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Non-helical type IV collagen polypeptides in human placenta

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Abstract

Our previous reports showed that cultured human cells secrete non-disulfide-bonded non-helical α1(IV) and α2(IV) chains under physiological conditions. In the present report we show that the α(IV) chains in non-helical form were reactive to lectin ABA (*Agaricus bisporus* agglutinin), whereas the α(IV) chains secreted in triple-helical form were not. These results indicate that ABA could be used to distinguish the two conformational isomers of type IV collagen polypeptides. An α1(IV) chain isolated from human placenta with an antibody-coupled column showed a positive reaction to ABA, indicating that gelatin form of the type IV collagen α1(IV) chain is produced and retained in the tissue in vivo. A possible significance of the gelatin form is discussed from the finding that the non-helical α1(IV) chain purified with EDTA-free buffer contained degraded polypeptides including NC1-size domain and showed an apparent inhibition against activated pro-MMP-9. This is the first report to show that a gelatin form of protein exists in vivo.

Keywords: Type IV collagen; Triple-helix; Glycosylation; Placenta; TIMP-like activity

Collagen is a major component of the extracellular matrix. It has a triple-helical structure consisting of three polypeptides called α chains. Denaturation of the collagen molecule results in the loss of its triple-helical structure and concomitantly yields separate single polypeptides with a random conformation. These denatured collagenous polypeptides are highly susceptible to proteolytic enzymes in vitro, and therefore, non-helical collagenous polypeptides are unexpected to exist stably in vivo [1,2].

Type I procollagen polypeptides that have failed to form stable triple-helical structures are prevented from leaving the endoplasmic reticulum (ER) and are degraded inside the cell through the quality control system or proteasome pathway [3–5]. Several groups have reported the production and secretion of non-helical α(IV) chains, but the results were considered to be attributable to artificial factors [6–8]. It has thus been considered to be a general biochemical rule that collagenous polypeptides without a triple-helical structure are not produced or secreted under physiological conditions.

However, in our studies on type IV collagen biosynthesis, under physiological conditions, all the cultured human cells that express the α1(IV) and the α2(IV) genes secreted non-helical, non-disulfide bonded α1(IV) and α2(IV) chains as well as triple-helical, disulfide-bonded type IV collagen molecules. Which conformational isomer of α1(IV) and α2(IV) chains is produced depends on the concentration of ascorbate in the cell culture medium [9,10]. Nevertheless, ascorbate does not affect the total amount of secreted α1(IV) or α2(IV) chains, since semi-quantification of the α1(IV) and α2(IV) chains by Western blotting is the same in the conditioned media with or without ascorbate. Ascorbate apparently influences post-translational events particularly the formation of the triple-helical type IV collagen but it does not affect the events prior to the translation. In addition, the secreted non-helical α(IV) chains remained without degradation in a culture medium for a prolonged period

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of several weeks (Yoshikawa et al, unpublished observations). These findings suggest for a possibility that the non-helical α(IV) chains are not only secreted but retained in the tissue. The purpose of the present study was to investigate the existence of the non-helical α(IV) chains in human placenta.

Conformation-specific antibodies have been used to identify two conformational isomers of type IV collagen polypeptides in culture media [10]. However, for the identification of an α(IV) chain isolated from tissue, the use of conformation-specific antibodies is inadequate, since the polypeptides might have suffered conformational change or damage during the extraction and analytical procedures. Any definitive method of identifying the conformation of tissue-derived α(IV) chains has not yet been established nor available.

In this report we show that only the non-helical α(IV) chains, but not α(IV) chains derived from the triple-helical type IV collagen molecule, were recognized by lectin ABA (*Agaricus bisporus* agglutinin). The lectin ABA reactivity was utilized to analyze by a biochemical method if the chain is isolated whether the non-helical α1(IV) chain in human placenta exists.

