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Distinct Glycosylation in Interstitial and Serum Tenascin-X

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We developed an easy and fast method to isolate extracellular matrix tenascin-X (TNX) from various tissues in mice based on TNX antibody affinity purification. We purified approximately 350-kDa cellular interstitial TNX (iTNX) from the spleen, liver and kidney as well as 200-kDa serum TNX (sTNX). Since the nature and significance of glycosylation in TNX remains to be elucidated, glycobiochemical properties of purified TNX were characterized by lectin blot analysis. Lectin blots by Con A, LCA, PHA-E4, RCA120 or WGA revealed the presence of *N*-glycan in the cellular TNX and especially complex-type *N*-glycan in the serum TNX. In addition, the iTNX from liver and kidney also possessed *O*-glycan based on the reaction to PNA. The binding to AAL indicated that iTNX from the three tissues possesses fucose linked α1,6 to a pentasaccharide core, whereas sTNX does not. The reaction to SSA but not to MAM suggested the presence of sialic acid linked α2,6 to galactose in both cellular and serum TNX. Lectin blots of trypsin-treated iTNX from the spleen also demonstrated that WGA alone reacts to the t300 product derived from the amino-terminal 300-kDa portion.

Key words glycosylation; lectin; purification; tenascin-X

The extracellular matrix (ECM) of multicellular organisms not only provides structural integrity to tissues but also acts as an informational entity in the sense for cell migration, growth and differentiation.[1] The ECM consists of a complex network of macromolecules that include glycoproteins, proteoglycans, and glycosaminoglycans (GAGs). Prominent members of non-collagenous extracellular glycoproteins are fibronectin, laminins and tenascins. The tenascin family constitutes a group of ECM glycoproteins with a characteristic structure. The four members of this family identified so far [tenascin-C (TNC), restrictin/J1-160/180 (tenascin-R, TNR), tenascin-X (TNX), and tenascin-W/tenascin-N (TNW/TNN)] have been found in vertebrates[2—6] and display a cysteine-rich segment at the amino terminus followed by epidermal growth factor (EGF)-like repeats, fibronectin type III (FNIII)-like repeats, and a fibrinogen-like domain at the carboxy terminus.

Tenascin-X (TNX) is the largest member of the tenascin family. Initially its gene was mapped in the class III region of the major histocompatibility complex (MHC), partly overlapping with the gene encoding steroid 21-hydroxylase.[7—9] TNX was also identified as flexilin by Lethias *et al.*[10] TNX is expressed abundantly during embryonic development and in the adult in a variety of tissues, as has been shown by immunohistochemistry[11] and *in situ* hybridization.[12] Accumulated experimental evidence has shown that TNX is involved in collagen fibrillogenesis. TNX is associated with collagen fibril-associated collagen types XII and XIV on collagen fibrils.[10] TNX deficiency in human patients causes a clinically distinct form of a heritable connective-tissue disorder, Ehlers–Danlos syndrome (EDS).[13—15] Biochemical analyses have shown that TNX not only regulates transcription of genes for collagen fibril-associated molecules, *e.g.*, types VI, XII and XIV collagens, decorin, lumican and fibromodulin[16] but also binds to type I collagen directly and modulates the rate and quantity of type I collagen fibril formation *in vitro*.[17] Studies in TNX-deficient (TNX−/−) mice demonstrated greatly disturbed biomechanical properties and reduced tensile strength of the skin.[18] TNX deficiency followed by impaired collagen fibrillogenesis would affect physical properties in the skin, leading to some abnormalities in mice. Actually mice deficient in TNX independently generated by us[19] showed alterations in triglyceride synthesis and the composition of triglyceride-associated fatty acids.[20] In addition, TNX deficiency results in the promotion of tumor invasion and metastasis by induction of matrix metalloproteinase-2 (MMP-2) *via* increased activation of c-Jun N-terminal kinase (JNK) and tyrosine phosphorylation of certain proteins.[21] Furthermore, morphological observations in skin biopsies of TNX-deficient EDS patients showed abnormalities of elastic fibers, indicating that TNX is involved in development and maintenance of the elastic fiber organization.[22] Recently, we identified the serum form of TNX (sTNX) with a molecular mass of 200 kDa in the mouse. The 200-kDa sTNX, which is generated by cleavage of spleen iTNX by spleen homogenate, contains 15 FNIII repeats and a fibrinogen domain identical to the C-terminal portion of interstitial TNX (iTNX).[23]

