibronectin was filtered through a 0.22 μm filter and stored at +8 °C, in 10 mM HEPES buffer, pH 7.4. The purity was checked by SDS-PAGE electrophoresis and western blotting; it was found to be around 98%. Protein concentration was determined from the absorbance at 280 nm using an extinction coefficient of 1.28 L g^{-1} cm^{-1} [34]. The molecular weight of plasma FN was estimated at 450 kDa.

2.2. Aggregation studies

FN stock solution was filtered through 0.22 μm filters. For the aggregation studies, FN was incubated at 70 °C under orbital rotation at 600 rpm for different durations. Different concentrations, from 0.25 mg mL^{-1} to 1.25 mg mL^{-1}, were tested. The absence of proteolysis was verified by SDS-PAGE. For the seeding experiments, 0.1% of pre-aggregated FN was added to fresh FN solutions at a concentration of 1 mg mL^{-1} and incubated at 70 °C under rotation. To obtain fibrils, purified FN was incubated 24 h at 70 °C under magnetic stirring at 200 rpm.

2.3. Circular dichroism measurements

Measurements were performed using a J-810 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a Pelletier temperature controller.

CD spectra were measured in 1 mm quartz cuvette with an accumulation of 10 scans at a scanning speed of 200 nm min^{-1}, and a bandwidth of 4 nm. Far-UV CD spectra were measured at a protein concentration of 50 μg mL^{-1} in 10 mM HEPES buffer, pH 7.4.

Thermal denaturation of fibronectin was monitored by far-UV CD spectroscopy at a wavelength of 227 nm in 1 cm quartz cuvette and at a concentration of 50 μg mL^{-1} in 10 mM HEPES buffer, pH 7.4. The unfolding was followed from 20 to 80 °C and from 80 °C to 20 °C with 1 °C min^{-1} steps. Similar results have been obtained by monitoring the denaturation at 218 nm and with different protein concentrations (from 30 to 75 μg mL^{-1}).

2.4. Electrospray ionization charge detection mass spectrometry (ESI-CDMS)

Experiments were performed on a custom-built charge detection mass spectrometer with an electrospray source, as described previously [35]. Briefly, aqueous solutions of fibronectin aggregates at around 1 mg mL^{-1} were injected at flow rates of typically 150 μL h^{-1}, and entered the electrospray chamber through a 0.1 mm internal diameter stainless steel capillary tube located inside the needle tip. Nitrogen drying gas was injected between the

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