Materials and methods

Antibodies and lectins. The monoclonal antibody JK132, which is specific for the primary sequence of the human α1(IV) triple-helical domain [11], and JK199, which is specific for the triple-helical conformation of human type IV collagen consisting of α1(IV) and α2(IV) chains [12], were obtained from the Shiseido Research Center (Japan). The monoclonal antibody H11, specific for the sequence of the human α1(IV) NC1 (non-collagenous 1) domain, and H21, specific for the sequence of the human α2(IV) NC1 domain, have been established as described previously [13]. Biotin-conjugated lectins, *Agaricus bisporus* agglutinin (ABA), concanavalin A (Con A), peanut agglutinin (PNA), *Ricinus communis* agglutinin (RCA), soybean agglutinin (SBA), and wheat germ agglutinin (WGA) were purchased from Seikagaku Corporation (Japan).

Cells and cell culture. Human mesangial cells (HMC), human umbilical vascular endothelial cells (HUVEC), and human aortic smooth muscle cells (HASMC) were purchased from Clonetics (USA). Fibrosarcoma cells, HT-1080, were obtained from the Japan Health Sciences Foundation. Human fetal lung fibroblasts, TIG-1 [14], established at the Tokyo Metropolitan Institute of Gerontology, were provided by Dr. Kiyotaka Yamamoto.

The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 μg/ml streptomycin and 50 U/ml penicillin at 37 °C under humidified 5% CO_2–95% air. The cells were grown to subconfluence or confluence in 10% serum-containing media. The cultured media were removed and washed out with phosphate-buffered saline (PBS). The cells were then cultured with or without 2.0 mM asc 2-p (L-ascorbic acid 2-phosphate) (Wako Pure Chemical Industries, Japan) in serum-free media. The culture periods were 5 days for HMC, TIG-1, and HASMC, and 24 h for HT1080 and HUVEC. The conditioned media collected were subjected to SDS–PAGE (SDS–polyacrylamide gel electrophoresis) followed by immunoblotting or lectin blotting, lectin precipitation, or purification by affinity chromatography.

SDS–PAGE, immunoblotting, and lectin blotting. SDS–PAGE was performed using polyacrylamide at concentrations of 3.5% and 4.5% for the stacking and separating gels, respectively, under reducing or non-reducing conditions. To detect the degraded products of the non-helical α1(IV) chain, SDS–PAGE was performed using a 4–20% gradient gel without a stacking gel (Daiichi Pure Chemicals, Japan). Protein bands were visualized by staining with Coomassie brilliant blue (CBB)-R250 (Nacalai Tesque, Japan). Immunoblotting with the monoclonal antibodies, JK132, H11, and H21, was carried out as described previously [10]. The procedures for lectin blotting were essentially the same as those for immunoblotting except that the PBS and monoclonal antibodies were replaced by Tris-buffered saline (TBS) and the biotin-conjugated lectins, ABA, Con A, PNA, RCA, SBA, and WGA, respectively.

Purification of the triple-helical type IV collagen molecule and the non-helical α1(IV) chain. To produce the triple-helical type IV collagen molecule, cells were cultured with 2 mM asc 2-p in a serum-free medium. To produce the non-helical α1(IV) chain, cells were cultured without asc 2-p in a serum-free medium. The conditioned media collected were applied to JK199-coupled column for purification of the triple-helical type IV collagen molecule, and JK132-coupled column for purification of the non-helical α1(IV) chain, respectively, as described previously [10].

ABA lectin precipitation. ABA–Sepharose was prepared by incubating avidin-conjugated Sepharose (Pierce, USA) with biotin-conjugated ABA, followed by washing out of excess biotin-conjugated ABA. The ABA–Sepharose was then incubated for 2 h at room temperature with the HMC-conditioned media cultured with or without 2 mM asc 2-p. The ABA–Sepharose incubated with the media was washed three times with Tween–PBS (PBS containing 0.1% Tween 20). The bound and unbound fractions on ABA–Sepharose were subjected to immunoblotting with the antibodies, JK132 and H21.

