Available online at www.sciencedirect.com
SCIENCE DIRECT®
Molecular Genetics and Metabolism 82 (2004) 144–153
Molecular Genetics and Metabolism
www.elsevier.com/locate/ymgme

Potential modifier role of the R618Q variant of proα2(I)collagen in type I collagen fibrillogenesis: in vitro assembly analysis

Anthony N. Vomund,[a] Stephen R. Braddock,[b] Gary F. Krause,[c] and Charlotte L. Phillips[a,b,*]

[a] *Department of Biochemistry, University of Missouri-Columbia, Columbia, MO 65212, USA*
[b] *Department of Child Health, University of Missouri-Columbia, Columbia, MO 65212, USA*
[c] *Department of Agronomy, University of Missouri-Columbia, Columbia, MO 65212, USA*

Received 31 March 2004; accepted 31 March 2004
Available online 10 May 2004

Abstract

An arginine to glutamine substitution in the triple helix of proα2(I)collagen (R618Q) was first reported in a patient with a variant of Marfan syndrome and later identified in conjunction with a second mutation in a patient with osteogenesis imperfecta (OI). The presence of the R618Q proα2(I)collagen allele in unaffected or mildly affected family members suggests that the R618Q allele is either a non-affecting polymorphism or a potential genetic modifier. Conservation of arginine$_{618}$ across species and fibrillar collagen types suggests it is functionally significant. To investigate the functional significance of the R618Q proα2(I)collagen allele, we isolated type I collagen from cultured dermal fibroblasts of control and two unrelated individuals heterozygous for the R618Q proα2(I)collagen allele and evaluated helical stability and fibrillar assembly. Type I collagen thermal stability analyzed by protease susceptibility and CD spectroscopy demonstrated no statistical difference between control and R618Q containing collagen molecules. In vitro fibril assembly analyses demonstrated that R618Q containing collagen exhibits rapid fibrillar growth with minimal fibril nucleation phase. Further, electron microscopy demonstrated that the diameter of assembled R618Q containing collagen fibrils was approximately 20% of control collagen fibrils. These findings suggest the R618Q variant does not impact triple helical stability but has a role in collagen fibril assembly, supporting the hypothesis that the R618Q proα2(I)collagen variant is a modifier of connective tissue structure/function and is potentially involved in disease pathogenesis.

Keywords: Type I collagen; Fibrillogenesis; Connective tissue; Assembly; Proα2(I)collagen; Circular dichroism; Dermal fibroblasts; Modifier gene

Introduction

Type I collagen is the predominant structural protein in the body, providing structural integrity to the skin, internal organs, and blood vessels as well as support for the mineralization of bone and teeth [1,2]. Type I collagen is normally a heterotrimer consisting of two α1(I) collagen chains and one genetically distinct α2(I) collagen chain that wind together to form a helix. Individual collagen molecules self-assemble into collagen fibrils through entropy-driven associations [3–5] and the assembly of collagen fibrils is a multi-step process involving the nucleation of monomers, followed by longitudinal and lateral assembly into the fibril [6–11].

Abnormalities in type I collagen and its post-translational modifications are known to cause various connective tissue disorders including osteogenesis imperfecta (OI) and certain forms of Ehlers–Danlos syndrome (for review [1]). These collagen defects can have effects ranging from mild joint laxity to severe skeletal and cardiovascular phenotypes.

In 1981, Byers et al. [12] identified an abnormal α2(I) collagen chain in an individual presenting with a variant of Marfan syndrome, another connective tissue disorder

* Corresponding author. Fax: 1-573-884-4597.
E-mail address: phillipscl@missouri.edu (C.L. Phillips).

1096-7192/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymgme.2004.03.004

commonly associated with defects in the glycoprotein, fibrillin [13]. Hollister et al. [14] identified a second patient with classic Marfan syndrome and normal fibrillin by immunofluorescence that also exhibited abnormal α2(I) collagen by gel electrophoresis. In both cases, the probands were subsequently shown to be heterozygous for a single amino acid alteration, an arginine to glutamine substitution at position 618 (R618Q) in the triple helical domain of the proα2(I) collagen chain [15].

In addition, the R618Q substitution was observed on the same allele (cis) as a glycine to aspartate substitution (G421D) in yet another patient presenting with lethal OI [16]. The glycine substitution was surely causative of the OI, and thus the effects of the R618Q variant were difficult to assess. The presence of the R618Q variant among unaffected or mildly affected family members suggests that the R618Q allele is either a non-affecting polymorphism or a genetic modifier [12,15,16].

A review of the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) indicates that the arginine residue at position 618 of the collagen triple helix is invariant among all fibrillar collagens (types I, II, III, and V) analyzed to date and across species, including human, mouse, rat, chick, and bovine. This high degree of evolutionary conservation suggests a functional role for this residue in the type I collagen molecule, but the significance of arginine$_{618}$ is currently unknown. Yang et al. [17] have demonstrated by host–guest peptide analyses that arginines in the Y-position of the Gly–X–Y triplet can enhance helical stability similar to Gly–Pro–Hyp. The Y-position arginine$_{618}$ may play a role in the stability of the type I collagen triple helix and/or a role in electrostatic interactions essential to molecular recognition events that lead to the alignment of collagen molecules for fibril assembly [17,18].

To gain insight into the putative role of arginine$_{618}$, we have developed an approach to systematically evaluate both the triple helical stability of type I collagen, at the molecular level, and the ability of the R618Q containing molecules to form collagen fibrils in vitro. To evaluate helical stability, type I collagen produced by control fibroblasts and fibroblasts that express the R618Q variant were analyzed for protease resistance to thermal denaturation and by circular dichroism (CD) spectroscopy [19–21]. To evaluate fibril-forming properties of the respective type I collagens, we used an in vitro fibril assembly assay [6,22]. The R618Q collagen containing fibrils generated during the in vitro fibril assembly assay were prepared for electron microscopy (EM) and compared to similarly prepared control collagen. Our results support the hypothesis that arginine$_{618}$ influences the collagen fibril assembly process, but has little impact on triple helical stability.

Materials and methods

Cultured dermal fibroblast cell lines

Cultured dermal fibroblasts, from two unrelated individuals heterozygous for the R618Q proα2(I)collagen variant, 92-430 and 93-327, were generously provided by Peter Byers (University of Washington, Seattle, WA) for this study. Type I collagen synthesized by 92-430 and 93-327 exhibited similar altered migration by urea SDS–polyacrylamide gel electrophoresis as the index patient [12,15]. Control fibroblasts (GMO2674) were obtained from the NIGMS Human Genetic Mutant Cell Repository at the Coriell Institute for Medical Research (Camden, NJ). Dermal fibroblasts were maintained and cultured as previously described [15].