A generally accepted role played by protein glycosylation, especially *N*-glycan, is in the promotion of proper folding of glycoproteins in the endoplasmic reticulum by serving as specific recognition structures that allow the glycoproteins to interact with a variety of lectins, glycosidases, and glycosyltransferases that in turn affect the folding and secretion of the protein.[24] Furthermore, some studies have demonstrated a direct apical sorting effect of *N*-glycans in some epithelial cells.[25] On the other hand, expression of aberrant carbohydrate determinants is associated with malignant transformation and involved in adhesion of cancer cells.[26]

In the tenascin family, TNR is exclusively analyzed on the carbohydrate conjugated to the many potential sites for *N*- and *O*-glycosylation. Zamze *et al.*[27] showed that the *N*-glycans in TNR are dominated by complex biantennary structures with core-fucosylation, a bisecting *N*-acetylglucosamine and abundant antennae truncation, whereas *O*-glycans in TNR are dominated by disialylated oligosaccharides. Furthermore, TNR is composed of chondroitin sulfate GAGs, which are involved in matrix interactions with fibronectin

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and TNC.[28)]

In this study, we focused on the carbohydrate characterization of TNX. Little is known about the molecular forms of the carbohydrate conjugated to the many potential sites for *N*- and *O*-glycosylation. The methodology used in this study is generally based on lectin blot analyses with plant lectins because they have the affinity for specific terminal sugars and some internal sugars, sugar linkages, or oligomers in complex carbohydrates. We purified mouse TNX from some tissues as well as from the serum and investigated the oligosaccharide composition of tenascin-X by lectin blot analyses.

MATERIALS AND METHODS

Animals and Tissues Adult C57/BL6 mice were bred under specific pathogen-free conditions. All animal experiments were carried out in accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of Hokkaido University.

Purification of iTNX and sTNX Affinity-purified anti-mouse TNX antibody pAb10d211[23)] was coupled to CNBr-activated Sepharose 4B according to the instructions of the manufacturer (Sigma-Aldrich, Tokyo, Japan). Ten grams of tissue from mouse spleen, liver or kidney was disintegrated with 50 ml of high-salt buffer containing 0.5 M NaCl and 20 mM Tris–HCl (pH 7.5) and protease inhibitor Complete (Roche Diagnostics, Indianapolis, IN, U.S.A.) by a Polytron homogenizer (Kinematica, Luzern, Switzerland). The sample was rotated overnight at 4 °C. After centrifugation for 20 min at 12000 rpm, the supernatant was collected and precipitated with 40% ammonium sulfate by stirring overnight at 4 °C. After centrifugation for 20 min at 12000 rpm, the pellet was dissolved in 35 ml of high-salt buffer and dialyzed overnight against the same buffer. The pAb10d211-coupled Sepharose 4B resin was suspended in the dialyzed sample and rotated overnight at 4 °C. The resin was washed extensively in the high-salt buffer. The antigen was eluted with 200 mM triethylamine in the high-salt buffer, and the eluate was immediately neutralized with 2 M Tris–HCl (pH 7.0). The eluate with iTNX from the tissues was analyzed by 7.5% SDS-PAGE and visualized by Coommasie Blue R-250. sTNX was extracted and purified from 20 ml of mouse serum as described in our previous paper.[23)] Finally, purified TNX from various organs and serum was dialyzed overnight against phosphate-buffered saline (PBS) at 4 °C.

Western Blot Analysis Purified iTNX from tissues or serum were separated by 7.5% SDS-PAGE, transferred onto Hybond ECL nitrocellulose membranes (Amersham Biosciences, Tokyo, Japan), and reacted with affinity-purified rabbit anti-TNX polyclonal antibodies (pAbM1, pAbM25, or pAbM31).[23)] The proteins on the membranes were then reacted with goat Alexa Fluor 680-conjugated secondary antibodies (Molecular Probes, Eugene, OR, U.S.A.) followed by visualization using an infrared imaging system (Odyssey, LI-COR, Lincoln, NB, U.S.A.).