Isolation of the non-helical α1(IV) chain from human placenta. Human placenta homogenate, 3 g wet weight, was repeatedly washed with PBS to remove any blood, followed by suspension in a 50 mM Tris–HCl buffer (pH7.5) containing 1% NP-40, 200 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1 μM pepstatin A, and 0.5 mg/ml sodium azide with stirring for 48 h at 4 °C. The placental tissue suspension was then centrifuged at 12,000*g* for 30 min. The supernatant was applied to JK132-coupled column, and the bound fraction was eluted with 0.2 M glycine–HCl (pH 2.5), containing 0.2 M NaCl, and subjected to blotting with JK132 and ABA.

Reverse zymography of the non-helical α1(IV) chain purified on an antibody-coupled column. The conditioned medium cultured without asc 2-p was applied to JK132-coupled column. The whole procedure of affinity column chromatography was done using an EDTA-free buffer. The eluted fraction was subjected to reverse zymography essentially according to the method developed by Hattori et al. [15]. The separating gel was composed of 12.5% acrylamide, 0.5 mg/ml fluorescein isothiocyanate (FITC) pre-labeled heat-denatured type I collagen, and 3 μg/ml pro-matrix metalloproteinase (MMP)-9 (Genzyme, USA). After electrophoresis, the gel was washed three times with 2.5% Triton-X for 20 min and then incubated with a reaction buffer composed of 50 mM Tris–HCl (pH 7.5), 200 mM NaCl, and 5 mM $CaCl_2$ at 37 °C. The gel was photographed under an UV illuminator when activated pro-MMP-9 digestion decreased the fluorescence intensity to an appropriate level.

Results

Reactivity of H11 and ABA to α1(IV) chains

The triple-helical type IV collagen molecule and the non-helical α1(IV) chain were purified from the HMC-conditioned media with JK199-coupled and JK132-coupled columns, respectively. The purified triple-helical

type IV collagen molecule and the purified non-helical α1(IV) chain were subjected to reactions with the monoclonal antibodies, JK132 and H11, and the lectins, ABA, Con A, PNA, RCA, SBA, and WGA. The monoclonal antibody JK132, which is specific for the primary sequence in the triple-helical region of the α1(IV) chain, reacted with both the triple-helical type IV collagen molecule-derived α1(IV) chain and the non-helical α1(IV) chain in the blotting analysis (Fig. 1Aa, lanes 1 and 2). As previously reported [10], the monoclonal antibody H11, which is specific for the NC1 domain of the α1(IV) chain, reacted with the α1(IV) chain derived from the triple-helical type IV collagen molecule, but not with the non-helical α1(IV) chain (Fig. 1Ac, lanes 1 and 2). The H11 epitope, KKPTPSTL [13], in the non-helical α1(IV) NC1 domain might be masked or removed. Since serine and threonine residues contiguous to proline residue in a primary sequence are known to be possible O-glycosylation sites [16], the reactivity of the non-helical α1(IV) chain to commercially available lectins was examined.

None of the lectins except ABA reacted with the α1(IV) chains (data not shown). Lectin ABA, specific for the oligosaccharides structure Galβ1-3GalNAc [17], reacted with the non-helical α1(IV) chain only (Fig. 1Ab, lane 2). It did not react with the α1(IV) chain derived from the triple-helical type IV collagen molecule (Fig. 1Ab, lane 1).

