Research Article

Effect of position-specific single-point mutations and biophysical characterization of amyloidogenic peptide fragments identified from lattice corneal dystrophy patients

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Corneal stromal dystrophies are a group of genetic disorders that may be caused by mutations in the transforming growth factor β-induced (TGFBI) gene which results in the aggregation and deposition of mutant proteins in various layers of the cornea. The type of amino acid substitution dictates the age of onset, anatomical location of the deposits, morphological features of deposits (amyloid, amorphous powder or a mixture of both forms) and the severity of disease presentation. It has been suggested that abnormal turnover and aberrant proteolytic processing of the mutant proteins result in the accumulation of insoluble protein deposits. Using mass spectrometry, we identified increased abundance of a 32 amino acid-long peptide in the 4th fasciclin-like domain-1 (FAS-1) domain of transforming growth factor β-induced protein (amino acid 611–642) in the amyloid deposits of the patients with lattice corneal dystrophies (LCD). In vitro studies demonstrated that the peptide readily formed amyloid fibrils under physiological conditions. Clinically relevant substitution (M619K, N622K, N622H, G623R and H626R) of the truncated peptide resulted in profound changes in the kinetics of amyloid formation, thermal stability of the amyloid fibrils and cytotoxicity of fibrillar aggregates, depending on the position and the type of the amino acid substitution. The results suggest that reduction in the overall net charge, nature and position of cationic residue substitution determines the amyloid aggregation propensity and thermal stability of amyloid fibrils.

Introduction

Transforming growth factor β-induced (TGFBI) corneal stromal dystrophies are a group of genetic disorders associated with the deposition of misfolded protein aggregates in various layers of the cornea. Genetic studies have identified several autosomal dominant mutations in the TGFBI gene to be responsible for the protein deposits and the resulting opacification of the cornea that can lead to severe visual impairment [1]. The age of onset for these dystrophies may vary from the first decade of life for severe phenotypes to the eighth or ninth decade for the less severe phenotypes. The nature of the protein deposits and the layer of the cornea affected depend on the type of mutations [2]. Based on slit lamp bio-microscopy and histological examinations, the protein deposits can be classified as amyloidogenic, where the protein deposits appear as thin and long amyloid fibers (lattice corneal
that the mutant TGFBIp protein is proteolytically processed differently in dystrophic cornea than control cornea [15–17]. It has been hypothesized that the proteolytic products with high aggregation propensity may act as fibrillation seeds and trigger an aggregation cascade [17]. The increased abundance of a short peptide fragment in the 4th FAS-1 domain of TGFBIp (Y571HIGDEILVSGGIGALVR588) in amyloid deposits and its ability to accelerate the fibrillation of the mutant protein confirmed the role of aberrant proteolytic fragments in accelerating the fibrillation of larger proteins [18]. The increased abundance of peptide fragments spanning the 4th FAS-1 domain of TGFBIp in the amyloid deposits of dystrophic patients further suggests the importance of this domain in pathophysiology [18].

Sørensen et al. [18] characterized the amyloid fibril forming properties of TGFBIp F515–N532 (E511SMLVAAILQGALTETLN522) and Y571–R588 (Y571HIGDEILVSGGIGALVR588) that were enriched in the amyloid deposits in LCD patients with R124C, V624M and A546D mutations. They further showed that the peptide fragment Y571–R588 accelerated the amyloid fibrillation of A546T mutant protein. These results implicated the possible role of amyloidogenic peptide fragments derived from large proteins in the pathology of aggregate formation. Previous studies by our group and the others have suggested that peptide fragments from the 4th FAS 1 domain of TGFBIp form amyloid fibrils under physiological conditions [19,20].

In the present study, we investigated the aggregation propensity of a previously reported 23-residue peptide [20] in the 4th FAS-1 domain (E511PVAEPDIMATNGVHVITNLQ635) and its clinically relevant variants which decrease the overall net charge of the peptide. The rationale for choosing this region was based on our proteomics studies that the peptide fragment E611PVAEPDIMATNGVHVITNLQPANRPQER642 was enriched in the amyloid deposits of the 4th FAS-1 mutant (R124C and H626R) of TGFBIp [Proteomic analysis of amyloid corneal aggregates from TGFBI-H626R lattice corneal dystrophy patient implicates serine–protease HTRA1 in mutation-specific pathogenesis of TGFBIp — manuscript submitted to Journal of Proteome Research]. Interestingly, mutations within this region are associated with amyloidogenic phenotypes [21–25] and majority of the mutations are associated with alteration of overall net charge of the sequence.

Materials and methods

Materials

The TGFBIp511–633 wild-type (WT) and mutant synthetic peptides were purchased from Synpeptides (Synpeptide Co. Ltd, Shanghai, China). The purity of peptides was greater than 95% when synthesized (Supplementary Figure S1). Formvar–carbon-coated nickel grids were bought from EMS (Electron Microscopy Sciences, PA, U.S.A.). Chemicals were bought from Sigma–Aldrich Inc. (MO, U.S.A.). Acetonitrile (ACN),
formic acid and water for mass spectrometric studies were obtained from Sigma–Aldrich (St Louis, MO, U.S.A.). UltraMicro Spin column was bought from The Nest Group (Southborough, MA, U.S.A.).

**Spectral counts of tryptic peptides from TGFBIp using LC–MS/MS**

Corneal sections were collected from three corneal dystrophy patients with amyloid lattice corneal dystrophy (two patients with H626R mutation and one patient with R124C mutation in the TGFBI gene) who were undergoing corneal transplantation at the Singapore National Eye Centre. Written informed consent was obtained from all patients prior to surgery. Ethical approval for the collection of patient corneas was granted by the Singhealth Institutional Review Board. Control tissues (n = 3), transplant-grade corneal samples from deceased individuals were obtained from Lion’s Eye Institute (Tampa, U.S.A.). The patient corneal sections were stained with Congo red to locate the amyloid fibril deposits. Positive Congo red sections were collected using laser capture microdissection (LCM) and proteins were extracted from the LCM cap. Three normal control corneas without epithelium were dissected into smaller pieces using a sharp scalpel and they were processed to extract corneal stromal proteins. The proteins from patients and controls were digested overnight with 10 μg of trypsin for 16 h at 37 °C and used for mass spectrometry analysis.