Detection of Glycoproteins on Nitrocellulose Membranes by PAS Staining Purified iTNX was resolved on a 7.5% SDS-PAGE under reducing conditions and transferred to a Hybond ECL nitrocellulose filter (GE Healthcare Bio-Sciences, Tokyo, Japan) according to the method described in our previous paper.[29)] An ECL glycoprotein detection system (GE Healthcare Bio-Sciences) was used to investigate glycosylation of TNX according to the manufacturer's instructions. Briefly, the protein with carbohydrates on the nitrocellulose filter was oxidized with 10 mM sodium metaperiodate for 20 min. After washing with PBS for 10 min three times, the filter was incubated with 0.25 μM biotin hydrazide in 100 mM acetate buffer (pH 5.5). Subsequently, the filter was washed with PBS for 10 min three times and was then incubated with horseradish peroxidase (HRP)-conjugated streptavidin. The blot was visualized using an ECL chemiluminescent Western blotting detection system (GE Healthcare Bio-Sciences).

Treatment of iTNX with *N*-Glycosidase F and/or *O*-Glycosidase Digestion of iTNX with *N*-glycosidase F (Roche Diagnostics, Tokyo, Japan) and/or *O*-glycosidase (Roche Diagnostics) was performed as follows. Ten microliters of 20 ng/μl iTNX from three tissues was added to 2.5 μl of denaturation solution (5% SDS and 5% β-mercaptoethanol). The samples were then heated for 5 min at 100 °C and cooled on ice for 5 min, followed by the addition of 2.5 μl of 20% Nonidet P-40 (NP-40). The sample was incubated with 2 μl of *N*-glycosidase F (1 unit/μl) or with 2 μl each of *N*-glycosidase F and *O*-glycosidase (500 unit/μl) in 163 mM phosphate buffer (pH 7.8) for 12 h at 37 °C. In the case of *O*-glycosidase alone, the sample was incubated with 2 μl of *O*-glycosidase in 163 mM phosphate buffer (pH 6.0) for 12 h at 37 °C. The purified or glycosidase-digested iTNX was subjected to 7.5% SDS-PAGE and transferred to Hybond ECL nitrocellulose membranes as described above. Then glycosidase-digested iTNX was detected by lectin blot analysis.

Lectin Blot Analysis The membrane onto which purified iTNX from tissues and sTNX were transferred was blocked with TBST [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% Tween-20] and then incubated with HRP- or biotin-conjugated lectin diluted 1000-fold with TBST for 1 h at room temperature. After washing with TBST three times, HRP-conjugated lectins with reactive proteins were detected using an ECL chemiluminescent detection system. For detection of biotin-conjugated lectin-reactive proteins, the membrane was further incubated with HRP-conjugated streptavidin and then washed with TBST followed by ECL chemiluminescent detection. Concanavalin A (Con A), *Lens culinaris* agglutinin (LCA), phytohaemagglutinin-E4 (PHA-E4), peanut agglutinin (PNA), *Ricinus communis* agglutinin 120 (RCA120) and wheat germ agglutinin (WGA) were used as HRP-conjugated lectins, and *Aleuria aurantia* lectin (AAL), *Maackia amurensis* lectin (MAM) and *Sambucus sieboldiana* agglutinin (SSA) were used as biotin-conjugated lectins. All lectins were purchased from Seikagaku Corp. (Tokyo, Japan).

Proteolysis of Spleen iTNX with Trypsin One microgram of spleen iTNX was incubated with 10 μl of 10 μg/ml trypsin (Wako, Osaka, Japan) at 37 °C. Twenty-microliter aliquots were removed at 0 h, 4 h, and 12 h (overnight) and the digestion was stopped by the addition of Laemmili buffer. Reactions were subsequently analyzed by 7.5% SDS-PAGE.

RESULTS AND DISCUSSION

Purification of iTNX from Various Tissues We devel-

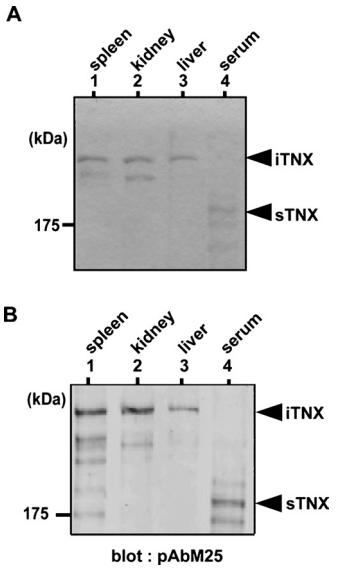


Fig. 1. Purification of Interstitial TNX (iTNX) and Serum TNX (sTNX)

(A) iTNX from mouse tissues (spleen, kidney and liver) or sTNX were affinity-purified with anti-TNX-specific 10d211. The protein was checked by 7.5% SDS-PAGE followed by Coomassie brilliant blue staining. (B) Immunoblot analysis with anti-M25 antibody (pAbM25).

