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Dual role of p53 amyloid formation in cancer; loss of function and gain of toxicity

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ABSTRACT

The tumor suppressor p53 plays an important role in genome integrity. It is frequently mutated in all types of human cancers, making p53 a key factor in cancer progression. Two phenotypic consequences of these alterations are dominant; a loss of function and a gain of function of p53, which, in several cases, accumulates in intracellular aggregates. Although the nature of such aggregates is still unclear, recent evidence indicates that p53 can undergo conformational transitions leading to amyloid formation. Amyloid diseases, such as, Alzheimer's disease, are characterized by the accumulation of insoluble aggregates displaying the fibrillar conformation. We decided to investigate the propensity of wild type p53 to aggregate and its consequent assembly into different amyloid species, such as oligomers and fibrils; and to determine if these changes in conformation lead to a loss of function of p53. Furthermore, we analyzed cases of Basal Cell Carcinoma (BCC), for the presence of p53 amyloids. Here, we show that p53 forms amyloid oligomers and fibrils, which coincide with p53 inability of binding to DNA consensus sequences. Both p53 amyloid oligomers and fibrils were detected in BCC cancer samples. Additionally, we demonstrate that p53 oligomers are the most cytotoxic to human cell cultures.

Our study reveals p53 amyloid formation and demonstrates its dual role in the pathogenesis of cancer by producing a loss of protein function and a gain of toxic function, extensively described in several amyloidogenic diseases. Our results suggest that under certain circumstances, cancer could be considered a protein-conformation disease.

1. Introduction

Mutations in the p53 gene are detected in ~50% of human cancers [1]. In addition to mutant p53 being inactive, several types of human cancers harbor a genetically wild-type, but a transcriptionally inactive form of p53 [2]. In these cases, wild-type p53 has been reported to accumulate in the cytoplasm and/or the nucleus [3]. Moreover, in these cancers, the inactive wild type p53 forms large protein aggregates [4], which may occur due to a conformational change of p53 [5].

A vast range of human diseases arise from the failure of a specific protein to adopt or remain in its native functional conformational state and change into a β-sheet conformation. These pathologic conditions collectively are referred to as protein conformational or amyloid diseases [6]. Recent research suggests that prefibrillar aggregates, soluble oligomers, rather than fibrils per se are the most potent mediators of cytotoxicity [7]. Amyloid fibril deposition has been described in patients with malignant diseases. It has been more frequently seen in hematological neoplasms and has also been noticed in patients with solid tumors. Previous studies demonstrated the presences of amyloid deposition in different types of cancer [8–10] . Amyliod deposition also occurs in numerous benign and malignant epidermal lesions such as basal cell carcinoma (BCC) [11]. The mechanism of amyloid formation in association with solid tumors is unknown and thus further investigation is necessary.

As mentioned above in some cancers, the inactive form of p53 shapes large protein aggregates [4], which can occur secondarily to a conformational change of p53 [5] and may possibly display amyloid-like structures. *In vitro* studies shows how p53 aggregates in amyloid assemblies and induces cytotoxicity in neuroblastoma cells [12], demonstrating that p53 amyloid can behave like any other amyloid disease [13]. So far, only a couple of studies have demonstrated cytoplasmatic inclusions of p53 in Tg mice expressing mutant p53 and in human colon carcinoma [14]. Another study shows the presence of p53 aggregates in human cases of breast cancer [15].

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The present study describes the dual role of p53 amyloid formation in cancer, where there is a loss of protein function and a gain of toxic function of p53. In order to display this "dual role" concept of p53, we analyzed human cases of BCC, which is the most commonly diagnosed malignant skin tumor [16]. Our data show that full length p53 can adopt diverse amyloid conformations such as oligomers and fibrils, and that these amyloid species lose the ability to bind to DNA. Also following a pro-inflammatory stimulus, p53 aggregates and forms amyloid oligomers and fibrils in BCC cell culture. Interestingly, these oligomers and fibrils are also present in human cases of BCC and p53 oligomers are associated with cell toxicity. Overall, our study reveals that p53 aggregation could contribute to disease pathology by not only a gain-of-toxic-function mechanism, but also a partial loss of function mechanism.

2. Materials and methods

2.1. Dot blot

Human recombinant p53 was purchased from Sigma–Aldrich (Cat #p6374). Fibrils and soluble oligomers were prepared as previously described [17]. Two microliters of each sample was applied to a nitrocellulose membrane, blocked with 5% non-fat milk overnight at 4 °C and then incubated again for 1 h at room temperature with the anti-oligomer antibody A-11 (1:1000) or the anti-fibril antibody OC (1:5000). Then the membranes were incubated with HRP conjugated anti-rabbit IgG (Promega) (1:10,000). Blots were developed with ECL chemiluminescence kit from Amersham-Pharmacia.

