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Tenascin-X increases the stiffness of collagen gels without affecting fibrillogenesis

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ABSTRACT

Tenascin-X is an extracellular matrix protein whose absence leads to an Ehlers-Danlos Syndrome in humans, mainly characterised by connective tissue defects including the disorganisation of fibrillar networks, a reduced collagen deposition, and modifications in the mechanical properties of dense tissues. Here we tested the effect of tenascin-X on *in vitro* collagen fibril formation. We observed that the main parameters of fibrillogenesis were unchanged, and that the diameter of fibrils was not significantly different when they were formed in the presence of tenascin-X. Interestingly, mechanical analysis of collagen gels showed an increased compressive resistance of the gels containing tenascin-X, indicating that this protein might be directly involved in determining the mechanical properties of collagen-rich tissues *in vivo*.

1. Introduction

Tenascin-X (TNX) is a huge extracellular matrix glycoprotein that has a multidomain structure consisting of an N-terminal region involved in oligomerisation, a series of epidermal growth factor (EGF)-like repeats, a variable number of fibronectin-type III (FNIII) modules and a C-terminal domain homologous to fibrinogen (Fbg) [1]. Several observations suggest that TNX is involved in the deposition and/or stabilisation of collagen fibrils, and in influencing the mechanical properties of connective tissues. Indeed, TNX deficiency leads to the appearance of an Ehlers-Danlos Syndrome in humans [2]. Major clinical symptoms consist of skin hyperextensibility and joint laxity, while ultrastructural analyses reveal abnormalities in collagen fibril networks and elastic fibre morphology. Mice deficient in TNX partly reproduced this phenotype [3], and fibroblasts isolated from these mice failed to deposit collagen in cell culture [4]. Moreover, we have previously demonstrated that TNX is localised at the surface of collagen fibrils within tissues [5]. This association might be explained by the interaction of TNX with several molecular components of these fibrils i.e., decorin, fibrillar and FACIT collagens [6–8]. More recently, it was shown that TNX has elastic properties, due to the extension/refolding of its constitutive FNIII modules [9]. Taken together, these data suggest that TNX might affect collagen fibrillogenesis and/or act as a bridge between collagen fibrils and consequently modulate the mechanical properties of connective tissues. In order to test these hypotheses, we analysed the consequences of TNX-collagen interaction *in vitro*, by studying the influence of TNX on collagen fibrillogenesis, and by measuring the mechanical properties of collagen gels polymerised in the presence of TNX.

2. Materials and methods

2.1. Production and purification of recombinant TNX

Recombinant full length bovine TNX was produced in mammalian HEK293 cells as previously described [7,10]. TNX was further purified from the culture medium by two chromatographic steps: firstly on heparin-Sepharose (GE Healthcare), and secondly on Q-Sepharose (GE Healthcare) [7]. TNX fractions were dialyzed against phosphate-buffered saline (PBS) and stored at −80 °C. Protein concentration was determined using the QuantiPro BCA assay kit (Sigma) according to the manufacturer's instructions.

2.2. Preparation of acid-soluble collagen

Acid-soluble collagen was extracted from tail tendons of young rats using standard procedures. All steps were performed at 4 °C. Tendons were dissected in 0.2 M NaCl, then dilacerated and transferred to 0.5 M acetic acid/0.2 M NaCl for 48 h. The extract was centrifuged at 20,000 g for 20 min, and the resulting supernatant was dialysed against 0.5 M acetic acid/0.9 M NaCl. After centrifugation (20,000 g, 20 min), the pellet was treated with 70% ethanol, dried in a

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sterile atmosphere, and dissolved in 0.001 M HCl. Collagen concentration was determined by both QuantiPro BCA assay kit and amino-acid analysis.

2.3. Collagen fibrillogenesis assays

Collagen solutions (3 to 5.6 mg/mL) were neutralised on ice using 0.5 M NaOH and diluted to a final concentration of 1 mg/mL by the addition of PBS containing recombinant TNX or control protein. Fibrillogenesis was initiated by warming the solutions to 37 °C in a thermostated cuvette, and gelation was detected by monitoring turbidity at 400 nm in a DU 640 spectrophotometer (Beckman). Assays were performed in triplicate and a minimum of three independent experiments were carried out. Pepsin-extracted bovine collagen was purchased from BD Biosciences (Le Pont de Claix, France).

2.4. Scanning electron microscopy

Collagen gels, prepared as described for the fibrillogenesis assay, were briefly rinsed in 0.15 M sodium cacodylate (Sigma) pH 7.4 then fixed for 1 h in 2% glutaraldehyde (Electron Microscopy Sciences) diluted in the same buffer. After further rinsing, gels were treated for 45 min with 1% osmium tetroxide diluted in 0.1 M sodium cacodylate. Samples were then dehydrated by a series of graded ethanol solutions, impregnated in hexamethyldisilazane (Sigma), and air-dried. After coating with gold-palladium by sputtering, the gels were observed with a Hitachi S800 microscope (operating at 15 kV) at the "Centre Technologique des Microstructures" (Université de Lyon). Collagen fibril diameters were measured using NIH Image J software.

2.5. Mechanical tests

Neutralised collagen solutions were prepared as described in the fibrillogenesis section, and 10.5 mL aliquots were transferred to 60 mm diameter cell culture Petri dishes (Falcon). Gelation was carried out for 30 min at 37 °C, then collagen gels were stored at 4 °C in the presence of PBS until mechanical testing. Collagen gel samples had a cylindrical form, with diameter ~50 mm and height ~3 mm, and their mechanical resistance was measured by compression testing in an unconfined configuration.