LC–MS/MS analysis was performed using a Q Exactive mass spectrometer coupled with the online Dionex Ultimate 3000 RSLC nano-LC system (Thermo Scientific, MA, U.S.A.). Samples were run on a Dionex EASY-spray column (PepMap® C18, 3 μm, 100 Å) using an 120 min gradient of mobile phase A (0.1% FA in 5% ACN) and mobile phase B (0.1% FA in ACN) with a flow rate of 300 nL/min. Separated peptides were passed through an EASY nanospray source with a source voltage of 1.5 kV. A full MS scan was set at 350–1600 m/z with a resolution of 70 000 at m/z 200. The maximum ion accumulation time and dynamic exclusion time were set as 100 ms and 30 s, respectively. The 10 most intense ions detected above a threshold of 1000 counts were selected for higher energy collisional dissociation fragmentation using 28% normalized collision energy with a maximum ion accumulation time of 120 ms. Other parameters used were automatic gain control 1e×10^6 for the full MS scan and 2e×10^5 for the MS2 scan, isolation width of 2 Da for the MS2 scan, and exclusion of single and unassigned charged ions from the MS/MS scan (with the underfill ratio set to 0.1%). Data acquisition was performed using the Xcaliber 2.2 software (Thermo Scientific, U.S.A.).

**Data analysis**

Raw data were processed using two different approaches to achieve high confidence of label-free quantitation. First, all raw data were converted into the mascot generic format (MGF) using Proteome Discoverer 1.4.1.14 (Thermo Fisher, MA, U.S.A.), and searches were performed using an in-house search engine (Mascot, version 2.4.1; Matrix Science, London, U.K.). The UniProt Knowledgebase (UniProtKB) of human proteins (downloaded on July 25, 2016, including 70 849 sequences and 23 964 784 residues) was used as the search database. The following settings were applied: static modification = carbamidomethyl at cysteine; dynamic modifications = methionine oxidation, asparagine/glutamine deamidation; digestion parameters = full trypsin with two missed cleavages, semi-trypsin with one non-specific cleavage; search parameters = #13C is 2; precursor mass is 10 ppm; fragment mass tolerance is 0.02 Da. Selection of proteins for final analysis was performed using a target-decoy search strategy with a false discovery rate (FDR) of ≤1% considering only those proteins identified with multiple peptides. An additional search was performed using semi-tryptic digestion as the enzyme parameter in order to identify non-tryptic peptide cleavage sites. The emPAI value reported by Mascot and spectral counting was used to perform quantification of proteins and peptides, respectively.

The raw data files were also analyzed using the Proteome Discoverer 1.4 software package. MS/MS spectra were searched against the same UniProt human database using the Mascot search engine with parameters identical with those described above. The peptide-to-spectrum matches were filtered by application of a <1% FDR threshold using Percolator. Only peptides identified with high confidence as assigned by the software were selected for further analysis. The precursor ion area detection node was enabled in order to obtain the area under the curve (AUC, or extracted ion chromatogram/XIC) of the LC elution profiles of the detected peptides. The AUCs of the same peptides obtained from both patient and control samples were used for quantitative analysis to determine the fold changes and extent of enrichment in patient tissues. Peptide/protein lists were exported to Excel and processed using in-house analytical scripts. All statistical calculations were performed using three technical replicates for each sample.
Circular dichroism spectroscopy to determine secondary structure of P1 and other mutant peptides

Far UV-circular dichroism (CD) spectra of the peptides were collected using a Jasco J-810 spectropolarimeter (Jasco, Inc., Easton, MD, U.S.A.) and Chirascan-plus (Applied Photophysics, U.K.) using a quartz cuvette with a path length of 0.1 cm (Hellma, Müllheim, Germany/Starna). The TGFBIp<sup>611-633</sup>WT and mutant synthetic peptides were reconstituted in PBS (pH 7.2) to a final concentration of 0.6 mg/ml and their CD spectra were recorded.

For time-dependent conformational transition of the model peptides, the peptides were shaken at 180 rpm at 37°C up to 5 weeks in a shaking incubator. The spectra were recorded from 260 to 190 nm with a scan rate of 50 nm/min for every 1°C. Initial and final spectra before and after heating and after cooling back to 20°C were recorded. The final spectrum used for plotting was an average of three scans. The CD data were expressed as the mean residual weight ellipticity (deg cm<sup>2</sup> dmol<sup>-1</sup>). The mean residual weight (MRW) ellipticity ([θ]<sub>MRW</sub>) was estimated using the following equation:

\[
[\theta]_{MRW} = \frac{[\theta]_{\lambda} \times MRW}{10 \times l \times c}
\]

where [θ]<sub>λ</sub> is the observed ellipticity, MRW is the mean residual weight and defined as M/N – 1 where M is the molecular mass and N is the number of amino acid residues, l is the path length of the cuvette and c is the concentration in mg/ml. The data obtained were plotted using the Origin 8.0 software.

To infer if the amyloid fibrils formed by the TGFBIp mutant peptides possess similar or varying thermal properties, we monitored the melting and reassembly of the preformed amyloid fibrils by variable temperature CD spectropolarimetry. To achieve homogeneous amyloid fibril preparations, the peptides were incubated in PBS buffer at 37°C for 5 weeks (10 weeks for P6) until the samples contain a significant abundance of amyloid fibrils. For thermal denaturation experiments, 0.6 mg/ml of the peptide fibrils in PBS (pH 7.2) were heated using the inbuilt Peltier heating system (Applied Photophysics, U.K.). The CD spectra were recorded as described earlier at various time intervals. Variable temperature scanning was done by heating the sample from 20 to 90°C at a rate of 1°C/min.

For studying the effects of ramping rates on the P5 peptide fibrils, the sample was analyzed by heating the fibrils to 90°C at various heating rates (0.5, 1 and 2°C/min).

Thioflavin T assay

The TGFBIp<sup>611-633</sup>WT and mutant synthetic peptides (0.6 mg/ml) were shaken at 180 rpm in a 37°C incubator, and samples were taken at various time points up to 36 h and were treated with 30 μM ThT in PBS buffer (pH 7.2) in a Greiner 96-well flat bottom polystyrol microplate (Greiner, Frickenhausen, Germany). The samples were excited at 445 nm and the resulting emission fluorescence at 485 nm was measured using a microplate reader (Tecan infinite M200 pro, Zanker Road, San Jose, U.S.A.).