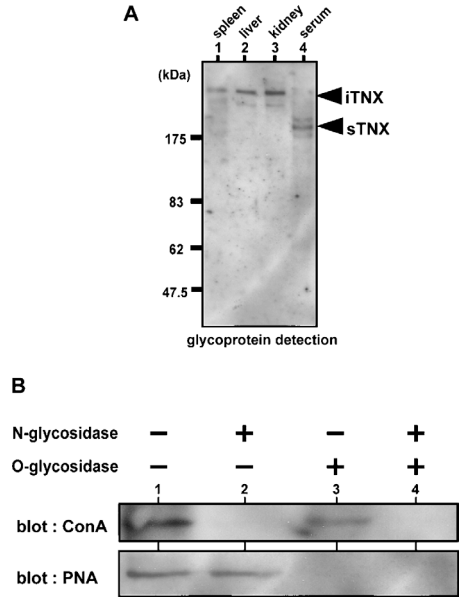


Fig. 2. Both iTNX and sTNX Are Glycosylated

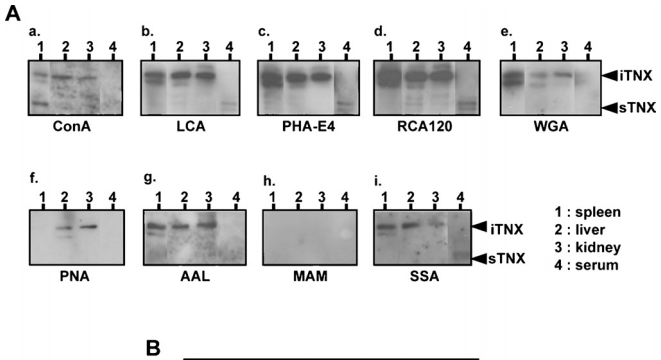
(A) Periodic acid-Schiff (PAS) staining. The glycosylation of iTNX and sTNX was detected by PAS staining. (B) Both *N*-linked and *O*-linked oligosaccharides are present on kidney iTNX. After digestion of kidney iTNX with *N*-glycosidase F and *O*-glycosidase, the iTNX protein was subjected to SDS-PAGE and the gels were stained for carbohydrate with either concanavalin A (Con A) or peanut lectin (PNA). A Con A or PNA-reactive band representing iTNX disappeared with *N*-glycosidase F or *O*-glycosidase, respectively.

oped an easy and fast method for purification of TNX from three tissues, spleen, kidney and liver tissues, and from serum in the mouse. Tissue homogenates with high-salt buffer were incubated with anti-TNX-specific 10d211 antibody-coupled Sepharose 4B beads, and iTNX from tissues and sTNX from serum were purified using a batch process. After binding to the beads, extensive washing with high-salt buffer was performed to eliminate nonspecific binding, followed by elution with 200 mM triethylamine. After five elutions with 200 mM triethylamine, the collected eluates were pooled and dialyzed against PBS. The purity and protein quantization of dialyzed iTNX and sTNX were checked by SDS-PAGE followed by both Coomassie brilliant blue staining (Fig. 1A) and immunoblotting with pAbM25 antibodies to mouse TNX (Fig. 1B). At the end of the purification process, about 60 μg of iTNX with the molecular weight of approximately 350 kDa or sTNX with a molecular weight of approximately 200 kDa in a final volume of 3 ml could be obtained from 10 g of tissues or 20 ml of serum, respectively. The smaller products in spleen and kidney TNX detected in immunoblot analysis (Fig. 1B) represent proteolytic products of iTNX.[23]

Glycosylation of Both iTNX and sTNX To demonstrate that TNX is glycosylated, purified iTNX from the three tissues and sTNX were subjected to SDS-PAGE under reducing conditions, followed by transfer of these proteins to nitrocellulose membranes, and visualization by periodic acid-Schiff reaction (PAS staining). As shown in Fig. 2A, both iTNX and sTNX were detected by PAS staining, indicating that TNX is glycosylated. Next, in order to examine which types of oligosaccharide linkages are present on kidney iTNX, lectin blot analysis was performed using HRP-conjugated Con A, which specifically recognizes *N*-linked oligosaccharides, or PNA, which interacts with *O*-linked oligosaccharides. As shown in Fig. 2B, both Con A and PNA reacted to kidney iTNX. Upon treatment of iTNX protein with *N*-glycosidase F, Con A reactive bands disappeared. On the other hand, upon treatment of iTNX protein with *O*-glycosidase, PNA reactive bands disappeared. These results indicate that both types of oligosaccharide linkages are present on kidney iTNX.