Protease resistance to thermal denaturation

Dermal fibroblasts were seeded at a density of 250,000/35 mm tissue culture dish, and the procollagens labeled with 2,3,4,5-[^{3}H]proline (101 Ci/mmol, Amersham–Pharmacia Biotech, Piscataway, NJ) in the presence of 50 μM ascorbic acid and harvested from the media as previously described by Phillips et al. [15]. [^{3}H]Proline-labeled collagen was aliquoted into nine tubes for the following temperature points: 25, 36–43 °C. Each tube was incubated for 5 min at the appropriate temperature. Trypsin and chymotrypsin were added to make a final concentration of 0.1 and 0.2 mg/ml, respectively, and the tube was then incubated at the indicated temperature for 2 min. The reaction was stopped by the addition of an equal volume of loading buffer (25% glycerol, 0.2 M Tris, pH 6.8, 2 M urea, and 2% SDS). The samples were then size fractionated on a 2 M urea, 8% polyacrylamide gel as previously described by Bonadio et al. [23]. The autoradiograph banding patterns of the α chains were assessed with the Quantity One software from Bio-Rad (Hercules, CA). Data points were taken in duplicate and averaged, and the experiments were repeated five times for each cell line. The statistical analysis model used was the test for parallelism using analysis of covariance while fitting a broken line to growth data [24].

In vitro fibril assembly assay

Confluent cultured dermal fibroblast cells were incubated in 0.5% calf serum in Dulbecco's modified Eagle's medium (DMEM) buffered to pH 7.4 with 24 mM sodium bicarbonate and 25 mM Hepes containing 100 μg/ml penicillin G sodium and 100 μg/ml streptomycin sulfate and supplemented with 100 μM ascorbic acid. The media were harvested daily and replaced with fresh media plus ascorbate. These treatments were continued for 4 days.

The collagen was purified from the media via an ethanol precipitation using 2 volumes of 95% ethanol to 1 volume of media [25]. Precipitation was allowed to occur overnight at 4 °C. Protein was collected by centrifugation for 30 min at 10,000*g*. The pellet was redissolved in 0.5 N acetic acid overnight at 4 °C. The preparation was centrifuged for 5 min at 10,000*g* with only the soluble material (supernatant) kept for further use. The soluble protein was dialyzed overnight with a 400-fold volume excess of 0.5 N acetic acid (3 changes), followed by pepsin digestion (50 μg/ml) on ice overnight to generate a homogeneous population of type I collagen monomers [26]. These samples were lyophilized and then dissolved in 5 mM acetic acid to a final concentration of 2.0 mg/ml, as determined with a modified Bio-Rad protein assay [27] and the hydroxyproline assay [28]. To initiate the in vitro fibrillar assembly assay, the collagen stock was diluted with 5 mM acetic acid to a volume of 500 μl and was then added to 500 μl of 30 mM NaP_i/100 mM NaCl, pH 7.0 (PS Buffer), to achieve final concentrations of 0.25, 0.40, 0.50, 0.60, 0.75, or 1.00 mg/ml. The pH was adjusted to 7.0 with NaOH, if needed. The turbidity of the solution was then analyzed in a spectrophotometer at 313 nm at 30 °C for 6 h at 5 min intervals.

Circular dichroism spectroscopy

During in vitro assembly collagen fibrils precipitate. The precipitate (fibrils) was collected by centrifugation (5 min at 10,000 rpm) and solubilized in 0.5% phosphoric acid (~pH 3.5) to a concentration of 1.0 mg/ml. This material was examined by circular dichroism (CD) spectroscopy using an Aviv 62DS CD spectrophotometer (Aviv, Lakewood, NJ) with a temperature controlled Peltier accessory. Preliminary scans of the sample were taken over a wavelength spectrum of 200–260 nm to determine sample readiness. Readings were obtained at 0.5 nm intervals, with 10 s signal. Thermal stability was determined by monitoring the ellipticity at 221 nm as the temperature was increased from 30 to 45 °C. Data points were obtained at 0.1 °C increments, with 20 s signal averaging.

Electron microscopy

After the turbidity maximum was reached, a portion of the incubated sample was retrieved. A drop was placed on a carbon film supported on a 300 mesh copper grid and after 90 s was drained slowly with filter paper. A drop of 1.3% phosphotungstic acid, pH 7.3, was then added and incubated at room temperature for 10 min [6]. The grid was slowly drained, then air-dried. The preparation was examined in a JEM 1200 EX electron microscope (JEOL, Ltd., Tokyo, Japan) operated at 1200 kV and fitted with a liquid nitrogen cooled decontamination device. Micrographs were taken at 40,000× magnification.

Results

Molecular stability: enzymatic susceptibility and CD spectroscopy

[^{3}H]Proline-labeled collagen synthesized by fibroblast cell cultures of control and R618Q containing cell lines (92-430 and 93-327) underwent an enzymatic assay for collagen triple helical integrity. In this assay, collagen is subject to digestion by trypsin/chymotrypsin as a function of temperature in order to assess triple helical integrity [21]. The data were analyzed via the test for parallelism using analysis of covariance while fitting a broken line to growth data [24]. This model gives us a value for the helical melting temperature and compares the rate of degradation of the individual collagen chains prior to melting. This type of analysis evaluates the data in three different areas (Fig. 1). The first region of analysis is the horizontal component of the thermal denaturation curve leading up to the breakpoint (from 25 to

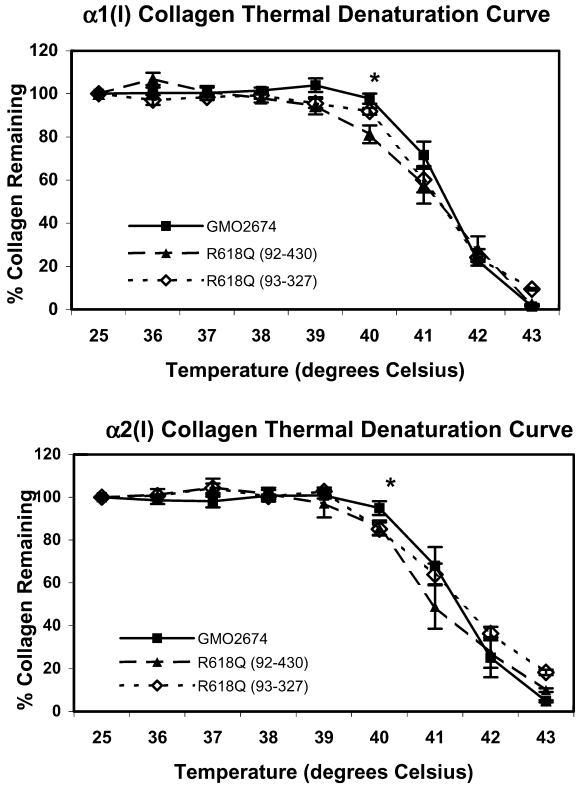

Fig. 1. Trypsin/chymotrypsin thermal stability assay suggests triple helical stability of control and R618Q containing collagen is similar ($n = 5$). The thermal denaturation curves of α1(I) collagen chain (top) and α2(I) collagen chains (bottom) were analyzed by assessing three regions of each curve: (1) the slope of the line before collagen unwinds and is degraded (36–40 °C); (2) the point of the curve at which the collagen starts to unwind and degrade (~40 °C); and (3) the downward sloping portion of the curve describing the rate of collagen degradation by trypsin and chymotrypsin (41–43 °C). Asterisk indicates significant difference in resistance to degradation from control at the individual temperature ($p < 0.05$).