Transmission electron microscopy

Transmission electron microscopic images of the peptide fibrils were acquired with a JEOL JEM-1010 transmission electron microscope using Digital Micrograph<sup>™</sup> 1.81.78 for GMS 1.8.0 (Gatan, Pleasanton, CA, U.S.A.) at the National University of Singapore Electron Microscopy facility. Aliquots of amyloid fibrils were obtained by shaking 0.6 mg/ml of each of the peptide at 37°C in a shaking incubator. About 500 μl of amyloid fibril samples were withdrawn at different time points and centrifuged gently. The supernatant was removed and 10 μl of amyloid fibril samples were applied onto Formvar-carbon-coated 300-mesh-size nickel grids with baticrin as the binding agent. Excess samples were blotted off the filter paper and negatively stained with 10% phosphotungstic acid, incubated for 5–10 min and washed with water. The grids were dried and observed at magnifications 8000–50 000× at 80 kV. For thermal denaturation studies on P5-derived fibrils, the fibrils were heated to 90°C and cooled back, centrifuged and examined using transmission electron microscopy (TEM) as described above. The length and mean diameter of the fibrils were calculated by the measurements obtained using the ImageJ software (National Institute of Mental Health, Bethesda, MA, U.S.A.). For enzyme-assisted proteolysis studies, the trypsin-digested fibril samples were examined using TEM as described.
LC–MS/MS analyses
Amyloid fibrils collected from P5 (300 μg) were digested with trypsin (Trypsin : Fibril = 1:25 ratio) for 12 h. The digested mixture was analyzed by LC–MS/MS [Ultimate 3000 nano-LC (Thermo Fisher Scientific/Dionex, Sunnyvale, CA, U.S.A.), coupled with AB SCIEX Triple TOF TM 5600 mass spectrometer] and monitored for the short fragment (E611R623) in the sequence. The peptide mixture was first desalted and pre-concentrated in a trap column (Acclaim PepMap 100 C18, 75 μm × 3 μm, 100 Å from Thermo Fisher Scientific/Dionex, Sunnyvale, CA, U.S.A.). A 35 min gradient was used at 300 nl/min. All data were acquired using the information-dependent acquisition mode with the Analyst TF 1.5.1 software (AB Sciex, U.S.A.). The Protein Pilot software (version 4.01, AB Sciex) was used to analyze the MS/MS data.

Peptide aggregation by one-dimensional solution NMR
For all NMR experiments, peptides P1 to P6 were selectively 13C- and 15N-labeled for amino acids (glycine, alanine, valine and isoleucine) and were purchased from Synpeptides (Synpeptide Co. Ltd, Shanghai, China) with purity greater than 95%. The amino acid residues that were 13C- and 15N-labeled for each peptide are highlighted in blue and given in Table 1.
To follow the aggregation profiles of peptides using one-dimensional (1D) solution NMR, 0.6 mg/ml peptides were dissolved in 1× PBS buffer along with 10% D2O and 5% 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). Samples were transferred to 5 mm NMR tubes and experiments were recorded at 37°C. Aggregation profiles were studied using the 600 MHz Bruker Avance II spectrometer by recording series of 1D NMR spectra. Spectra were collected every 30 min until a clear drop in amide peak intensity was observed in the 1D NMR spectra that served as an indirect readout of aggregation. The NMR data were processed using TopSpin 2.0 (www.bruker-biospin.com) and analyzed using CARA (cara.nmr.ch).
1D NMR intensities between 6.8 and 8.8 ppm for each time point for each peptide were obtained by using the integral function of the Topspin software and normalized to the integral values of the internal standard, DSS. The normalized intensity values were fit using a single-phase decay exponential function. The aggregation rate (K) values for each of the peptides were tabulated.

Packing of fibrils in the rotor for solid-state NMR
About 6 mg of 13C- and 15N-labeled peptides were dissolved in PBS to attain a final concentration of 0.6 mg/ml. The peptides were incubated at 37°C in a shaking incubator in PBS buffer for amyloid fibril formation. The fibrils were collected after 5 weeks. The presence of amyloid fibrils was confirmed by TEM. The fibrils were spun gently and the pellet was collected and sent to RIKEN Institute for solid-state NMR experiments. The pellet sample was filled into the NMR sample rotor by centrifugation in a swing rotor and using a pipetteman tip as a funnel.

Table 1 Peptide sequence of the TGFβIp–4th FAS-1 domain (amino acids 611–633) WT and various disease-associated mutants
The underlined residues are labeled with 13C and 15N isotopes. The total number of amino acids labeled for each residue is also tabulated.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Number of amino acids</th>
<th>Reference in this manuscript</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFβIp611–633WT</td>
<td>EPVAEPDMATNGWVHVTNLQ</td>
<td>10</td>
<td>P1</td>
</tr>
<tr>
<td>TGFβIp611–633M619K</td>
<td>EPVAEPDKATNGWVHVTNLQ</td>
<td>10</td>
<td>P2</td>
</tr>
<tr>
<td>TGFβIp611–633N622H</td>
<td>EPVAEPDMATGWVHVTNLQ</td>
<td>10</td>
<td>P3</td>
</tr>
<tr>
<td>TGFβIp611–633N622K</td>
<td>EPVAEPDMATGKWVHVTNLQ</td>
<td>10</td>
<td>P4</td>
</tr>
<tr>
<td>TGFβIp611–633G623R</td>
<td>EPVAEPDMATNWRWVHVTNLQ</td>
<td>9</td>
<td>P5</td>
</tr>
<tr>
<td>TGFβIp611–633H626R</td>
<td>EPVAEPDMATNGWVHVTNLQ</td>
<td>10</td>
<td>P6</td>
</tr>
</tbody>
</table>
Solid-state NMR for the amyloid fibrils

1D and two-dimensional (2D) $^{15}$N–$^{13}$C correlation experiments were performed on a 700 MHz Bruker (Bruker BioSpin GmbH, Rheinstetten, Germany) Avance III spectrometer with a magic-angle spinning (MAS) rate of 15.75 kHz. Cross-polarization magic-angle spinning (CP-MAS) $^{13}$C SS NMR experiments were performed at 25°C. 2D $^{13}$C–$^{13}$C dipolar-assisted rotational resonance (DARR) experiments for the P5 and P1 peptides were performed at 25 and 10°C, with a mixing time of 20, 60, 200 and 600 ms. The spectral width ($f_2$ and $f_1$) was set to 270 ppm and center ($f_2$ and $f_1$) was 97.7 ppm. The number of time domain (TD) data points along $f_2$ and $f_1$ dimensions were 1024 and 768, respectively. The recycling delay was set to 1.5 s with 64 scans. For the NCO and NCA experiments, the spectral width ($f_2$ and $f_1$) was set to 270 and 74 ppm and the center ($f_2$ and $f_1$) was 97.7 and 115 ppm, respectively. The recycling delay was set to 2 s with 384 scans. For all $^{13}$C and $^{15}$N experiments, chemical shifts were calibrated from the gyromagnetic ratio based on the H$_2$O signal. The data were processed using TopSpin 2.0 (www.bruker-biospin.com) and analyzed using CARA (cara.nmr.ch).

Cytotoxicity of amyloid fibrils on cultured human corneal fibroblast cells by xCELLigence

The xCELLigence system (ACEA, San Diego, CA, U.S.A.) was used to investigate the effects of the peptide fibrils of TGFBIp on cultured human corneal fibroblast (HCSF) proliferation and cell death. The E-plate 96 is incorporated with sensor arrays at the bottom of each well, which detects and translates cell attachment in the form of electronic impedance. A parameter termed cell index is derived which corresponds to the relative density and adherence strength of cells in each well. HCSF cells were seeded 7000 cells per well on an E-plate 96, in fibroblast media (DMEM with 10% FBS). The cells were allowed to attach onto the E-plate until the impedance curves reached a plateau. The matured fibrils were formed from 0.6 mg/ml monomeric peptides dissolved in PBS and shaken at 37°C at 180 rpm for 4 weeks. These peptide fibrils were centrifuged and diluted in culture media and treated with the cells. Culture was maintained for 48 h in the xCELLigence real-time cell analysis (RTCA) SP Station, placed in an incubator at 37°C with 5% CO$_2$. There was no change in media during the 48 hour time period to prevent any fluctuations in the impedance values caused by removing the plate from the RTCA system.