Lectin Blot Analysis of iTNX and sTNX Furthermore, various kinds of lectins were used to investigate possible *N*- and *O*-glycosylation of iTNX from the spleen, liver and kidney, and from sTNX. The iTNX and sTNX samples were subjected to SDS-PAGE under reducing conditions and proteins were transferred to nitrocellulose filters. The blotted filters were stained with HRP-conjugated Con A, LCA, PHE-E4, PNA, RCA120 and WGA, and with biotin-conjugated AAL, MAM and SSA (Fig. 3).

When the blotted filters were incubated with Con A, which mainly recognizes high mannose-type *N*-linked oligosaccharides, iTNX from the three tissues reacted to the lectin, while sTNX did not. However, PHA-E4, which is specific for GlcNAc linked β1,4 to core mannose, and RCA120, which is specific for terminal Gal linked β1,4 to GlcNAc, reacted to sTNX. These results indicated the presence of *N*-glycan in iTNX from the spleen, liver and kidney, and especially complex-type *N*-glycan in sTNX. Liver iTNX as well as kidney iTNX reacted to not only Con A but also PNA, indicating that liver iTNX and kidney iTNX also possess *O*-glycans. Furthermore, when the blotted filter was incubated with AAL, which mainly interacts α1,6-linked fucose residue, iTNX from all three tissues reacted strongly to the lectin, while sTNX did not. These results indicated that fucose which is α1,6-linked to pentasaccharide is present in iTNX from the three tissues but not in sTNX. The lack of fucose residue in sTNX was confirmed by a very faint reactivity of sTNX to LCA, which recognizes the α1,6-fucosylated tri-



B

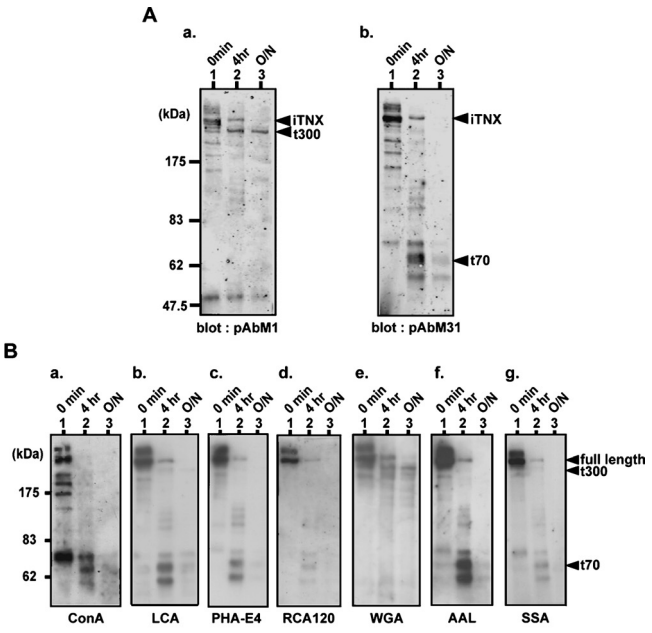
	spleen	liver	kidney	serum
ConA	+	+	+	−
LCA	++	++	++	±
PHA-E4	++	++	++	+
RCA120	++	++	++	+
WGA	++	+	+	−
PNA	−	+	+	−
AAL	++	++	++	−
MAM	−	−	−	−
SSA	++	++	+	+

Fig. 3. Lectin Blot Analysis of iTNX from the Spleen, Liver, Kidney and sTNX

(A) Lectin blot profiles of purified iTNX and sTNX. Purified iTNX and sTNX were characterized by lectin blots using (a) Con A, (b) LCA, (c) PHA-E4, (d) RCA120, (e) WGA, (f) PNA, (g) AAL, (h) MAM, or (i) SSA. (B) Lectin binding properties of iTNX from the spleen, liver, kidney and sTNX from serum. ++, strong positive staining; +, positive staining; ±, faint staining; −, negative staining.

mannosyl core structure. Moreover, the staining pattern with MAM and SSA suggested the type of sialylation of iTNX and sTNX. MAM specifically recognizes sialic acid linked α2,3 to Gal, whereas SSA exclusively binds sialic acid linked α2,6 to Gal. iTNX from all three tissues and sTNX reacted to SSA but not to MAM. These results indicated that sialic acid linked α2,6 to galactose is present in both interstitial and serum TNX.