~40 °C). There was no statistical difference between the slope of this line in either the α1(I) or α2(I) collagen chains from control or the R618Q containing cell lines. An important part of the denaturation curve is the breakpoint of the curve (~40 °C, Fig. 1). It is important to note that the standard melting point of type I collagen with this assay is approximately 41 °C [23]. The breakpoint analysis fits a smooth curve to the data as a whole and therefore does not precisely define the melting point, but approximates it. The breakpoints of the thermal denaturation curves are similar in all cell lines for both the α1(I) and α2(I) collagen chains and show no statistically significant differences (Fig. 1). Finally, the third region of the thermal denaturation curve analyzed is the downward slope of the curve, which describes the rate of degradation of the collagen chains (Fig. 1). The slopes of these curves were compared and tested for parallelism. Though the α2(I) chain data exhibited a trend toward a steeper slope for the denaturation curves of R618Q containing collagens when compared to control collagen, this was not statistically significant and suggested that the rates of degradation for the R618Q containing collagen are similar to control collagen. Based on the standard error associated with the slope data, the power of this study indicates that these experiments would need to be repeated 12 times to see a significant (20%) difference in the slopes of R618Q containing collagens as compared to control [29]. Analysis of covariance at individual temperature points along the denaturation curve shows that the relative amount of α1(I) and α2(I) collagen chains resistant to degradation at each temperature point is not statistically different between R618Q containing collagen and control collagen with the exception of 40 °C. At 40 °C the R618Q containing cell line, 92-430, α1(I) and α2(I) collagen chains were significantly less resistant than control α1(I) and α2(I) collagen chains ($p = 0.0005$ and $p = 0.045$, respectively; $n = 5$), and the R618Q containing cell line, 93-327, α2(I) collagen chain was significantly less resistant than the control α2(I) collagen chain ($p = 0.0349$; $n = 5$). This decrease in thermal stability of the R618Q containing collagen at 40 °C is consistent with the concept that Y-position arginines contribute to helical stability and the loss of a Y-position arginine may decrease stability [17]. Though the area around the breakpoint shows some peculiar traits, this phenomenon does not seem to affect the overall type I collagen helical stability. Overall, these results demonstrate that R618Q containing collagens exhibit similar thermal denaturation characteristics as control collagen and suggest that R618Q does not appear to greatly affect helix stability.

Type I collagen from the control cell line and the R618Q containing cell lines, 92-430 and 93-327, were also examined by CD spectroscopy (Fig. 2). The harvested collagen gave characteristic CD spectra with a single peak with a maxima at 221 nm indicating that collagen was present and that there was little if any protein from contaminating sources. Sample purity was also confirmed by SDS–PAGE for all samples. Monitoring the ellipticity at 221 nm as a function of temperature indicated that the collagen samples demonstrate similar thermal stability (Fig. 2). Melting temperatures were determined by choosing the point along the curve where the first derivative is equal to zero. In all cases, melting temperatures were around 36.5 °C [control $T_m = 36.42 \pm 0.24$ °C (means ± SE; $n = 4$); 92-430 $T_m = 36.72 \pm 0.11$ °C ($n = 4$); 93-327 $T_m = 36.70 \pm 0.17$ °C ($n = 3$)].

In vitro fibril assembly

The assembly of type I collagen fibrils from pepsin-treated collagen synthesized by either control fibroblasts or R618Q fibroblast cell lines was analyzed by monitoring the turbidity of the collagen solution as a function of time [19,20]. As collagen forms fibrils in solution, the aggregates no longer remain soluble and form a light precipitate in solution. This property is easily measured as a change in optical density at 313 nm (Fig. 3). The slope of the assembly curve during initial linear growth is then plotted versus concentration of collagen to generate rate curves (Fig. 3). The rate of fibril formation appears altered in the R618Q collagen containing cell lines as compared to controls; the R618Q containing collagens formed fibrils more rapidly. Fibrils formed from R618Q collagens appeared to nucleate very rapidly upon addition of PS buffer; there was no noticeable lag period. Control collagen though shows a lag period followed by a period of sustained fibril formation. At the lowest concentration (250 μg/ml) the rates of fibril formation were similar between control type I collagen and the R618Q containing collagens [control: Δoptical density/s $= 12.6 \times 10^{-6}$; 92-430: Δoptical density/s $= 8.8 \times 10^{-6}$; and 93-327: Δoptical density/s $= 6.4 \times 10^{-6}$]. However, as the concentration of collagen increased the rates of formation diverged significantly (Fig. 3); for example the following rates were observed when fibril assembly was performed with a concentration of 400 μg/ml of type I collagen, control: Δoptical density/s $= 3.6 \times 10^{-5}$; 92-430: Δoptical density/s $= 9.8 \times 10^{-5}$; and 93-327: Δoptical density/s $= 8.2 \times 10^{-5}$. As a result, the shapes of the fibril formation curves at all concentrations were statistically different, resulting in rate curves that suggest a fundamental difference in the associative properties of R618Q collagen.