Results

Identification of C-terminal peptide fragments in the amyloid deposits of H626R and R124C mutant

The amyloid deposits from patients’ cornea were isolated by LCM and digested with trypsin (a detailed protocol is provided in Materials and Methods). To identify the proteolytic fragments that form the core regions of the amyloid fibrils, we analyzed the total percentage of peptide spectra derived after tryptic digestion of mutant TGFBIp and compared with the fragments present in control TGFBIp. The results of the combined spectral counts of peptides from the three patients and control TGFBIp (Figure 1) show that peptides $^{G511}DNFRSMLVAAIQSAGLTELNR^{533}$, $^{V571}HIGDEILVSGGIGALVR^{588}$, $^{E611}PVAEPDIMATNGVHVITNVLPANRPQER^{642}$, $^{L497}TPPMGTVMVLKDNRFSMLVAAIQSAGLTELNR^{533}$, $^{A350}ISNKDILATNGVIHYIDELLPSAK^{377}$, $^{I296}LGDPEALDLNNHILK^{313}$ and $^{H187}GMTLTSMYQNSIQHYPNGIVTVNCAR^{216}$ were significantly enriched in the dystrophic cornea but were absent from the control samples. We next assessed if the peptide fragments contained the altered amino acid residues in the deposits isolated from individual dystrophic patients. In R124C amyloid deposits, we were able to identify two mutant peptides, $^{G96–K127}$ (G96CPAALPLSNLYETLGVVGTSSQDCTEK$^{127}$) and $^{V91–K127}$ (V91PGEKGCPAALPLSNLYETLGVVGTSSQDCTEK$^{127}$), which carry the cysteine residue. In one of the amyloid deposits collected from H626R patient, we could detect the presence of two peptide fragments, N603–R626 ($^{N603}NVVSVEPVAEPDIMATNGVVR^{626}$) and E611–R626 (E611PVAEPDIMATNGVVR$^{626}$), with the altered amino acid sequence.

The change in tryptic peptide profiles between the patient samples and normal control samples may be due to the fact that, in a normal WT protein, the protein is well folded and the hydrophobic residues are not exposed, whereas in the mutant protein, the mutations may cause the proteins to mis/unfold, thus exposing the hydrophobic residues. The hydrophobic residues serve as substrates for various proteolytic enzymes, which result in shorter peptides. The change in the protein folding between the WT and mutant thus produces a
difference in the accessibility of the proteolytic enzyme action, and hence the peptide generation pattern varies between the WT and mutant proteins.

Cumulative analysis of the mass spectrometric data from three dystrophic patients suggests that, in addition to the previously reported amyloidogenic peptide fragments G\(^{511}\)DNFSMLVAIQSAGLTETLNR\(^{533}\) and Y\(^{571}\)-HIGDEILVSAGGALVR\(^{588}\), the C-terminal fragment E\(^{611}\)–R\(^{642}\) (E\(^{611}\)PVAEPDIMATNGVVHVITNVLQPPANRPQR\(^{642}\)) was also enriched significantly in the amyloid deposits. This region is clinically important as more than 16 different mutations have been reported and many of them alter the overall net charge of the protein, which leads to the formation of amyloid deposits in the cornea. We recently reported a 23-residue peptide from this region (E\(^{611}\)PVAEPDIMATNGVVHVITNVLQ\(^{633}\)) that forms amyloid fibrils under physiological conditions [26,27]. Thus, we synthesized five different peptides from clinically significant mutations for the present study (Table 2).

**Table 2 Peptide sequence of the TGFβp-4th FAS-1 domain (amino acids 611–633) WT and various disease-associated mutants**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Net charge</th>
<th>Reference in this manuscript</th>
</tr>
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<tbody>
<tr>
<td>TGFβp(^{611-633})WT</td>
<td>EPVAEPDIMATNGVVHVITNVLQ</td>
<td>−3</td>
<td>P1</td>
</tr>
<tr>
<td>TGFβp(^{611-633})M619K</td>
<td>EPVAEPDIMATNGVVHVITNVLQ</td>
<td>−2</td>
<td>P2</td>
</tr>
<tr>
<td>TGFβp(^{611-633})N622H</td>
<td>EPVAEPDIMATHGVHVITNVLQ</td>
<td>−3</td>
<td>P3</td>
</tr>
<tr>
<td>TGFβp(^{611-633})N622K</td>
<td>EPVAEPDIMATKBVHVITNVLQ</td>
<td>−2</td>
<td>P4</td>
</tr>
<tr>
<td>TGFβp(^{611-633})G623R</td>
<td>EPVAEPDIMATNRHVITNVLQ</td>
<td>−2</td>
<td>P6</td>
</tr>
<tr>
<td>TGFβp(^{611-633})H626R</td>
<td>EPVAEPDIMATNGVRVITNVLQ</td>
<td>−2</td>
<td>P6</td>
</tr>
</tbody>
</table>

Bold indicates the type of amino acid change (cationic or anionic substitution).
Secondary structural analysis and time-dependent conformational transition in model peptides

It has been shown that the conversion from random coil or α-helix into β-sheet conformation is a hallmark of amyloid fibril formation [28,29]. It has been reported that, for β-structured amyloid fibrils, the amplitude of the maximum ∼195–200 nm increased tremendously, higher than the native β-structured protein [30,31]. Therefore, the effect of cationic amino acid substitution on conformational properties of the peptides was investigated by CD spectropolarimetry. We studied the temporal changes in secondary structure of P1–P6 in PBS at 37°C (Figure 2). Figure 2A–F displays temporal changes in the secondary structure of the peptides over a period of 5 weeks. At time 0, P1 (Figure 2A) showed an initial minimum ∼200 nm, typical of an unfolded peptide in equilibrium with a β-sheet structure [28,29,31] and with increase in incubation time, the negative minimum shifts from ∼195–200 nm at 0 h to 210–212 nm at 5 weeks.