2.2. Atomic force microscopy (AFM)

The morphology of oligomers and fibrils preparations were assessed by AFM by a non-contact tapping method (ScanAsyst-air) using a Multimode 8 AFM machine (Veeco, CA).

2.3. Thioflavin T assay

Twenty μl of samples (3.3 μM) were incubated 15 min at room temperature in 50 mM glycine, pH 9.2 and 2 μM ThT. Fluorescence was measured at excitation wavelength of 435 nm with emission at 485 nm.

2.4. DNA binding assay

Fibrils and oligomers were prepared as described above. DNA binding was measured by the Panomics ELISA transbinding kit (Panomisc #EK1050).

2.5. Detection of p53 amyloid oligomers and fibrils in Basal Cell Carcinoma cell culture

BCC cells were treated with 0.0005% formaldehyde for 72 h. Then, the cells on the coverslip were fixed and permeabilized. The samples were blocked for 1 h in 5% goat serum and incubated overnight at 4 °C with A-11 (1:1000) or OC (1:5000). Sections were then washed and incubated with an Alexa 568-conjugated goat anti-rabbit antibody (1:700; Invitrogen) for 1 h at room temperature. For co-localization studies, the sections stained with conformational antibodies were then incubated overnight at 4 °C with anti-p53 (1:2000), then washed and incubated with an Alexa 488-conjugated goat anti-mouse antibody (1:700). For nuclear staining, DAPI was used (1:4000, Invitrogen). Sections were then washed for 30 min and cover slipped. Single images of immunostained cells were acquired on an LSM510 Zeiss laser scanning confocal microscope.

2.6. Human histology and immunohistochemistry

Biopsy tissue was obtained from the skin of 6 patients with BCC. H&E staining was performed to examine general skin morphology and basal cell carcinoma pathology. Congo red and Thioflavin-S was used to analyze the deposition of amyloid in patients with BCC. The bright field and polarized images were acquired using a Nikon Multizoom AZ100 microscope.

2.7. Immunohistochemistry and Immunofluorescence

Immunohistochemistry was performed on paraffin-embedded sections. In brief, primary antibodies were detected with biotinylated goat anti-mouse IgG (1:2000, Jackson ImmunoResearch) or biotinylated goat anti rabbit IgG (1:1800) and visualized with Avidin–Biotin Complex kit (catalog no. PK-6200; Vector Laboratories), according to the manufacturer's recommendations. The following antibodies were used for immunostaining: OC (1:5000), A-11 (1:1000), and anti-human p53 (1:2000).

For immunofluorescence sections using A-11, OC and p53, the same protocol used for BCC cells was performed. Other sections stained with conformational antibodies were then incubated with ApopTag Fluorescein in situ kit (Cat #S7110, Chemicon) to label apoptotic cells according to manufacture specifications.

2.8. Toxicity assays

AlamarBlue assay: SH-SY5Y cells and fibroblasts from basal cell carcinoma were grown in 96-well plates. Cells were treated with Aβ, α-synuclein and p53 monomers, oligomers or fibrils (10 μM) or with 1X PBS for untreated controls. All measurements were performed in triplicate. Cytotoxicity was measured using an Alamar-Blue assay kit (Serotec). Cell images were acquired using a EVOSxl microscope from AMG. Statistical analyses were based on a Two-Way ANOVA test, performed using Origin-8 software (Origin Lab).

3. Results

3.1. P53 forms oligomers and amyloid fibrils that cannot bind to DNA

To characterize recombinant p53 oligomers and fibrils, we performed a dot blot assay using the conformation-specific A-11 antibody, which recognizes toxic oligomers formed by a variety of amyloidogenic proteins [17] and OC antibody, which recognizes amyloid fibrils independent of the aminoacidic sequences [18]. Recombinant p53 freshly dissolved in phosphate buffer (p53 monomer) and p53 fibrils are not recognized by the A-11 antibody. However, recombinant p53 oligomers exhibits A-11 immunoreactivity (Fig. 1A). In the case of the p53 fibril sample was only detected with the OC antibody, confirming the presence of fibrilar amyloids in the preparation. For further characterization, AFM was used to investigate the morphological features of the aggregates. In the sample positive for A-11, a homogenous population of spherical oligomers of p53 with an average size of 2.6 ± 0.2 nm (Fig. 1B) was present. In Fig. 1C it is possible to see fibrillar structures of p53 which were also positive for OC. The formation of fibrils was also monitored using Thio-T dye-binding assay [19], which confirmed the formation of p53 fibrils (Fig. 1D).

To investigate whether the formation of p53 oligomers and/or fibrils leads to a loss of DNA binding, we utilized an ELISA that detects p53 when bound to consensus duplex deoxyologonucleotide