In order to assess the quality of fibrils generated by the in vitro fibril assembly reaction, the collagen fibrils were pelleted and collected for electron microscopy (EM). The EM results indicate that not only is the rate of assembly altered for the R618Q collagen, but the resulting fibril diameter is altered as well (Fig. 4). R618Q containing collagen fibrils are approximately 20% of the diameter of control fibrils (Fig. 5). By calculating the

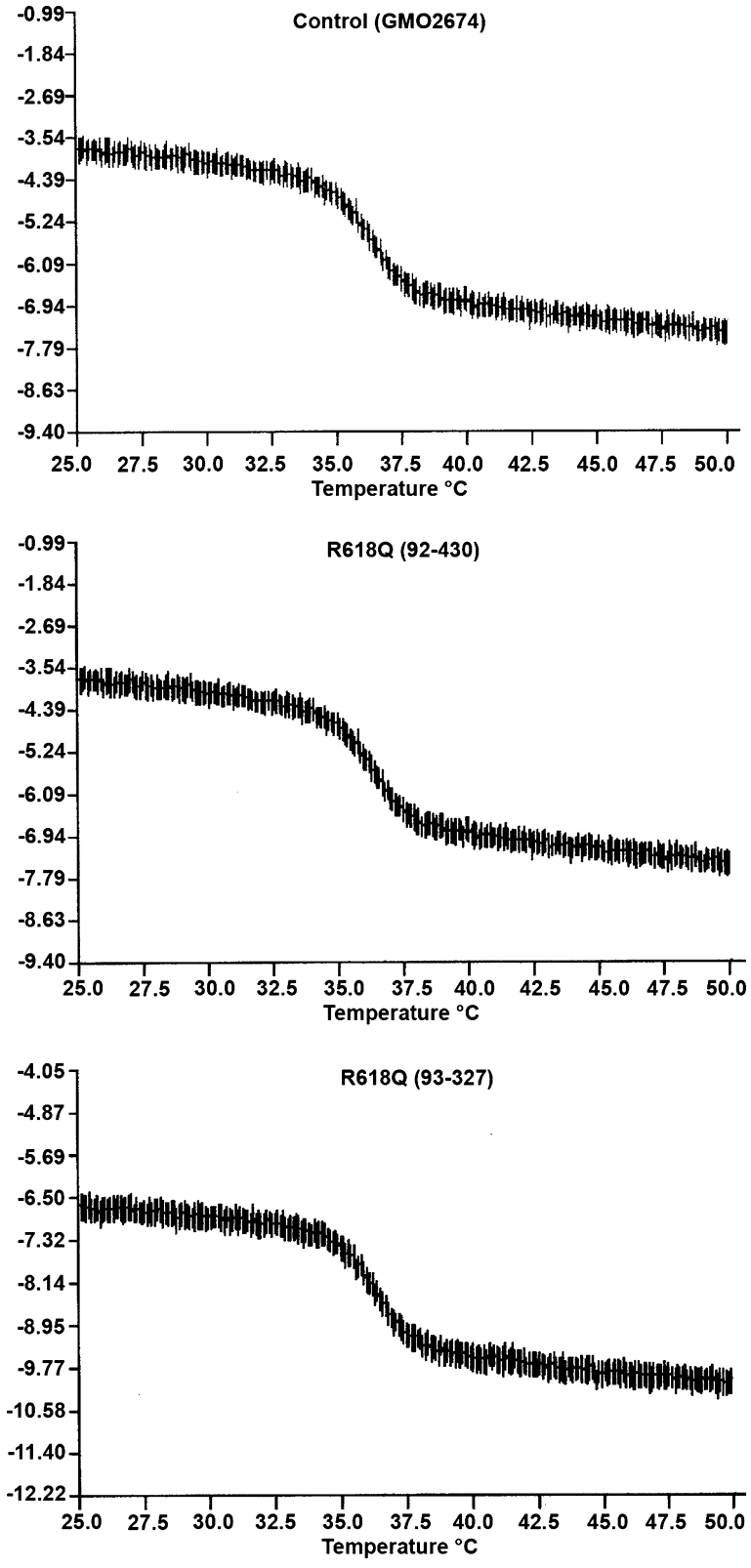

Fig. 2. Thermal denaturation circular dichroism scans of type I collagen harvested from fibroblast cell lines suggest the triple helical stability of control and R618Q containing collagen is similar. Shown are thermal scans representing collagen from human control cells (top), 92-430 (middle), and 93-327 (bottom). Thermal scans were performed using an Aviv CD spectrometer fitted with a temperature controlled Peltier apparatus. The samples were monitored for changes in ellipticity every 0.1 °C with readings averaged over 20 s. Error bars for individual data points are indicated.

