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Bioactive $TTR_{105-115}$-based amyloid fibrils reduce the viability of mammalian cells

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ABSTRACT

A growing number of protein-based fibrous biomaterials have been produced with a cross-β amyloid core yet the long-term effect of these materials on cell viability and the influence of core and non-core protein sequences on viability is not well understood. Here, synthetic bioactive TTR1-RGD and control TTR1-RAD or TTR1 fibrils were used to test the response of mammalian cells. At high fibril concentrations cell viability was reduced, as assessed by mitochondrial reduction assays, lactate dehydrogenase membrane integrity assays and apoptotic biomarkers. This reduction occurred despite the high density of RGD cell adhesion ligands and use of cells displaying integrin receptors. Cell viability was affected by fibril size, maturity and whether fibrils were added to the cell media or as a pre-coated surface layer. These findings show that while cells initially interact well with synthetic fibrils, cellular integrity can be compromised over longer periods of time, suggesting a better understanding of the role of core and non-core residues in determining cellular interactions is required before TTR1-based fibrils are used as biomaterials.

1. Introduction

Protein-based fibrous structures, including amyloid fibrils, have recently been targeted as novel biomaterials for use in tissue regeneration [1–5] or as functional scaffolds within miniature devices [6–8]. While many structures are rich in β-sheet, amyloid fibrils differ due to the arrangement of protein within a cross-β core [9]. The molecular organisation within this core involves β-sheets stacked along the fibril axis bound by a network of protein-backbone hydrogen-bonding, with adjacent β-sheets packed across the fibril axis.

The cross-β core structure of amyloid fibrils is considered responsible for a range of physical properties, such as high mechanical strength, which can provide the fibril with functionality. In the oocyte and developing embryo of silkmoth, for example, amyloid fibrils offer protection from environmental hazards [10]. Fibrils are also thought to contribute to the adhesive cement used by barnacles for surface attachment [11]. Other functional amyloid fibrils promote melanin synthesis or act as a store of peptide hormones in mammals [12,13], suggesting that these assemblies form a different class of structures to those fibril forming sequences known to be associated with protein misfolding diseases [14].

The factors that distinguish functional fibrils from those known to be associated with disease is not yet clear and the molecular mechanism by which amyloid fibrils can be toxic to mammalian cells is not well understood [15]. It is also not known whether amyloid fibrils designed to mimic naturally occurring fibrils are compatible with cells or whether they will potentially decrease cell viability over long periods of time. Several studies have shown favourable interactions between fibrils and cells in short or longer term assays, this includes amyloid nanofibrils from crystalline proteins [16], fibrils used as nanoparticles for cell transfection [17], fibrils used as scaffolds for biomimetic bone growth [18] and fibrils used as biomaterial surface coatings [19,20]. A greater understanding of the interactions between fibrils and cells and the role of core and non-core protein sequences in these interactions will facilitate the potential use of synthetic self-assembling sequences and other fibril forming proteins as materials and in food [21].

Several features are thought to contribute to the potential toxicity of mature amyloid fibrils. Firstly, exchange may occur between the fibril end and the bulk solution, resulting in the release of

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protein monomers, oligomers or protofilaments [22–24]. Fragmentation can also increase toxicity [25]. Non-core sequences exposed on the fibril surface may be critical as their flexibility allows interaction with the cell membrane. Indeed, precursor aggregates that are less compact and more hydrophobic have been shown to interact with cell membrane lipids [15]. Reports of the toxicity of mature fibrils in cell culture differ and range from no detectable toxicity [26–28] to moderate [25] and high levels of toxicity [29,30]. Comparisons between studies are made harder by the use of different cell lines and culture conditions.

