Tenascin-X, Collagen, Elastin and the Ehlers-Danlos Syndrome

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Abstract

Tenascin-X is an extracellular matrix protein initially identified because of its overlap with the human CYP21B gene. Because studies of gene and protein function of other tenascins had been poorly predictive of essential functions in vivo, we used a genetic approach that critically relied on an understanding of the genomic locus to uncover an association between inactivating tenascin-X mutations and novel recessive and dominant forms of Ehlers-Danlos syndrome. Tenascin-X provides the first example of a gene outside of the fibrillar collagens and their processing enzymes that causes Ehlers-Danlos syndrome. Tenascin-X null mice recapitulate the skin findings of the human disease, confirming a causative role for this gene in Ehlers-Danlos syndrome. Further evaluation of these mice showed that tenascin-X is an important regulator of collagen deposition in vivo, suggesting a novel mechanism of disease in this form of Ehlers-Danlos syndrome. Further studies suggest that tenascin-X may do this through both direct and indirect interactions with the collagen fibril. Recent studies show that TNX effects on matrix extend beyond the collagen to the elastogenic pathway and matrix remodeling enzymes. Tenascin-X serves as a compelling example of how human "experiments of nature" can guide us to an understanding of genes whose function may not be evident from their sequence or *in vitro* studies of their encoded proteins.

Introduction

Tenascin-X is one member of a family of extracellular matrix glycoproteins that share a common general structure, but are distinguished by their patterns of expression in development and disease [1]. There are 4 mammalian tenascins, each identified by more than one laboratory, leading to a variety of names depending on the context of discovery. For clarity, the mammalian genes are now called tenascin-C (TNC, formerly cytotactin, hexabrachion), tenascin-R (TNR, restrictin, januscin), tenascin-X (TNX) and the recently described tenascin-W (TNW, also tenascin-N) [2-10]. While many possible functions for these proteins have been proposed based on patterns of expression and studies in vitro, their functions *in vivo* remain poorly understood. The findings that the TNC, TNR and TNC/TNR double knockout mice lack an overt phenotype was both surprising and disappointing[11-13], and it remains uncertain whether this occurs because the tenascins share redundant functions or whether their most important functions are not during development but in adult responses to stress or injury. It was on this background, that the initial association of TNX deficiency with the Ehlers-Danlos syndrome (EDS) was made[14]. This observation was of particular interest not only because it revealed unique functions for at least one tenascin, but also because it demonstrated that genes beyond the fibrillar collagens and collagen modifying enzymes can cause EDS[15, 16]. In this review we discuss the discovery of TNX, studies supporting a causative role in Ehlers-Danlos syndrome in humans, and recent studies of its role in matrix biology.

Discovery of tenascin-X

TNX was initially cloned serendipitously because of its 3' overlap with the human CYP21b gene[5]. CYP21B encodes steroid 21-hydroxylase an essential enzyme in cortisol and mineralocorticoid biosynthesis, deficiency of which leads to the recessive disorder congenital adrenal hyperplasia[17]. While trying to clone to a mutant CYP21B cDNA from an adrenal library prepared from the adrenal gland of an affected fetus, we instead cloned a low-level antisense transcript that would be obscured by the 100-fold greater expression of CYP21B in the normal adrenal. Further analysis of this locus on chromosome 6 showed that this transcript arose from a gene (TNX) that was partially duplicated along with CYP21 and C4 encoding the 4th component of serum complement. As shown in Fig. 1, these genes are arranged in the order: C4A/CYP21A/XA/C4B/CYP21B/TNX (Fig 1). Both C4 genes are functional, while only the CYP21B gene is functional in humans. The duplication event truncated the duplicate TNX gene (called XA) and XA also contains a 121 bp deletion that interrupts its open reading frame rendering it non-functional [18]. Surprisingly, the XA gene is transcribed abundantly in the adrenal gland, but XA does not appear to encode protein [19].