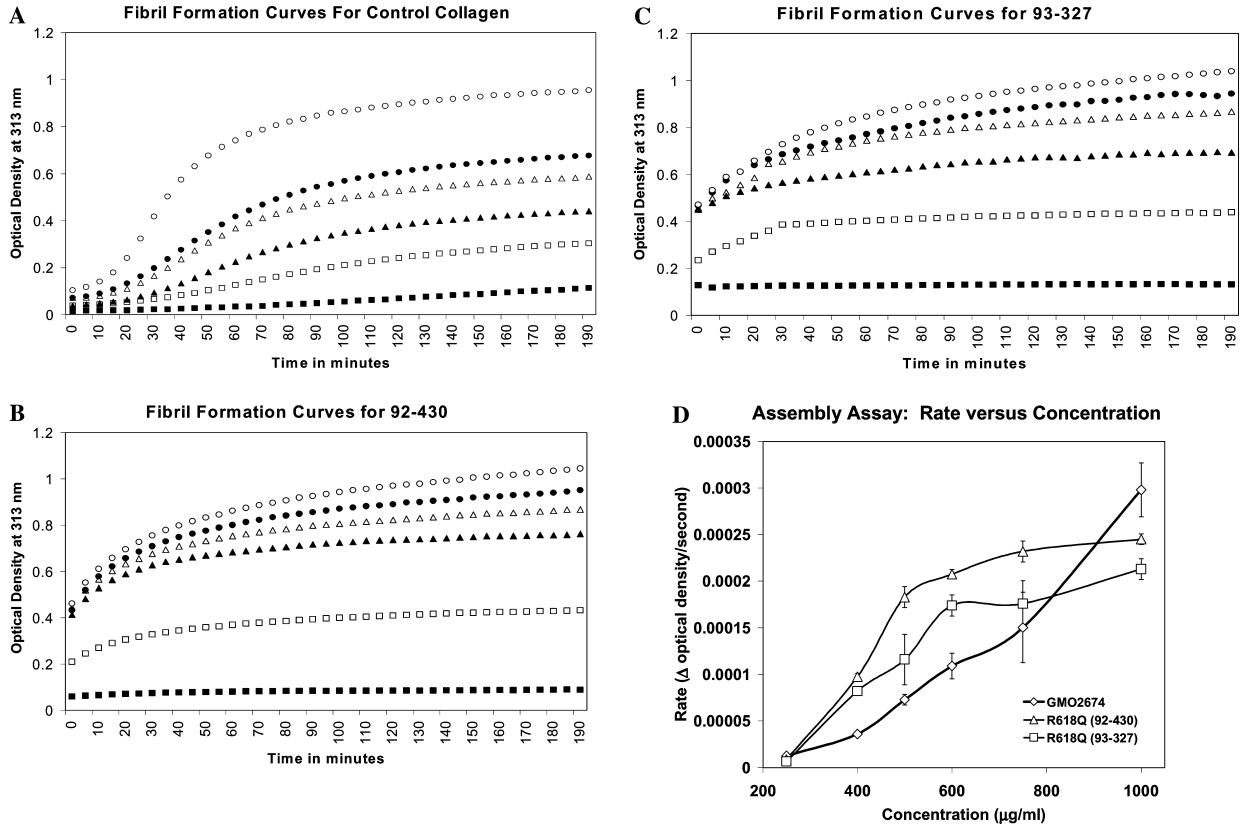

Fig. 3. The rate of fibril formation is altered in R618Q containing collagen, which exhibit more rapid fibril formation than control collagen during in vitro fibrillar assembly analyses. Control (A) and R618Q containing collagen (92-430, B; 93-327, C) in vitro assembled fibrils were generated by mixing the collagen with an equal volume of PS buffer and fibril formation was monitored by optical density at 313 nm every 300 s [final collagen concentrations: 1.00 mg/ml (open circle), 0.75 mg/ml (filled circle), 0.60 mg/ml (open triangle), 0.50 mg/ml (filled triangle), 0.40 mg/ml (open square), and 0.25 mg/ml (filled square); $n = 2$]. To assess if the rate of fibril formation was altered in the R618Q containing collagens, the rate of fibril formation was determined from the slopes of the early linear growth phase of the initial fibril formation curves (A–C). (D) Comparison of the rates of collagen fibril assembly of the control (open diamond) and R618Q containing collagens [92-430 (open triangle); 93-327 (open square)] relative to the concentration of collagen demonstrated that R618Q containing collagen have a distinctly different shape than control type I collagen.

cross-sectional area of the assembled fibril based on the diameter (67 nm for control collagen, 17 nm for R618Q collagen) versus that of the triple helix (1.5 nm in cross-section), these data suggest that approximately 1600 collagen triple helices participate in the formation of control fibrils, while only ~100 molecules of the R618Q collagen triple helix aggregate in cross-section. The diameter of our assembled normal control collagen fibrils is consistent with the normal range for type I collagen fibrils (between 50 and 70 nm depending on the tissue) associated with connective tissues [30].

The R618Q containing fibrils were very fine and the resolution of the electron micrographs was not sufficient to confirm periodicity of banding. In addition, even though the R618Q containing collagen fibrils produced thin fibrils, their appearance does not correspond to other previously described collagen aggregates such as segment long-spacing (SLS) and fibrous long-spacing (FLS) collagen fragments [31,32].

Discussion

Type I collagen is a protein that is strongly conserved throughout evolution with a high degree of invariant amino acids [33,34]. The functional necessity of a glycine residue for every third amino acid in the triple helical domain has clearly been established by the number of unique glycine substitutions in both α1(I) and α2(I) collagen chains responsible for OI [35,36]. The strong evolutionary conservation of arginine$_{618}$ among fibrillar collagens and across species suggests a function for this residue that has yet to be determined.

Preliminary models indicate that arginine$_{618}$ is located on the outer surface of the collagen triple helix with the charged side chain protruding from the surface of the molecule [37]. Therefore, one would not hypothesize a major role in the helical stability of the type I collagen for arginine$_{618}$, but rather a role in protein–protein interactions and/or fibrillar assembly. However, there is also evidence that arginines in the Y-position of the Gly–X–Y

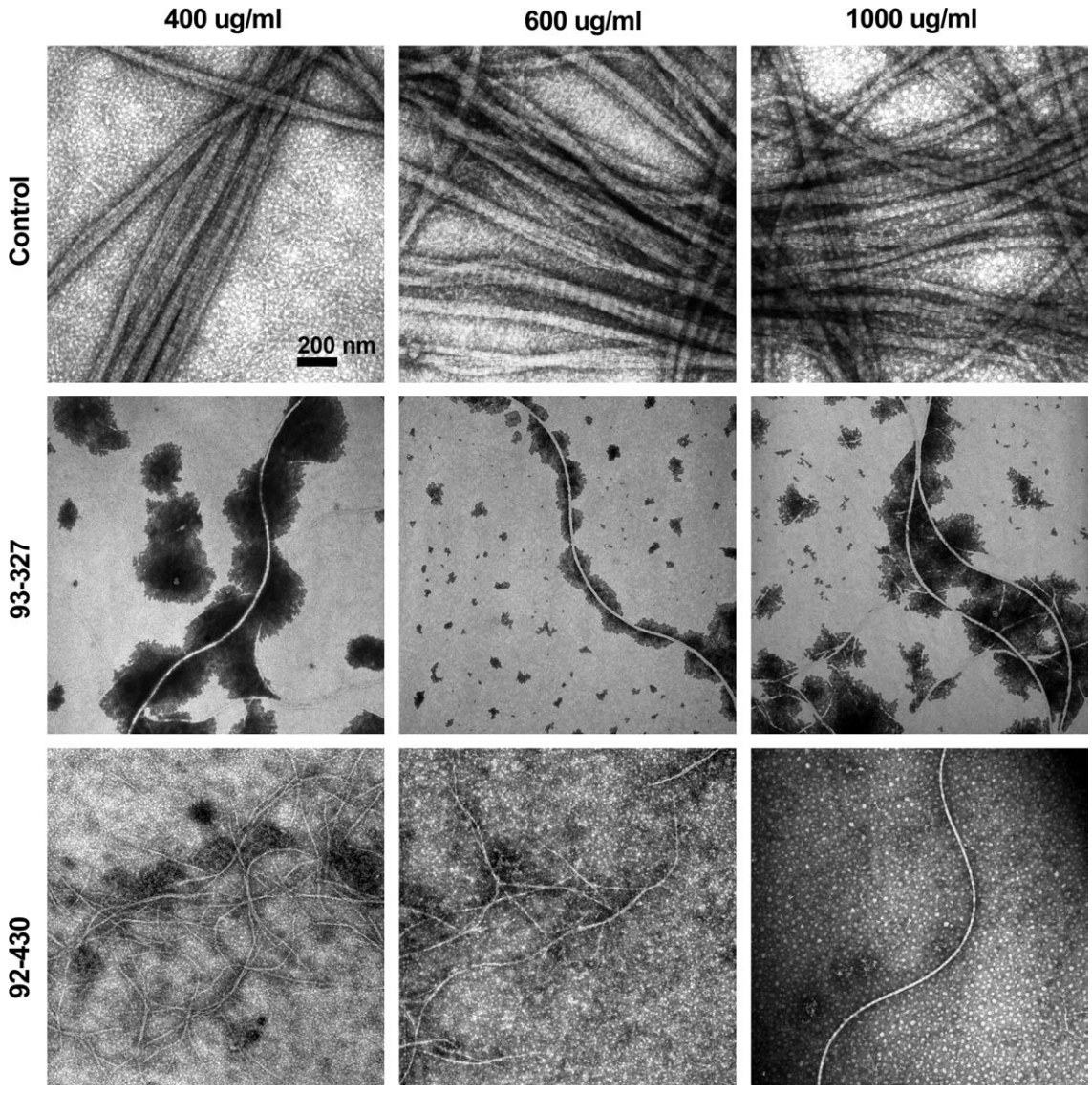

Fig. 4. Transmission electron micrographs demonstrate that in vitro assembled R618Q containing collagen fibrils are markedly thinner than control fibrils. Collagen fibrils from control (top) and R618Q [93-327 (middle); 92-430 (bottom)] were harvested and prepared as described. Micrographs of control collagen exhibit characteristic size and periodicity, whereas the R618Q containing collagen fibrils are markedly thinner. In vitro assembly analyses were performed with the indicated concentrations of collagen. Bar = 200 nm (top left).