Lectin Blot Analysis of Tryptic Digests of Spleen iTNX In order to determine the positions of oligosaccharides that react to each lectin, spleen iTNX was subjected to tryptic digestion. The tryptic digests of this protein were fractionated by SDS-PAGE followed by reaction with polyclonal antibodies, pAbM1 or pAbM31 (Fig. 4A). The time-dependent profile for the generation of tryptic products from spleen iTNX was determined. The pAbM1 antibody recognizes other some FNIII repeats of iTNX as well as its own M1 repeat, which is used as the antigen, whereas pAbM31 specifically reacts to only the M31 FNIII repeat located in the carboxyl-terminal portion of mouse TNX.[23] As shown in Fig. 4Aa, t300 with a molecular weight of approximately 300 kDa obtained with tryptic digestion overnight from iTNX was detected by pAbM1 antibodies, but the remaining products were not identified by this antibody. On the other hand, fortunately, pAb31 detected a 70-kDa tryptic product, t70 (Fig. 4Ab), indicating that the t70 product is comprised of a carboxyl-terminal 70-kDa fragment. Subsequently, lectin blot analysis of tryptic products from spleen iTNX was performed (Fig. 4B). The various lectins used (Con A, LCA, PHA-E4, RCA120, AAL and SSA) other than WGA, which binds strongly to GlcNAc and its β1,4 oligomers, reacted to the t70 product. On the other hand, WGA alone reacted to the t300 product. These results suggest that t300 and t70 products are derived from different portions of spleen iTNX



C

	t300	t70
ConA	−	++
LCA	−	++
PHA-E4	−	++
RCA120	−	+
WGA	++	−
AAL	−	++
SSA	−	+

Fig. 4. Lectin Blot Analysis of Tryptic Digests of Spleen iTNX

(A) Digestion of spleen iTNX with trypsin for 0 min, 4 h, and overnight (O/N). (a) Tryptic products of spleen iTNX detected by anti-M1 antibody (pAbM1) that recognizes other some FNIII repeats of iTNX as well as its own M1 repeat. After the digestion of iTNX, a product with the molecular weight of approximately 300 kDa (referred to as t300) was detected by Western blot analysis. (b) Tryptic products of spleen iTNX detected by anti-M31 antibody (pAbM31) that recognize the 31st FNIII repeat (M31) of TNX. A product with a molecular weight of approximately 70 kDa (referred to as t70) was detected by Western blot analysis. (B) Lectin blot profiles of tryptic digests of spleen iTNX using (a) Con A, (b) LCA, (c) PHA-E4, (d) RCA120, (e) WGA, (f) AAL, or (g) SSA. (C) Lectin binding properties of tryptic digests of spleen iTNX. ++, strong positive staining; +, positive staining; −, negative staining.

and that t300 is from the amino-terminal 300-kDa portion. Only WGA reacted to the t300 fragment and most of lectins other than WGA reacted to the t70 fragment.

Recently, we showed that 200-kDa sTNX was generated by proteolytic cleavage of spleen iTNX by spleen homogenate.[23] The 200-kDa sTNX contains C-terminal 15 FNIII repeats and a fibrinogen domain. Thus the t70 fragment from iTNX is included in the 200-kDa sTNX. However, the lectin reactivities as for Con A, LCA, and AAL to t70 fragment and sTNX are different. These results indicate that the cells in which sTNX is produced may be distinct from those of iTNX.

It is known that core fucosylation affects the biological functions of adhesion molecules and growth factors. Wang *et al.*[30] reported that core fucosylation regulates epidermal growth factor receptor (EGFR)-mediated intracellular signaling by governing affinity binding of EGFR to EGF. In the present study, we demonstrated that iTNX from the three tissues possesses α1,6-fucosylated trimannosyl core structures of *N*-glycan, whereas sTNX does not. There might be different binding affinities of iTNX and sTNX to some receptors.

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