All the other mutant peptides, except P6, displayed substantial changes in the secondary structure at time 0 and faster ability to form β-structured assembly as the time progressed when compared with P1 peptide. P2 (Figure 2B) and P3 (Figure 2C) displayed a minimum ∼200 nm and a broad shoulder around n–π* region. A relatively rapid progression was observed for P2 as the β-sheet structures could be seen as early as at 48 h of incubation. The final conformation of P2 appeared to consist of a mixture of secondary structures, as the peptide displayed a broad minimum spanning the region 210–220 nm, indicating the possibility of mixed populations of amyloid oligomers/fibrils. Similar to P2, a rapid conversion into β-structure was apparent for P3 within 48 h. For P4 (Figure 2D), a broad minimum between 205–220 nm and a weak positive peak ∼198 nm was observed, indicating the presence of an ordered secondary structure at time 0 [28,29]. P4 showed a significant increase in the amplitude of the maximum ∼198 nm and the minimum ∼218 nm with an increase in incubation time. CD spectra of P5 (Figure 2E) indicated the presence of an intense minimum ∼218 nm and a strong maximum ∼198 nm, which are characteristics of β-sheet structure at time 0 [28,29]. With increase in time, P5 showed a red shift in both minima and maxima, and the amplitude of π–π* maximum was increased by more than four times the peak intensity at zero time. For P6, the secondary structure was similar to P1 with an intense minimum ∼200 nm (Figure 2F). With increase in time, P6 did not show a transition into β-structured assembly even after 8 weeks of incubation. Thus, substitution of positively charged residues in the
model peptide had variable effects on the secondary structure and conversion into β-structured assembly, depending on the position and nature of the amino acid substitution.

### Aggregation propensity of the amyloid-forming peptides

To analyze the effect of amino acid substitution on the kinetics of amyloid formation, we determined the temporal changes in fluorescence intensity of amyloid-binding dye Thioflavin T (ThT), incubated with the peptides [32–34]. Figure 3 shows the time-dependent changes in the ThT fluorescence intensity. With increase in time, all the mutant peptides (P2–P6) showed a progressive increase in the ThT fluorescence intensity without any lag phase, indicating the presence or formation of amyloid fibrils (Table 3). Peptides P2, P3 and P5 displayed higher $k_{\text{agg}}$ and lower $t_{1/2}$ values, whereas P4 showed similar values when compared with P1. Among all the peptides, P6 displayed the lowest $k_{\text{agg}}$ and the highest $t_{1/2}$, confirming the CD results.

To gain insights into conformational transitions during the early stages, we used 1D proton NMR (Supplementary Figure S2). 1D NMR spectra was recorded at several time points with conditions similar to the ThT experiment. For all the peptides, there was a clear decrease in peak intensity (or broadening of peaks)

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**Figure 3.** Aggregation kinetics of TGFBIp611–633WT (P1) and mutant peptides (P2–P6) probed by ThT fluorescence.

Aggregation kinetics of TGFBIp611–633WT (P1) and mutant peptides (P2–P6) probed by ThT fluorescence. Fluorescence intensity is plotted along the Y-axis against the observed time in seconds along the X-axis. All the mutant peptides show an increased propensity to form amyloid fibrils compared with the TGFBIp611–633WT peptide. ThT intensity data from two independent experiments were averaged for the determination of kinetic parameters.
The broadening of 1D NMR peak intensity, due to formation of oligomer, was observed during early stages. At the end of 2 weeks, clear fibrillar assembly was obtained. All the three peptides showed long unbranched fibrils among the three peptides followed by P3 (Figure 5C). Most of the fibrils of P1 were thin and short in length with a few fibrils extending up to 1–1.2 μm. For P2, more matured fibrils of shorter lengths (100–200 nm) and stacks could be seen during early stages (at 4 days), which then converted into long (1–1.4 μm) unbranched fibrillar assembly after 2 weeks (Figure 5B). For P3, P4 and P5, higher amounts of matured fibrils could be seen in 4 days, with P5 showing the highest abundance of matured fibrils among the three peptides followed by P3 (Figure 5C–E), in accordance with the kinetics of aggregation. All the three peptides showed long fibrillar (2–4 μm) assembly after 2 weeks of incubation. P6, the P5 peptide was the fastest to aggregate and tended to form oligomers as soon as the peptide was dissolved in the buffer. The amide peaks were not traceable even at time 0. The broadening of 1D NMR peak intensity, due to formation of oligomer, was plotted against time to obtain the rate constant (k) of aggregation. For each of the time points, the integral values between 6.5 and 8.5 ppm, which showed a maximum change in peak intensity upon aggregation, were collected and normalized with the internal control DSS. The integral values at each time point were used to fit a single-phase decay curve (Figure 4) and the rate constant (K) values were summarized as in Table 2. We could not obtain rate constant (K) values for peptides P1 and P5 as there were not enough points for curve fit. Among the four peptides which could be used for curve-fit analysis, peptide P2 was the fastest to aggregate and peptide P6 was the slowest to form amyloid fibrils. The absence of a lag phase in both ThT and 1D NMR experiments suggests that the aggregation process is spontaneous with the formation of larger oligomers or fibrillar species within a short period of time. Overall, these results are in agreement with the kinetics of aggregation determined by ThT assays. The experiments support the fact that P5 has the fastest rate of aggregation while P1 and P6 peptides displayed slowest rates of aggregation.

**Morphology of the amyloid fibrils by TEM**

The morphology of amyloid fibrils formed by various peptides was examined by TEM. Upon visual examination, gel-like deposition on the inner walls of the falcon tubes at the rims of the liquid surface with varying levels of transparency and translucency were observed from week 2 onwards. While P3 and P4 displayed nearly transparent deposition, P5 showed more translucent deposition along the inner walls of the tube. The results showed that while all the peptides formed long unbranched fibrils as early as at 2 weeks of incubation, there were differences observed in their morphology, densities, stacking and distribution during the early stages (Figure 5). Images of fibrils formed after 4 days were examined to study the oligomeric, proto-fibrillar or prefibrillar structure and morphologies. It was clear that the oligomers and the protofibrils of the different mutants displayed different morphologies, lengths and diameters. For P1, a mixed population of fibrils and oligomers were observed during early stages. At the end of 2 weeks, clear fibrillation was observed and the oligomeric species were completely absent, indicating partial or complete conversion into amyloid fibrils (Figure 5A). Most of the fibrils of P1 were thin and short in length with a few fibrils extending up to 1–1.2 μm. For P2, more matured fibrils of shorter lengths (100–200 nm) and stacks could be seen during early stages (at 4 days), which then converted into long (1–1.4 μm) unbranched fibrillar assembly after 2 weeks (Figure 5B). For P3, P4 and P5, higher amounts of matured fibrils could be seen in 4 days, with P5 showing the highest abundance of matured fibrils among the three peptides followed by P3 (Figure 5C–E), in accordance with the kinetics of aggregation. All the three peptides showed long fibrillar (2–4 μm) assembly after 2 weeks of incubation. P6, maturated fibrils could be seen in 4 days, with P5 showing the highest abundance of matured fibrils among the three peptides followed by P3 (Figure 5C–E), in accordance with the kinetics of aggregation. All the three peptides showed long fibrillar (2–4 μm) assembly after 2 weeks of incubation. P6, P5, higher amounts of matured fibrils could be seen in 4 days, with P5 showing the highest abundance of matured fibrils among the three peptides followed by P3 (Figure 5C–E), in accordance with the kinetics of aggregation. All the three peptides showed long fibrillar (2–4 μm) assembly after 2 weeks of incubation. P6,
however, displayed similar morphological features as those of P2, showing a mixed population of oligomers and truncated amyloid fibrils in 4 days, which then formed long amyloid fibrils with increase in incubation time up to 2 weeks (Figure 5F). Estimation of diameters of matured fibrils formed by the peptides did not show any significant differences among the various fibrillar assemblies. These results indicated that despite the changes in kinetics of aggregation and nature of amino acid substitution, all the matured fibrils displayed nearly identical morphologies and diameter distribution.