triplet enhance helical stability [17]. To test these hypotheses we evaluated the triple helical stability of the R618Q containing collagen and its ability to assemble in vitro into collagen fibrils. We utilized two methods for assessing helix stability, and neither method demonstrated a significant difference in overall helical stability between R618Q containing collagen and control collagen, suggesting that triple helical stability is not significantly influenced by the substitution of a glutamine for arginine$_{618}$. However, since the 92-430 and 93-327 cell lines are heterozygous for the R618Q variant, one cannot rule out the possibility that the presence of normal proα2(I) collagen chains from the normal COL1A2 allele may dilute or mask some of the R618Q response.

Based on current understanding, the information necessary for collagen monomers to form native-like D-staggered fibrils is encoded by the triple helical domain and that the role of the telopeptides is to accelerate collagen fibril nucleation [38,39]. To test if arginine$_{618}$ has a potential role in the alignment of collagen molecules and/or fibril formation we chose to use pepsin-treated collagen. Pepsin treatment allowed (1) for efficient preparation of a homogeneous type I collagen population from tissue culture media and (2) for direct evaluation of the impact of arginine$_{618}$ on collagen–collagen interactions in fibril assembly [38,39]. Kuznetsova and Leikin [38] demonstrated that with pepsin treatment the kinetics of collagen fibril assembly is slowed, but the information essential for monomer association, fibrillar

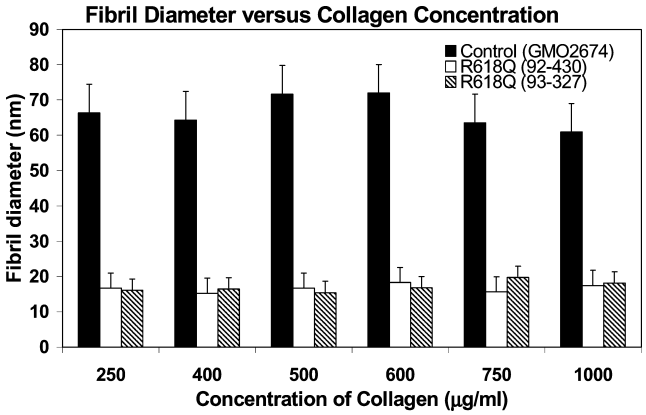

Fig. 5. In vitro assembled R618Q collagen fibrils have significantly reduced fibril diameter. Analysis of collagen fibril diameter of in vitro assembled collagen fibrils from control (solid bar), and the R618Q [92-430 (open bar), 93-327 (diagonal bar)] fibroblasts [$n = 3$; for each experiment: three unique fibrils per collagen concentration were measured; 10 measurements per fibril]. Within a particular collagen type, the diameter of assembled fibrils did not differ regardless of the initiating collagen concentration. However, the diameter of the R618Q containing collagen fibrils was 20–25% of that of control collagen fibrils.

elongation, lateral growth, and the ability to form native-like D-staggered fibers is encoded by the triple helical region. To evaluate the fibril-forming properties of the R618Q containing collagen we used an in vitro fibrillar assembly assay and the R618Q containing type I collagen demonstrated altered fibril formation in vitro as compared to control type I collagen. The R618Q containing collagen fibrils assembled more rapidly than control collagen fibrils, and when examined by electron microscopy, the R618Q containing fibrils were much thinner with a decreased diameter as compared to control collagen fibrils assembled under the same conditions. These changes to fibril formation and diameter cannot be accounted for as simply a consequence of reduced amounts of available collagen or the lack of the R618Q containing α2(I) collagen chains participating in fibril formation. If this were the case, the normal allele in these cell lines would still produce normal functional α2(I) collagen chains and the non-functioning variant allele would only serve to decrease the total amount of collagen available for fibril formation by as much as 50%, simulating the response of reduced levels of control collagen. However, even at 50% lower concentrations, the control collagen fibril assembly curves do not resemble the phenomena observed with the R618Q containing collagens.

One hypothesis is that the positively charged arginine$_{618}$ residue is vital for alignment with a hydroxylated residue of an adjacent triple helix, thus establishing the proper orientation for collagen alignment, lateral growth and/or cross-link formation. The charge difference resulting from the substitution of glutamine for arginine$_{618}$ in half the collagen molecules may disrupt this alignment and prohibit assembly beyond a certain size. The R618Q variant may demonstrate a preference for rapid monomer association at the expense of proper alignment that propagates fibril expansion. Perhaps even, a small, localized abnormality in the triple helix is sufficient to alter the shape of the helix in a region critical for molecular recognition, lateral growth or cross-link formation.

In vitro studies of the self-assembly of DNA indicate that it is based on a delicate balance of attractive and repulsive forces between helices [40]. Similar studies with helical proteins, particularly collagen, have demonstrated similar forces acting on the protein [41]. The repulsive forces seem to center around the energetically and entropically important release of water around surface groups on the protein as protein molecules associate. Leikin et al. [41] conclude that as polar molecules are brought together, they repel exponentially as a result of forcing water molecules to leave the apposing hydrophilic surfaces. Either electrostatic polarization or hydrogen bonding could cause this exponential perturbation in water structure surrounding polar or charged groups. Therefore, alterations in charge or hydrogen bonding patterns may have a lasting effect on collagen ultrastructure.

Another potential explanation for the thinner R618Q containing collagen fibrils may lie in the observation that the R618Q containing collagen appears post-translationally overmodified when evaluated by urea/SDS–polyacrylamide gel electrophoresis [12,15]. Type I collagen fibril assembly experiments by Torre-Blanco et al. [42] demonstrated that type I collagen containing increased hydroxylysine content formed fibrils with significantly smaller diameters than normal type I collagen. Perhaps the thinner fibril formation of the R618Q containing collagen is a reflection of alterations in post-translational processing.