Following this initial report, Matsumoto et al identified a cluster of fibronectin type III repeats in the class III region of the MHC that clearly represented an upstream portion of the TNX gene [20]. Reports of the complete human gene and mouse cDNA soon followed, and included studies of expression that were consistent with important roles in development and matrix biology [2, 4, 21]. However, the large size of the encoded mRNA (12 kb) and protein (450 kD) impeded functional studies and suggested to us that genetic strategies were most likely to identify unique functions for TNX.

Association of tenascin-X with recessive Ehlers-Danlos syndrome

We based our search for patients that might have disease caused by TNX on extensive prior work concerning the mechanism of disease in congenital adrenal hyperplasia (CAH). Specifically, it was well known that 25% of CAH alleles carry deletions within the C4/CYP21/TNX locus that are usually inherited, but can arise de novo from unequal crossover events. In this model, there is misalignment of the 30 Kb regions of homology in the A and B regions of the locus and recombination may occur anywhere in this region [22]. If recombination occurs between C4 genes, an allele is produced that contains a hybrid C4A/B gene, normal CYP21B and TNX genes, and which lacks CYP21A and XA. This allele does not produce disease because the remaining C4 gene, CYP21B and TNX genes are all functional, although reduced C4 gene dosage may be associated with autoimmune disease [23]. When recombination occurs between CYP21 genes a hybrid CYP21 gene is produced. This allele is a CAH allele because the CYP21A/B hybrid will contain one or more truncating mutations found in the CYP21A pseudogene, while a normal TNX gene persists[24]. Many examples of these deleted C4 and CYP21 alleles exist, as well as the reciprocal triplicated alleles .

Based on these findings, we postulated that recombination also occurs between TNX and XA. Such a recombination event would be a CAH allele because CYP21B is deleted and should also alter function of the TNX gene if the 3'end of XA (containing the 121 bp deletion) were retained. We reasoned that such an allele might produce a contiguous gene syndrome consisting of CAH and a connective tissue disorder. A brief search identified such a patient: a 25 year-old man with salt-wasting CAH and Ehlers-Danlos syndrome consisting of hyperextensible skin and joints, accompanied by prominent bruising and absence of atrophic scarring [2]. The index patient was found to share the predicted hybrid TNX/XA allele with his father and a sister, both

of whom were phenotypically normal. Further studies showed complete absence of TNX mRNA and protein in the proband. These studies were most consistent with a recessive pattern of inheritance, but no mutation was identified on the proband's maternal TNX allele and a complex interaction between CAH or its treatment and TNX haploinsufficiency was not formally excluded.

In order to assess whether isolated TNX deficiency occurs and is associated with Ehlers-Danlos syndrome, we needed to screen large numbers of patients for TNX deficiency. To make this possible, Schalkwijk developed a single dilution TNX ELISA that is presently in use in our laboratories for routine screening. Screening was also facilitated by the observation that a 140 kD C-terminal fragment of TNX circulates in serum and its absence is uniformly associated with absence of TNX mRNA and protein in dermal fibroblasts. We then screened serum from 151 patients with isolated forms of EDS and found 5 patients to be TNX-deficient [25]. Absence of TNX mRNA and protein was confirmed in skin fibroblasts and all had clinical findings of classical Ehlers-Danlos except that they had normal wound healing and lacked atrophic scars. Allele specific PCR demonstrated one patient (with CAH) was homozygous for the TNX deletion allele and another was heterozygous for the deletion. The remaining 3 patients carried homozygous inactivating mutations. This study confirmed the association of TNX deficiency with a recessive form of EDS and strongly suggested that TNX mutations were causative. Subsequent testing in our laboratory identified 4 additional patients with TNX-deficient EDS and the clinical findings of all known patients are summarized in Table 1.