Thermal denaturation studies of amyloid fibrils
Many previous studies have shown that amyloid fibrils are resistant to thermal challenges when compared with the monomeric protein [35,36]. However, to infer if the amyloid fibrils formed from various peptides display similar or different thermal properties, we recorded the variable temperature-circular dichroism (VT-CD) of the solution containing matured fibrils. Reversible thermal changes in the CD ellipticity at 218 nm ($\theta_{218}$) corresponding to the $\beta$-sheets [28,29,37] were monitored by heating the amyloid fibrils (0.6% w/v) from 20 to 90°C. For P1, the negative maxima $\theta_{218}$ increased with increase in temperature, and began to decrease after 70°C and reached a plateau after 85°C. Upon cooling, no clear transition was observed and $\theta_{218}$ followed a linear decrease without achieving the original $\theta_{218}$ values. Subsequent heating produced traces which followed the $\theta_{218}$ values observed during the cooling cycle (Figure 6A). Far UV-CD spectra confirmed a clear transition from $\beta$-sheet conformation to a disordered conformation after heating/cooling cycles (Figure 6B). We also monitored the change in intensity of ThT-bound amyloid fibrils with temperature. As shown in Figure 6C, a smooth sigmoidal transition was observed with maximum loss of ThT intensity above 70°C, consistent with the transition observed in the VT-CD experiments. On cooling, no reversible transition was
observed, confirming the conversion of amyloid fibrils into monomeric forms (Figure 6C and inset). Similar changes were observed for the peptides P2, P3 and P6 (Supplementary Figure S3). However, for the peptides P4 and P5, a marked reversibility in the thermal transition was observed. For P4, the initial increase in
temperature increased the \([\theta]_{218}\) intensity, and at elevated temperature the \([\theta]_{218}\) intensity decreased considerably (Figure 6D). On cooling, the values remained identical and reheating did not alter the \([\theta]_{218}\) intensity values. CD spectra indicated that the \(\beta\)-sheet structure remained intact even after the heating/cooling cycle, although a slight decrease in the amplitude of the negative maximum \(\sim 218\) nm was observed (Figure 6E). After second heating/cooling cycle, no obvious change in the secondary structure was observed and the peptide remained intact in \(\beta\)-amyloid conformations. The ThT assay indicated a similar sigmoidal transition upon heating P4 and partial retention of the intensity upon cooling (\(~12\%\) of the original ThT intensity; Figures 6F and inset).

The amyloid fibrils prepared from P5 displayed even more remarkable thermal reversibility as shown by the absence of any clear transition upon heating (Figure 6G), although a slight decrease in the CD intensity was observed upon cooling. Reheating did not change the \([\theta]_{218}\) intensity and it remained similar to the values obtained during the cooling cycle over the entire temperature range. CD spectra confirmed the presence of a \(\beta\)-amyloid structure that remained intact even after two heating/cooling cycles, suggesting significant stability of the fibrils compared with the amyloid fibrils derived from other peptides (Figure 6H). The P5 fibril was examined further by performing heating and cooling of the fibrils for five cycles (Supplementary Figure S4). Reheating in the subsequent cycles did not show any changes in intensities. Thermal denaturation using ThT
fluorescence studies indicated a sharper decrease in the ThT intensity upon heating, and moderate recovery (>50%) was observed upon cooling (Figure 6I and inset) the amyloid fibrils.

TEM was used to further confirm the thermal stability of the amyloid assemblies derived from P1, P4 and P5. For P1, after the thermal denaturation cycle, no amyloid fibrils could be detected, confirming complete disassembly of the fibrils (Figure 7A,B). However, for both P4 and P5, the amyloid structure remained intact (Figure 7C–F). For P5, many fibrils coalesced along the entire length that resulted in a 2-fold increase in the average diameter (18.1 ± 1.52 to 31.71 ± 4.01 nm) of the fibrils. For P4, though the fibrils remained intact, there was little or no coalescence observed and the mean fibrillar diameter remained unaltered (17.5 ± 1.9 nm before heating to 18.8 ± 1.4 nm). These results confirmed the stability achieved by the amyloid fibrils formed from P5 and corroborate the results observed in the VT-CD studies and the ThT assay. Together, these results suggest the possible position-sensitive influence of electrostatic interactions on thermal stability of the amyloid fibrils.

To examine if the thermal ramping rates of heating had any effects on the fibrils formed, we also studied the thermal denaturation of P5 by heating the fibrils to 90°C at various heating rates (0.5, 1 and 2°C/min). In all experiments, the β-sheet structure corresponding to the fibrils remained intact without an apparent change in the temperature at the midpoint, $T_m$ (Supplementary Figure S5).

Since the cationic residue substitution (Gly to Arg) in P5 introduced a trypsin proteolytic site which is not present in P1, we determined the proteolytic stability of the amyloid fibrils derived from P5. The P5 fibrils (300 μg) were digested with trypsin (Trypsin:Fibril = 1:25 ratio) for 12 h and examined by LC–MS/MS and TEM. No discernible peak, which corresponds to the proteolytic fragment (E$^{611}$PVAEPDIMATNVHVITNVLQ$^{633}$), could be detected in the LC–MS spectra. TEM images (Supplementary Figure S6) further confirmed no apparent changes in the morphology of the fibrils before and after trypsin digestion, indicating that Arg 623 residue may form the amyloid fibril core in P5.
Solid-state NMR for the amyloid fibrils

1D and 2D solid-state NMR was used to study the structure of amyloid fibrils. $^{13}$C CP-MAS spectra were recorded to check the quality of the fibrils and structural homogeneity of the samples (Supplementary Figure S7). The $^{13}$C cross-polarization spectra indicated that the fibrils formed were uniform and with the resolved $^{13}$C resonances and line width smaller than 100 Hz, which warranted further studies using the 2D solid-state experiments.