The effects of the R618Q variant on in vivo structure and function are unknown, and may involve alternative mechanisms reflecting the consequences of interactions with other components of the extracellular matrix. Charged amino acid residues, particularly arginines, have also been postulated to be involved with molecular chaperone recognition and/or in recruitment of other extracellular matrix proteins, which are in turn responsible for the alignment of collagen during fibrillogenesis [18,43,44]. Sequence analysis reveals that arginine$_{618}$ is located along what may be a decorin-binding site on the collagen triple helix [45]. In vitro, the addition of decorin to fibrillogenesis assays has impeded the formation of collagen fibrils while increasing the thermal stability of the fibril [46,47].

Recently, Di Lullo et al. [48] generated a map of ligand-binding sites and disease-related mutations for type I collagen. There are four non-OI mutations reported in the triple helical region of type I collagen (one of which is the original R618Q variant reported by Phillips et al. [15]), that are associated with coronary artery disease, Marfan syndrome, connective tissue

weakness, and Ehlers–Danlos syndrome. As the authors noted, these four mutations cluster together with certain osteoporosis and osteopaenia mutations in two zones of the fibril "D" pattern. Consequently, these four non-OI mutations may directly interfere with one or more classes of type I collagen interactions leading to a relatively mild connective tissue phenotype. Though the actual mutations themselves may not cause a direct affect on type I collagen, these variants may indeed modify the activity of other matrix proteins necessary for collagen alignment, fibril assembly, or binding to other matrix molecules.

The R618Q variant has not been found in any other Marfan syndrome patients beside the original two described [12,14,15]. However, one OI patient had the variant on the same allele as a glycine mutation [16]. The contribution of the R618Q variant in this case is masked by the severity of the glycine substitution, which almost certainly is responsible for the OI phenotype. The presence of the R618Q variant among unaffected or mildly affected family members suggests that the R618Q allele is either a non-affecting polymorphism or a genetic modifier. The effect of the R618Q allele on in vitro assembly more strongly favors a role for the R618Q variant as a modifier of collagen expression and fibril assembly. Currently, we hypothesize that the R618Q variant of type I collagen may belong to a family of modifier alleles that impact connective tissue structure and function and may significantly influence the pathogenesis of acquired and inherited connective tissue disorders.

Acknowledgments

The authors thank Dr. Peter Byers (University of Washington) for generously providing the R618Q variant fibroblast cell lines. The authors sincerely thank the National Marfan Foundation and the American Heart Association Heartland Affiliate (9910193Z, A.N. Vomund, pre-doctoral fellow) for their support of this research.

References

[1] P.H. Byers, Disorders of collagen biosynthesis and structure, in: C. Scriver, A. Beaudet, W. Sly, D. Valle (Eds.), The Metabolic and Molecular Basis of Inherited Disease, eighth ed., McGraw-Hill, New York, 2001, pp. 5241–5285.

[2] C. Kielty, I. Hopkinson, M. Grant, The collagen family: structure, assembly, and organization in the extracellular matrix, in: P.M. Royce, B. Steinmann (Eds.), Connective Tissue and its Heritable Disorders, Wiley-Liss, New York, 1993, pp. 103–147.

[3] A. Cooper, Thermodynamic studies of the assembly in vitro of native collagen fibrils, Biochem. J. 118 (1970) 355–365.

[4] K.E. Kadler, Y. Hojima, D.J. Prockop, Assembly of collagen fibrils de novo by cleavage of the type I pC-collagen with procollagen C-proteinase, J. Biol. Chem. 262 (1987) 15696–15701.

[5] N. Kuznetsova, S.L. Chi, S. Leikin, Sugars and polyols inhibit fibrillogenesis of type I collagen by disrupting hydrogen-bonded water bridges between the helices, Biochemistry 37 (1998) 11888–11895.

[6] B.R. Williams, R.A. Gelman, D.C. Poppke, K.A. Piez, Collagen fibril formation. Optimal in vitro conditions and preliminary kinetic results, J. Biol. Chem. 253 (1978) 6578–6585.

[7] R.A. Gelman, B.R. Williams, K.A. Piez, Collagen fibril formation. Evidence for a multistep process, J. Biol. Chem. 254 (1979) 180–186.

[8] R.A. Gelman, K.A. Piez, Collagen fibril formation in vitro. A quasielastic light-scattering study of early stages, J. Biol. Chem. 255 (1980) 8098–8102.

[9] D.F. Holmes, M.J. Capaldi, J.A. Chapman, Reconstitution of collagen fibrils in vitro: the assembly process depends on the initiating procedure, Int. J. Biol. Macromol. 8 (1986) 161–167.

[10] K.E. Kadler, Y. Hojima, D.J. Prockop, Collagen fibrils in vitro grow from pointed tips in the C- to N-terminal direction, Biochem. J. 268 (1990) 339–343.

[11] M. Gale, M.S. Pollanan, P. Markiewicz, M.C. Goh, Sequential assembly of collagen revealed by atomic force microscopy, Biophys. J. 68 (1995) 2124–2128.

[12] P.H. Byers, R.C. Siegel, K.E. Peterson, D.W. Rowe, K.A. Holbrook, L.T. Smith, Y.-H. Chang, J.C.C. Fu, Marfan syndrome: abnormal alpha 2 chain in type I collagen, Proc. Natl. Acad. Sci. USA 78 (1981) 7745–7749.

[13] R.E. Pyeritz, Marfan syndrome and other disorders of fibrillin, in: D. Rimoin, J. Connor, R. Pyeritz (Eds.), Principles and Practice in Medical Genetics, Churchill-Livingston, New York, 1997, pp. 1027–1081.

[14] D.W. Hollister, M. Godfrey, L.Y. Sakai, R.E. Pyeritz, Immunohistologic abnormalities of the microfibrillar–fiber system in the Marfan syndrome, N. Engl. J. Med. 323 (1990) 152–159.

[15] C.L. Phillips, A.W. Shrago-Howe, S.R. Pinnell, R.J. Wenstrup, A substitution at a non-glycine position in the triple-helical domain of pro alpha 2(I) collagen chains present in an individual with a variant of the Marfan syndrome, J. Clin. Invest. 86 (1990) 1723–1728.

[16] A. Forlino, D.R. Keene, K. Schmidt, J.C. Marini, An alpha2(I) glycine to aspartate substitution is responsible for the presence of a kink in type I collagen in a lethal case of osteogenesis imperfecta, Matrix Biol. 17 (1998) 575–584.

[17] W. Yang, V.C. Chan, A. Kirkpatrick, J.A. Ramshaw, B. Brodsky, Gly-Pro-Arg confers stability similar to Gly-Pro-Hyp in the collagen triple-helix of host–guest peptides, J. Biol. Chem. 272 (1997) 28837–28840.

[18] M. Tasab, L. Jenkinson, N.J. Bulleid, Sequence-specific recognition of collagen triple helices by the collagen-specific molecular chaperone HSP47, J. Biol. Chem. 277 (2002) 35007–35012.