While the number of TNX-deficient patients known world-wide remains small [25, 26], several generalizations about the clinical features can be made. The first is that all known affected individuals have both hyperextensible skin and hypermobile joints and none have

atrophic scars. In most patients, bruising has been a prominent feature. Together, these features strongly suggest TNX deficiency and should trigger genetic evaluation and serum testing for TNX deficiency. Some female heterozygotes will have isolated joint hypermobility (vide infra), so these findings in an affected patient's mother or daughter does not exclude the diagnosis of recessive TNX-deficiency. In our experience, the absence of affected first-degree relatives more often slows referral for genetic evaluation and many of our referrals now come directly from rheumatology and hematology clinics.

Arthralgias, subluxations and degenerative arthritis are the most frequent debilitating findings in patients identified to date, although a variety of important complications including mitral valve prolapse and pulmonary disease have been seen. In our clinic we recommend annual physical exam and biennial echocardiography to evaluate the mitral valve. We have not found aortic root dilation or dissection in any TNX-deficient patient.

Association of tenascin-X with hypermobility-type Ehlers-Danlos syndrome

Following publication of these findings, we had the opportunity to examine many members of these families not originally available for study. Heterozygous deficient family members were found to have TNX levels ~50% of those of normal individuals and 9 of 20 had isolated joint hypermobility, representing nearly two-thirds of heterozygous women in the families [27]. No male TNX heterozygous were affected. We then evaluated TNX levels by ELISA in 80 unrelated Dutch patients with hypermobility-type EDS and found 6 individuals with TNX levels more than 2.5 standard deviations below the mean, a 10-fold excess over what might be expected by chance. Not unexpectedly, these patients had frequent joint subluxation and chronic arthralgias. Allele-specific PCR and sequencing of coding regions revealed two

inactivating mutations in this group, supporting a causative role for TNX. The absence of coding-sequence mutations in the remaining 4 patients was surprising, but it was suggested that regardless of cause, the 50% reduction in TNX was likely to be responsible for disease. This study raised important questions of whether undefined TNX regulatory mutations might play an important role in this disorder, and why only women TNX heterozygotes were affected.

Evidence that tenascin-X is causative of Ehlers-Danlos syndrome

Demonstration of TNX protein deficiency and inactivating mutations in patients with a clinically distinct form of Ehlers-Danlos syndrome provided strong presumptive evidence that the association was causal, and proof-positive was ultimately provided by producing a mouse phenocopy of human TNX deficiency through gene targeting of mouse Tnx [28]. Tnx null mice are viable and are grossly normal at birth, but by weaning, their skin is noticeably hyperextensible. Formal biomechanical testing confirmed increased deformability and reduced tensile strength of Tnx null skin compared to wildtype littermates. Ultrastructural examination showed relatively normal size and shape of collagen fibrils, but the density of fibrils in dermis was significantly reduced, leading to a 30% reduction in collagen content in skin. Tnx null skin fibroblasts were studied in cell culture and found to have near normal collagen synthesis, but a significant deficit in the amount of collagen deposited into insoluble matrix. This led directly to the hypothesis that TNX-deficiency causes Ehlers-Danlos syndrome not by interfering with collagen synthesis or processing, as has been described in other forms, but rather through regulation of fibril deposition into matrix by dermal fibroblasts. It has long been known that collagen is deposited into widely varying tissue-specific forms, and while recent studies confirm that this process is highly regulated in fibroblasts, the molecular details of this process remains

unknown [29, 30]. TNX is postulated to participate in this pathway and it is proposed that other genes playing a role in this process may be responsible for other Ehlers-Danlos variants [16].