The synthetic TTR1-based family of peptides provides a useful system for probing the interactions between designed fibrils and cells. Six sequences have been shown to form fibrils with a well characterised structure [8,19,20,31]; fibrils from the TTR1-RGD peptide display the cell adhesion ligand GRGDS that supports cell attachment and growth, TTR1-RAD displays a control sequence (GRADS) that does not promote cell adhesion, while TTR1 forms the base self-assembling scaffold. The kinetics of fibril formation are known for these peptides, the conversion of free peptide to mature fibrils is high (>80% by mass) [19,20,31] and the mature fibrils stable [32], indicating low levels of dissociation of the monomer from the mature fibril. The full length of the TTR1 sequence is incorporated within a cross-β core that has been well characterised [33] and the C-terminal amino acids of the TTR1-RGD and TTR1-RAD peptides are excluded [20], allowing comparisons between the effect of core and non-core residues. These fibrils also display a hydrophilic surface [19].

This study evaluates the effect of synthetic fibrils on mammalian cell viability following extended exposure in cell culture. TTR1-RGD fibrils were expected to provide a more favourable growth substrate for adherent cells that express integrin cell surface receptors, compared to TTR1-RAD or TTR1 fibrils [34] but the long term viability of cells was unknown. Our experiments aimed to test cell viability of cells exposed to thin layers of fibrous coatings and fibres presented to cells in solution to determine the potential of these synthetic amyloid fibrils as materials for cell support and growth.

2. Materials and methods

2.1. Fibril formation

The peptides TTR1, TTR1-RGD or TTR1-RAD were synthesised and purified to >95% by CS BioCo. (Menlo Park, USA). The amino acid sequence for each peptide and conditions used to prepare mature fibrils have been reported previously [20].

Fragmented lysozyme fibrils produced from chicken hen egg white lysozyme protein (Sigma–Aldrich, Australia), were used as positive control for cell death and were prepared by following the procedure in Xue et al. 2009 [25]. Briefly, lysozyme protein was dissolved into 10 mM HCl. This solution was syringe-filtered (0.2 μm Millipore), incubated at 60 °C for 48 h then left at room temperature for 2 months. Fragmented lysozyme fibrils, referred to herein as lysozyme seed fibrils, were produced by stirring a 500 μL aliquot of the mature fibril sample with a magnetic flea 8 mm in length at 1000 rpm for 72 h.

2.2. Fibril coated surfaces

Sterilised glass coverslips 22 × 22 mm^2 in size (Menzel-glaser, Germany) were coated with fibrils as described previously [19]. The flat bottoms of 96- or 384-well tissue culture polystyrene (TCPS) plates (Greiner Bio-one, USA) were also coated with mature fibrils or freshly dissolved peptide diluted (1:1) in MilliQ water at the density indicated. The coated TCPS plates were air-dried in a laminar flow cabinet and protected from dust before use.

2.3. Transmission – Fourier transform infrared spectroscopy

The secondary structure of a thin hydrated film of TTR1-based peptides was examined on the Infrared (IR) beamline at the Australian Synchrotron (Victoria, Australia). The preparation of spectra, data collection and instrument settings were as described previously [19]. Briefly, a 5 μL aliquot of freshly dissolved TTR1 peptide, TTR1-RGD peptide or TTR1-RAD peptide at a concentration of 10 mg ml^{-1} in MilliQ water was spread on a 0.5 mm thick, flat CaF_2 window (Crystran Pty Ltd, Dorset, U.K.) over an area of approximately ~10 × 10 mm^2 then left to air-dry for 2 h before IR measurements. Spectrum were collected at multiple positions on the calcium fluoride window (n = 20) and normalised to the amide I peak. The peak means for the peptide or fibril were compared using a one-way ANOVA.

2.4. Transmission electron microscopy

Transmission electron microscopy (TEM) was used to examine the morphology of ANS fibril preparations including dye binding TTR1-based aggregates and mature or short lysozyme fibrils. TEM grids were prepared, images were acquired and the instrument used is as described previously [19]. Briefly, TTR1-RGD or TTR1-RAD peptides were incubated under conditions known to induce fibril formation (§2.1). A 3 μL aliquot of the solution was added to a prepared TEM grid once the maximal ANS fluorescence was reached, as indicated in Fig. 5, then the preparation imaged. A 3 μL aliquot of lysozyme mature fibrils or lysozyme seed fibrils was added to a prepared TEM grid then imaged. Fibril dimensions were measured using ImageJ software (NIH, Bethesda, MD, USA) and the pixel size was calibrated using the imprinted scale bar.