Among all peptides, the P5 peptide had greater propensity to form amyloid fibrils and the fibrils displayed excellent thermal stability, hence we investigated the possible structural differences in the amyloid fibrils formed from P5 and P1 by solid-state NMR. The DARR [38,39] experiment with a short mixing time was used to identify all intra-residue and 2 bond carbon–carbon correlations. Additionally, 2D NCA and NCO spectra were recorded to aid in the DARR assignment. Figure 8B shows the NCA spectrum of peptide P5 where the $^1$H to $^{15}$N via cross-polarization and then selectively to the $^{13}$C using specific cross-polarization. (C) A plot of the C-alpha and C-beta chemical shifts from the rigid part of DARR assignment of the P5 peptide subtracted from the random coil shifts ($\Delta C^\alpha$ or $\Delta C^\beta = [C^\alpha or C^\beta -$ random coil shifts$]$). The plot shows the presence of a $\beta$-sheeted secondary structure.

Figure 8. (A) Expansion of the aliphatic 2D $^{13}$C–$^{13}$C chemical shift correlation DARR spectra for fibrils derived from the P5 peptide recorded with a 200 ms mixing time. Out of the nine amino acids that were uniformly $^{13}$C- and $^{15}$N-labeled, peaks were visible for seven amino acids; four valine, two isoleucine and one alanine residues were identified readily based on their characteristic fingerprint region and intra-residue cross-peaks; one valine (V613) and one alanine residue (A614) were not visible for assignment as they might be highly dynamic. (B) NCO/NCA plot for peptide P5 used to assign nitrogen chemical shifts. In an NCA experiment, magnetization is transferred from $^1$H to $^{15}$N via cross-polarization and then selectively to the $^{13}$C using specific cross-polarization. (C) A plot of the C-alpha and C-beta chemical shifts from the rigid part of DARR assignment of the P5 peptide subtracted from the random coil shifts ($\Delta C^\alpha$ or $\Delta C^\beta = [C^\alpha or C^\beta -$ random coil shifts$]$). The plot shows the presence of a $\beta$-sheeted secondary structure.

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were uniformly $^{13}$C-labeled, one valine (V613) and one alanine residue (A614) were not visible, presumably due to the high intramolecular mobility. The other four valine, two isoleucine and one alanine residues were readily identified based on their characteristic fingerprint chemical shifts and intra-residue cross-peaks.

A plot of the C-α and C-β chemical shifts [41,42] from the rigid part of DARR assignment of the P5 peptide subtracted from the random coil shifts showed a clear β-sheet secondary structure (Figure 8C). The DARR experiment with longer mixing time did not give many extra cross-peaks and the inter-residue connectivities were not observed except between residues V627 and I628, suggesting that the amyloid fibrils may be in a parallel β-sheet arrangement. Thus, solid-state nuclear magnetic resonance (ssNMR) results for the P5 peptide were in agreement with the CD experiments, suggesting that the peptide formed clear β-sheet amyloid fibrils with a parallel β-sheet arrangement. However, for P1, the NCA experiment showed multiple sets of cross-peaks (suggesting the presence of polymorphs) with various confirmations of the amino acids (Supplementary Figure S8A,SB), and hence it was difficult to exhaustively assign the DARR peaks [38–40]. It is likely that the glycine residue in P1 may allow the formation of dynamic β-structured assembly, whereas the presence of arginine in P5 might have stabilized the fibrils through electrostatic interactions, thus producing a homogeneous assembly.

**Cytotoxicity of amyloid fibrils on HCSF cells**

CD, ThT fluorescence and NMR studies indicated a gradual transition from predominantly unordered conformations to a β-structured fibrillar assembly with time for all the peptides (except P4 and P5). Figure 9 compared the effect of various freshly prepared peptide solutions and amyloid fibrils on the cell index values derived from xCELLigence. While none of the peptides/amyloid fibrils were cytotoxic to the HCSFs, a significant decrease in the proliferation rate was observed, depending on the nature of the amino acid modifications. For the peptide P1, there was no change in the proliferation rate and the cell index value observed was

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Figure 9. The cell survival following the treatment of the peptides and peptide fibrils of TGFBIp against HCSF cells monitored for 48 h. HCSF cells seeded at 7000 cells per well on an E-plate 96, in fibroblast media with 5% FBS, were treated with the peptides and peptide fibrils of TGFBIp. Culture was maintained for 48 h in the xCelligence RTCA SP Station, placed in an incubator at 37°C with 5% CO₂. Cell index generated was taken for further analysis.
form fibrils more rapidly compared with the other peptides within 72 h, and it is likely that peptide may have already formed protofibrils during measurements. Together, these results highlight that the soluble and insoluble forms of peptide assemblies display variable interactions with the proliferating fibroblasts.

**Discussion**

In the normal cornea, the full-length TGFβlp is processed into many shorter fragments by serine proteases [10,16]. The C-terminal truncated isoforms of TGFβlp are the most abundant in the cornea. Other fragments have molecular mass ranging from 29 to 64 kDa. Mutations in amino acid 124, which is located at the 1st FAS-1 domain, lead to a 1.5-3-fold increase in the level of full-length mutant protein in the affected cornea. In addition, R124C generates abnormal accumulation of many proteolytic products in the dystrophic cornea, which are absent from the control populations [10]. Thus, our first objective was to identify tryptic peptide fragments that were enriched in the amyloid fibrils isolated from dystrophic patients relative to normal control.

Spectral counts of the tryptic peptides derived from corneal deposits of three lattice dystrophy patients (two patients with a H626R mutation and one patient with a R124C mutation) showed enrichment of several peptides in the 4th FAS-1 domain that were either absent from control cornea or present in lower abundance. A 32-residue peptide (E611PVAEPDIMATNGVVHITNVLPQANRPQER642) was one of the most abundant peptide in the corneal deposits, indicating that this region could form the core of the amyloid fibrils. Mass spectrometric analysis of amyloid fibrils isolated from dystrophic mutants also showed higher abundance of E611R626 in the semi-tryptic fragments, suggesting the possible pathological association of this fragment in corneal dystrophies [15].

Interestingly, the majority of the amino acid alterations in this region affects the overall net charge of the protein (Supplementary Table S1). A significant number of mutations along this peptide region are associated with clinically relevant phenotypes that result in lattice corneal dystrophy. Since the amyloid-forming properties of any native or mutant peptides derived from this region have not been characterized before, we determined the aggregation properties of a 23-residue peptide (E611PVAEPDIMATNGVVHITNVLPQANRPQER633) encompassing this region (E614PVAEPDIMATNGVVHITNVLPQANRPQER642) and clinically relevant amino acid alterations (M619K, N622K, N622H, G623R and H626R) which decreased the overall net charge. It is likely that the accumulation of clinically relevant mutant peptides in this region could potentially act as amyloid fibril seeds, triggering the amyloid deposition in the affected patients. In support of this argument, bioinformatic analysis predicted that this region of TGFβlp has significant amyloidogenic potential as well. Therefore, we investigated the amyloidogenic potential, thermal stability and structure of the amyloid fibrils and their interaction with HCSF cells.

Formation of amyloid fibrils from native proteins is an intrinsic characteristic of the constituent amino acid sequence. Consistent with the prediction algorithms, we were able to show that the WT peptide (P1) clearly formed well-defined amyloid fibrils even in the absence of any external additives or other harsh biophysical conditions, e.g. sonication or organic solvents, demonstrating intrinsic amyloidogenic potential of the peptide.