Tenascin-X and the collagen fibril

The simplest mechanism by which TNX might regulate the spacing between fibrils in the dermis is through direct binding to collagen fibrils, and several lines of evidence support this concept. First, Lethias et al cloned bovine TNX as a fibril-associated protein in dermal collagen preparations [31]. Second, recent studies suggest that native TNX binds collagen *in vitro* and the native protein increased both the rate and extent of fibril formation [32]. Third, Lethias showed that TNX is also capable of binding decorin, a small leucine-rich proteoglycan that binds collagen avidly and regulates fibrillogenesis [33]. Finally, immunogold labeling localizes TNX to the fibril surface in human dermis, while staining is absent from TNX-deficient skin (Fig. 2a,b).

Together these data strongly support physical interaction between TNX and collagen fibrils. However, if TNX specifically regulates interfibrillar distance, the protein must interact directly or indirectly with multiple fibrils. The simplest way for this to occur is if TNX is multimeric. This is the case for both TNC and TNR and sequences at the N-terminus of these molecules that mediate oligomerization do appear to be conserved in TNX [2, 8, 34]. However, we and others have been unable to demonstrate multimeric TNX complexes in extracts of tissue or fibroblast conditioned medium. Monomeric TNX might fulfill this requirement through interaction of multiple domains of the molecule with collagen fibrils. Recent evidence suggests that this may be the case. Lethias has shown that the 10^{th} and 11^{th} fibronectin repeats of TNX interact with the dermatan sulfate chains of decorin, while Egging et al have shown that the

recombinant C-termius of TNX is capable of interacting directly with fibrils, leading to the model depicted in Fig 2c [35].

Tenascin-X, elastogenesis and matrix remodeling

Zweers et al followed up molecular observations on TNX-deficient patients with ultrastructural analysis of their skin. They were able to confirm normal appearing fibrils and reduced collagen content, but also noticed irregular and immature elastin fibers containing few or no microfibrils [36]. Elastin staining showed absence of fine elastic fibers in papillary dermis and fragmented or clumped elastic fibers in reticular dermis. Elastin abnormalities have not been shown in other forms of EDS, so it seems likely that the elastic fiber abnormalities are a direct result of TNX-deficiency and not a secondary consequence of altered collagen metabolism. What is still unclear is the extent to which the TNX-deficient phenotype is altered by co-existing elastin abnormalities.

We recently performed a microarray-based analysis of expression differences between Tnx null and wildtype mouse fibroblasts that may shed light in this phenomenon. We found significant up-regulation of fibrillin-2 and stromelysin-3 (M. Collier and J. Bristow, unpublished). Stromelysin-3 is a protease whose primary known target is the α-1 proteinase inhibitor that in turn, normally binds and inhibits elastase [37]. The net consequence of these alterations may be activation of dermal elastic fiber remodeling that could account for the observed changes *in vivo*.

Recently reports of additional matrix alterations in Tnx null mice have emerged. Matsumoto et al have reported altered expression of collagen VI, fibril associated proteoglycans and matrix metalloproteases 2 and 9 in independently derived Tnx null mice [38, 39]. While the

implications of these findings for TNX-deficient Ehlers-Danlos syndrome are uncertain, these observations together suggest that TNX has effects on matrix synthesis and remodeling that extend beyond the fibrillar collagens.

Future directions

Recent genetic investigations of TNX-deficiency in humans provided evidence for the first essential functions of any of the tenascins. The extent to which this role is shared with other tenascins in other contexts is uncertain and worthy of investigation. Similarly, study of TNX-deficient humans and mice has uncovered a novel mechanism of disease in Ehlers-Danlos syndrome. Identification of other genes and proteins that participate in the organization of tissue-specific forms of collagen will provide additional candidate genes for testing in the 30-50% of Ehlers-Danlos patients for whom no present molecular explanation exists. Finally, because TNX is frequently up-regulated during fibrosis that follows tissue injury, it may facilitate collagen deposition in fibrotic diseases like cirrhosis, hypertensive cardiac fibrosis, and idiopathic pulmonary fibrosis. Interrupting the interaction between TNX and collagen fibrils might limit fibrotic damage to tissues without altering collagen turnover in normal tissues that do not normally express TNX.

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