2.5. Cell culture

Mouse 3T3 embryonic fibroblast cells were the kind gift of St Vincent's Hospital, (Melbourne, Australia) and monkey MA104 embryonic kidney epithelial cells (CRL-2378.1) were from ATCC (Rockville, MD). Cells were cultured at 37 °C, 5% CO_2 and 95% relative humidity in tissue culture flasks (growth area of 25 cm^2, Greiner Bio-One, U.S.A). Dulbecco's modified Eagle's medium with sodium pyruvate and high glucose or low glucose was used for 3T3 or MA104 cells, respectively. The medium was supplemented with 10% (v/v) foetal bovine serum, 50 units mL^{-1} penicillin, 50 mg mL^{-1} streptomycin, 2 mM L-glutamine. For MA104 cells only, the medium was also supplemented with 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). Cultures were routinely split (1:3) at ~80% confluency by rinsing the cells with Dulbecco's phosphate buffered saline (DPBS, 10 mM) without calcium or magnesium and released for sub-cultivation using 0.05% (v/v) trypsin-EDTA without phenol red. All cell culture reagents were from Gibco (Invitrogen, Mount Waverley, Australia) unless otherwise specified.

2.6. Tetrazolium salt reduction assay

The affect of TTR1-based mature fibrils and other aggregate species on cell viability was assessed using the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma Aldrich, Castle-Hill, Australia) tetrazolium salt conversion assay. The intensity of the formazan produced from enzyme cleavage of the tetrazolium salt by metabolically active cells is proportional to the number of viable cells (Sigma–aldrich).

TTR1-based peptide and fibril samples were tested using two different methods of exposure. Samples were either suspended in the cellular media at a concentration of 240 μM per well or coated onto the assay surface as described in Section 2.2 prior to the addition of cells. Cells at ~80% confluency were detached from culture using either 0.05% trypsin-EDTA (1 mL per flask) or an enzyme-free cell dissociation buffer containing ethylenediaminetetraacetic acid (EDTA, 1 ml per flask) as indicated (Invitrogen Pty Ltd, Australia).

Cells were plated at a density of 5000 cells per well in 100 μL of medium in a 96-well plate (Greiner Bio-One, U.S.A) for 72 h time course experiments or at a density of 10,000 cells per well for the 24 h time course experiments. The cells were added in six replicates to a set of three 96-well plates, where each plate was used as a 24, 48 or 72 h time point. The appropriate initial cell number was determined using calibration curves to ensure that the final cell number was within a linear absorbance range of the assay (data not shown).

Cells were allowed to equilibrate for at least 24 h at 5% CO_2 and 37 °C in a humidified incubator before the addition of medium containing the suspended samples or were added directly to the TTR1-based peptide or fibril coated surfaces. Untreated cells and cells lysed with 9% (v/v) Triton X-100 were used as controls for the expected maximum and minimum MTT reduction, respectively. Cells were incubated with staurosporine at a concentration of 0.5 μM overnight to induce apoptosis following a previously described method [35]. Lysozyme samples were added to cells at a final concentration of 21 μM as a second control known to induce toxicity [25]. The background absorbance reading for TTR1-based samples in the presence of the tetrazolium salt without cells was also measured. The data is expressed as the percentage of MTT reduction compared to an untreated cell control corrected for the absorbance of a blank sample containing MTT and TTR1-based samples without cells.

The MTT compound was used according to the protocol described by the manufacturer. Briefly, cells were incubated in DPBS (10 mM) and 0.5 mg ml^{-1} MTT per well for 2 h at 37 °C. Dimethyl sulfoxide (DMSO, AR grade) was added to solubilise the formazan product and the plate was gently mixed for 10 min at 500 rpm before the absorbance was measured at 570 nm ± 10 using a plate-reader (Bio-Rad Laboratories, USA). Cells lysed with Triton X-100 were used to assess the minimum absorbance after incubation with the MTT salt and the absorbance for these samples was <10% of untreated control cells ($p < 0.001$) for each time point tested (data not shown).