[19] D.J. McBride, K.E. Kadler, Y. Hojima, D.J. Prockop, Self-assembly into fibrils of a homotrimer of type I collagen, Matrix 12 (1992) 256–263.

[20] D.J. McBride, V. Choe, R.J. Shapiro, B. Brodsky, Altered collagen structure in mouse tail tendon lacking the alpha 2(I) chain, J. Mol. Biol. 270 (1997) 275–284.

[21] P. Bruckner, D.J. Prockop, Proteolytic enzymes as probes for the triple-helical conformation of procollagen, Anal. Biochem. 110 (1981) 360–368.

[22] G.C. Na, L.J. Phillips, E.I. Freire, In vitro collagen fibril assembly: thermodynamic studies, Biochemistry 28 (1989) 7153–7161.

[23] J.E. Bonadio, K.A. Holbrook, J. Jacob, P.H. Byers, Altered triple helical structure of type I procollagen in lethal perinatal osteogenesis imperfecta, J. Biol. Chem. 260 (1985) 1734–1742.

[24] K.R. Robbins, A method, SAS program, and example for fitting the broken line to growth data, Research Report 86-09, The University of Tennessee Agriculture Experiment Station, Knoxville, Tennessee, 1986.

[25] M.K. Wirtz, R.W. Glanville, B. Steinmann, V.H. Rao, D.W. Hollister, Ehlers–Danlos syndrome type VIIB. Deletion of 18 amino acids comprising the N-telopeptide region of a pro-alpha 2(I) chain, J. Biol. Chem. 262 (1987) 16376–16385.
[26] R.J. Wenstrup, A.W. Shrago-Howe, L.W. Lever, C.L. Phillips, P.H. Byers, D.H. Cohn, The effects of different cysteine for glycine substitutions within alpha 2(I) chains. Evidence of distinct structural domains within the type I collagen triple helix, J. Biol. Chem. 266 (1991) 2590–2594.
[27] R.C. Duhamel, E. Meezan, K. Brendel, The addition of SDS to the Bradford dye-binding protein assay, a modification with increased sensitivity to collagen, J. Biochem. Biophys. Methods 5 (1981) 67–74.
[28] H. Stegemann, K. Stadler, Clin. Chim. Acta 18 (1967) 267–273.
[29] B.S. Everitt, Advanced Methods of Data Exploration and Modeling, Heinemann Educational Books, Exeter, NH, 1983.
[30] B. Eyden, M. Tzaphlidou, Structural variations of collagen in normal and pathological tissues: role of electron microscopy, Micron 32 (2001) 287–300.
[31] K. Kühn, Segment-long-spacing crystallites, a powerful tool in collagen research, Collagen Rel. Res. 2 (1982) 61–80.
[32] J.H. Highberger, J. Gross, F.O. Schmitt, Electron-microscope observations of certain fibrous structures obtained from connective-tissue extracts, J. Am. Chem. Soc. 72 (1950) 3321–3322.
[33] K. Kühn, The classical collagens: types I, II, and III, in: R. Mayne, R.E. Burgeson (Eds.), Structure and Function of Collagen Types, Academic Press, Orlando, 1987, pp. 1–52.
[34] B.S. Emanuel, L.A. Cannizzaro, J.M. Seyer, J.C. Myers, Human alpha 1(III) and alpha 2(V) procollagen genes are located on the long arm of chromosome 2, Proc. Natl. Acad. Sci. USA 82 (1985) 3385–3389.
[35] R. Dalgleish, The human type I collagen mutation database, Nucleic Acids Res. 25 (1997) 181–187.
[36] R. Dalgleish, The Human Collagen Mutation Database 1998, Nucleic Acids Res. 26 (1998) 253–255.
[37] J.E. Scott, Proteodermatan and proteokeratan sulfate (decorin, lumican/fibromodulin) proteins are horseshoe shaped. Implications for their interactions with collagen, Biochemistry 35 (1996) 8795–8799.
[38] N. Kuznetsova, S. Leikin, Does the triple helical domain of type I collagen encode molecular recognition and fiber assembly while telopeptides serve as catalytic domains? Effect of proteolytic cleavage on fibrillogenesis and on collagen–collagen interaction in fibers, J. Biol. Chem. 274 (1999) 36083–36088.
[39] K. Sato, T. Ebihara, E. Adachi, S. Kawashima, S. Hattori, S. Irie, Possible involvement of aminotelopeptide in self-assembly and thermal stability of collagen I as revealed by its removal with proteases, J. Biol. Chem. 275 (2000) 25870–25875.
[40] D.C. Rau, B.K. Lee, V.A. Parsegian, Measurement of the repulsive force between polyelectrolyte molecules in ionic solution: hydration forces between parallel DNA double helices, Proc. Natl. Acad. Sci. USA 81 (1984) 2621–2625.
[41] S. Leikin, D.C. Rau, V.A. Parsegian, Direct measurement of forces between self-assembled proteins: temperature-dependent exponential forces between collagen triple helices, Proc. Natl. Acad. Sci. USA 91 (1994) 276–280.
[42] A. Torre-Blanco, E. Adachi, Y. Hojima, J.A.M. Wootton, R.R. Minor, D.J. Prockop, Temperature-induced post-translational over-modification of type I procollagen, J. Biol. Chem. 267 (1992) 2650–2655.
[43] J.E. Scott, Supramolecular organization of extracellular matrix glycosaminoglycans, in vitro and in the tissues, FASEB J. 6 (1992) 2639–2645.
[44] J.E. Scott, Proteoglycan–fibrillar collagen interactions in tissues: dermatan sulphate proteoglycan as a tissue organizer, in: J.E. Scott (Ed.), Dermatan Sulphate Proteoglycans: Chemistry, Biology, Chemical Pathology, Portland Press, London, 1993, pp. 81–102.
[45] D.R. Keene, J.D. San Antonio, R. Mayne, D.J. McQuillan, G. Sarris, S.A. Santoro, R.V. Iozzo, Decorin binds near the C terminus of type I collagen, J. Biol. Chem. 275 (2000) 21801–21804.
[46] P.J. Neame, C.J. Kay, D.J. McQuillan, M.P. Beales, J.R. Hassell, Independent modulation of collagen fibrillogenesis by decorin and lumican, Cell. Mol. Life Sci. 57 (2000) 859–863.
[47] N.P. Ward, J.E. Scott, L. Coster, Dermatan sulphate proteoglycans from sclera examined by rotary shadowing and electron microscopy, Biochem. J. 242 (1987) 761–766.
[48] G.A. Di Lullo, S.M. Sweeney, J. Korkko, L. Ala-Kokko, J.D. San Antonio, Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type I collagen, J. Biol. Chem. 277 (2002) 4223–4231.