Step-wise compression tests were performed with an Advanced Rheometric Expansion System (ARES) equipped with a maximum cell load of 20 N and controlled gap (hi) (TA Instruments). The load assembly consists of two stainless steel 50 mm diameter parallel plates above and below the sample. In order to control the boundary conditions, the stainless steel platens were sonicated twice for 10 min at 25 °C in ethanol before use. For each test, a cycle of 4 strains was performed at an imposed speed deformation of 0.02 mm/s, the compression level representing 5% of the sample thickness. Every compression strain ramp was followed by a period of stress relaxation of 600 s. This period of stress relaxation was necessary to reach the balance of stress equilibrium characteristic to cartilage biphasic materials [11].

The normal force and the gap thickness were directly measured by the rheometer sensors. To reproduce the same initial load condition, the test began after having submitted each sample to a compressive force of 0.02 N followed by a 600 s relaxation period. Stress-relaxation curves were generated for each sample, including compressive and relaxation stresses, calculated as normal force divided by gel area, and were drawn as a function of time. Stress–strain curves were generated for each sample by taking only into consideration the compression peak stress, where the compressive strain was calculated as $(h_0 - h_i)/h_0$ (where h_0 is the initial gap). The linear regression slope of the stress–strain curve was used to estimate the apparent elastic modulus of each gel.

For each type of gel (collagen only, collagen and BSA, collagen and TNX), four samples were tested, and each test was repeated three times to verify the reproducibility of measurements.

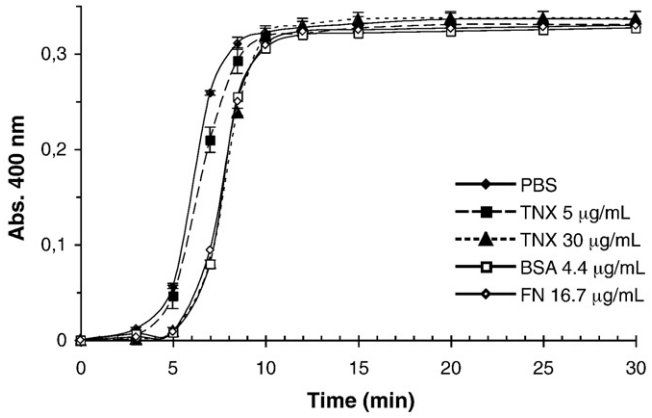

Fig. 1. Effect of TNX on collagen fibrillogenesis. Fibril formation was measured by monitoring collagen solution turbidity at 400 nm. The different curves represent control experiment with collagen alone (♦), collagen plus TNX at 5 µg/mL (■) or 30 µg/mL (66.6 nM, ▲), collagen plus BSA at 4.4 µg/mL (66.6 nM, □), or collagen plus fibronectin at 16.7 µg/mL (66.6 nM, ◊). Each point is the mean of triplicate values and error bars are SEM. The data shown are representative of three independent experiments.

3. Results and discussion

3.1. Influence of TNX on collagen fibrillogenesis

The effect of TNX on collagen fibril formation was tested *in vitro* by monitoring turbidity during the gelation process. As shown in Fig. 1, TNX did not seem to affect collagen fibrillogenesis except at high concentrations where a slight delay in the initiation step was apparent. However, the same effect was obtained by the addition of a control protein (BSA), which does not specifically interact with fibrillar collagens. A careful examination of the fibrillogenesis parameters led to the same conclusion (Table 1). Neither the lag phase, nor the fibrillogenesis rate or the absorbance plateau were significantly modified by the presence of TNX. These results are somehow contradictory with previous results [12] showing a diminished lag phase and an increased absorbance plateau of collagen solutions when fibrillogenesis assays were done in the presence of TNX.

The first point of divergence that might explain the discrepancy between our results and those of Minamitani et al. [12] concern the source of the collagen. Their collagen solution was from a commercial source, and gave rise to slow fibrillogenesis in control conditions (2 h for their lag phase instead of 5 min in our experiments and elsewhere [13,14]). One explanation could be that the commercial collagen was pepsinised and consequently devoid of telopeptides, which have been shown to be important for fibrillogenesis [15]. To test this hypothesis, we performed fibrillogenesis assays with bovine pepsinised collagen and indeed observed a slower fibrillogenesis. However, even in these conditions, the presence of TNX did not modify the fibrillogenesis parameters compared to the control protein BSA (data not shown).

The differences observed might also result from the concentration and presence of FNIII repeats present in the recombinant TNX used during the fibrillogenesis assays. These discrepancies seem not to be due

Table 1
Collagen fibrillogenesis parameters corresponding to Fig. 1 experiments. Lag time, rate of fibrillogenesis, and absorbance plateau were calculated according to the method published by Sullivan et al. [12].

	Lag time (min)	Rate of fibrillogenesis (1×10^{-2}/min)	Absorbance plateau (400 nm)
Control	5.1 ± 0.2	6.5 ± 0.1	0.34 ± 0.08
TNX (5 µg/mL)	5.5 ± 0.1	5.8 ± 0.4	0.34 ± 0.04
TNX (30 µg/mL)	6.6 ± 0.4	6.3 ± 0.3	0.35 ± 0.01
BSA (4.4 µg/mL)	6.6 ± 0.4	6.3 ± 0.4	0.34 ± 0.07
Fibronectin (16.7 µg/mL)	6.6 ± 0.4	6.2 ± 0.2	0.33 ± 0.01