The effect of substitution of positively charged residues had significant effects on the secondary structures and the rate of amyloid formation. It was clear that substitution of residues that possess a low intrinsic β-sheet propensity with residues that have higher propensity to form β-sheets caused a substantial increase in the rate of amyloid formation. This effect was clearly evident for P5 where substitution of glycine by arginine caused a considerable increase in the β-sheet propensity and a higher rate of amyloid formation. Glycine residue lacks side chains and thus confers higher conformational flexibility and low β-sheet propensity, whereas arginine residue could easily be accommodated in a β-sheet structure owing to its ability to form salt bridges with other negatively charged amino acid residues [43]. These results suggest that localized perturbation of the secondary
structure together with a decrease in the overall net charge and the nature of amino acid substitution would possibly accelerate the amyloid fibril formation.

TEM studies indicated that all the peptides formed unbranched amyloid assemblies with increase in incubation time. Morphologically, no apparent changes in the fibrillar assemblies were observed among the fibrils derived from various peptides. However, significant changes in the thermal denaturation profiles were observed as peptides P4 and P5 displayed reversible thermal stability. Their fibrillar structures remained intact and retained the β-sheet structures even after five heating/cooling cycles. The amyloid fibrils formed by other peptides dissociated irreversibly to the disordered monomeric forms. Morel et al. [35] showed that the amyloid fibrils formed by an N47A mutant of the α-spectrin SH3 domain dissociated upon heating to 90°C and reassociated with identical morphology when cooled to ~65°C. In our study, there was no dissociation of the fibrils or hysteresis observed for P5. Furthermore, we have shown that the thermal denaturation was not affected by the rate of heating, suggesting that the process is not kinetically controlled and appears to occur under equilibrium, confirming remarkable stability achieved by the amyloid fibrils from P5. It is likely that the presence of a multidentate guanidine side chain of the arginine residue could enhance the stability of the fibrils through hydrogen bonding and electrostatic interactions [44].

Solid-state NMR of the amyloid assembly formed by P5 gave insights into the secondary structure of the amyloid fibril formation. Since all the amino acids were not 13C- and 15N-labeled, a complete structure of the peptide could not be determined. Nevertheless, the results suggested that the P5 peptide formed stable and homogenous amyloid fibrils as indicated by the spread of DARR signals when compared with WT peptide. The secondary structures elucidated from the chemical shifts index were in agreement with the other biophysical experiments. However, the DARR spectrum of P1 appeared complex, less-resolved and heterogeneous, presumably due to population of other secondary structures or polymorphisms; therefore, we could not assign the spectra for secondary structure determination of the peptide. The above observations further support the excellent thermal stability of the amyloid fibrils derived from P5, whereas fibrils derived from P1 dissociated completely after heating.

Mutants associated with corneal dystrophies exhibit tissue damage in disease conditions. We have shown previously [27] that β-oligomeric aggregates of TGFBIp mutants display cytotoxicity in HCSF cells. Analysis of the cytotoxicity of peptides on corneal fibroblasts by label-free xCELLigence in both the amyloidogenic and the prefibrillar forms indicated considerable differences in the inhibition of cell proliferation between the two states, although no cytotoxic effect was observed. Interestingly, amyloid fibrils formed by mutant peptides displayed an identical increase in proliferation rates when compared with PBS, whereas amyloid fibrils from native peptide P1 showed a strong inhibitory effect on the proliferation rates. However, in the prefibrillar forms, all the mutant peptides displayed inhibition of cell proliferation when compared with the native P1 peptide, indicating that the oligomers of P1 did not affect the cell proliferation. Among the mutant peptides in their prefibrillar form, P6 displayed the strongest inhibitory effect. The identification of peptide fragments around the amino acid sequence of P6 and accumulation of oligomers in patients with the H626R phenotype could be responsible for the lesions observed in them.

The formation of amyloid fibrils and the phenotype of the disease depend on two main factors [45], the position of the mutation and the type of amino acid change compared with the native form. While the critical length of peptides/proteins that constitute amyloid deposits in dystrophic patients remains unknown, our results suggest that subtle decrease in the overall net charge, nature of amino acid substitution and the ability to form β-sheet structure could confer enhanced propensity to form amyloid aggregates or may accelerate the fibrillation of TGFBIp proteins/peptides.

Previous reports from Enghild and colleagues identified the presence of smaller peptide fragments of the protein accumulating in the cornea possibly due to aberrant proteolytic processing [15, 16, 18]. In particular, an increased abundance of peptide fragment L128–R172 in patients’ cornea carrying the R124C mutation was reported. Schmitt-Bernard et al. [46] studied the amyloidogenicity of a 22 amino acid-long L110-Q131 peptide which partly overlaps with the L128–R172 peptide fragment and showed that the mutant peptides accelerated the amyloid fibrillation in comparison with native peptides. Together with their studies, our studies shed light into the importance of investigating the amyloid propensity of pathologically relevant peptide fragments.

**Conclusion**

Using mass spectrometry, we identified a short peptide fragment in the 4th FAS-1 domain that was enriched in the amyloid deposits of the dystrophic patients with H626R and R124C mutations. The amyloid propensity of
this fragment along with five other clinically relevant substitutions, which decreased the overall net charge, was investigated by various biophysical studies. We showed that a decrease in the overall net charge of the peptide altered the secondary structure and accelerated the amyloid formation, depending on the nature of cationic residues and position of the substitution. In terms of morphology, the matured amyloid fibrils formed by the various peptides were indistinguishable, although considerable differences were observed in the thermal denaturation scans and ssNMR structure. The latter revealed the intact population β-induced; TGFBIp, transforming growth factor beta-induced protein; ThT, Thioflavin T; VT-CD, variable temperature-circular dichroism; WT, wild type.

Abbreviations

1D, one-dimensional; 2D, two-dimensional; ACN, acetonitrile; AUC, area under the curve; CD, circular dichroism; CP-MAS, cross-polarization magic-angle spinning; DARR, dipolar-assisted rotational resonance; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; emPAI, exponentially modiﬁed protein abundance index; FA, formic acid; FAS-1, fasciclin-like domain-1; HCSF, cultured human corneal ﬁbroblast; LCD, lattice corneal dystrophy; LCM, laser capture microdissection; LC-MS, liquid chromatography-mass spectrometry; MRW, mean residual weight; PBS, phosphate-buffered saline; RTCA, real-time cell analysis; ssNMR, solid-state nuclear magnetic resonance; TD, time domain; TEM, transmission electron microscopy; TGFBI, transforming growth factor beta; TGFBIp, transforming growth factor beta-induced protein; ThT, Thioflavin T; VT-CD, variable temperature-circular dichroism; WT, wild type.

Author Contribution


Acknowledgements

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

References


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