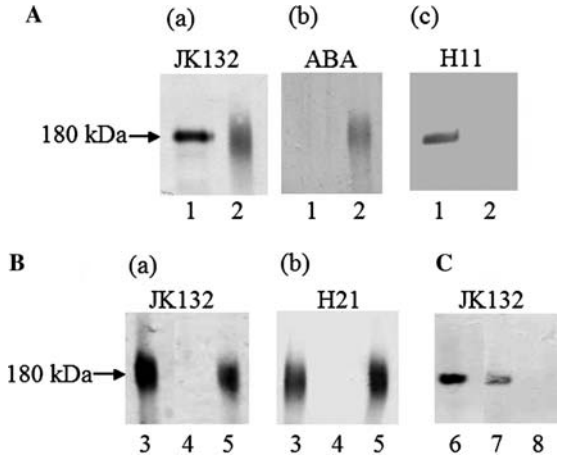

Fig. 1. Reactivity of α(IV) chains with monoclonal antibodies and lectin ABA. Human mesangial cells were cultured for 5 days either with 2 mM asc 2-p to accumulate the triple-helical type IV collagen molecule in the medium (conditioned medium asc+), or without asc 2-p to accumulate the non-helical α(IV) chains in the medium (conditioned medium asc−). (A) The triple-helical type IV collagen molecule and the non-helical α1(IV) chain were purified from the conditioned media with antibody coupled columns and subjected to blotting analysis with JK132 (a), lectin ABA (b), and H11 (c) under reducing conditions. Purified triple-helical type IV collagen molecule (lane 1); Purified non-helical α1(IV) chain (lane 2). (B) The conditioned medium asc− was mixed with ABA–Sepharose. The starting material (lane 3), ABA-unbound fraction (lane 4), and ABA-bound fraction (lane 5) were subjected to immunoblotting with JK132 (a) and H21 (b) under reducing conditions. (C) The conditioned medium asc+ was mixed with ABA–Sepharose. The starting material (lane 6), ABA-unbound fraction (lane 7), and ABA-bound fraction (lane 8) were subjected to immunoblotting with JK132 (c) under reducing conditions. (SDS–PAGE was performed using 4.5% acrylamide for the separating gel).

The conditioned medium of HMC cultured without asc 2-p contained only the non-helical α1(IV) and α2(IV) chains, as shown in Fig. 1Ba and b, lane 3. Contrary to the non-reactivity of H11 to the non-helical α1(IV) chain, the monoclonal antibody H21, which is specific for the NC1 domain of human α2(IV) chain, reacted with the non-helical α2(IV) chain as well as with the α2(IV) chain derived from the triple-helical type IV collagen molecule, as previously noted [10]. The conditioned medium of HMC cultured with 2 mM asc 2-p contained only the triple-helical type IV collagen molecule, as shown in Fig. 1C, lane 6. ABA–Sepharose was added to each of the conditioned media to fractionate them into ABA-bound and ABA-unbound fractions. The fractions were then subjected to blotting with the antibodies JK132 (Figs. 1Ba and C) and H21 (Fig. 1Bb). The non-helical α1(IV) and α2(IV) chains were recovered from the bound fraction, but not from the unbound fraction (Fig. 1Ba and b, lanes 5 and 4). In contrast, the triple-helical type IV collagen molecule was recovered only from the unbound fraction, not from the bound fraction (Fig. 1C, lanes 7 and 8). These results indicate that there is a strict reciprocal relationship between the triple-helical structure of type IV collagen and ABA reactivity. ABA bound with the non-helical α(IV) chains, but not with the triple-helical type IV collagen molecule nor with the heat-denatured α1(IV) and α2(IV) chains.

Reactivity of ABA to α1(IV) chains produced by other human cells

The reactivity of ABA to α1(IV) chains produced by cells other than HMC was examined. The triple-helical type IV collagen molecule and the non-helical α1(IV) chain were purified with JK199-coupled and JK132-coupled columns, respectively, and subjected to ABA lectin blotting (Fig. 2B) together with JK132 immunoblotting (Fig. 2A) after culturing human cells other than HMC; i.e., embryonic lung fibroblasts, TIG-1 (Fig. 2, lanes 3 and 4); fibrosarcoma cells, HT-1080 (Fig. 2, lanes 5 and 6); umbilical endothelial cells, HUVEC (Fig. 2, lanes 7 and 8); and aortic smooth muscle cells, HASMC (Fig. 2, lanes 9 and 10). ABA reacted with the non-helical α1(IV) chains secreted by all the cell types (Fig. 2B, lanes 2, 4, 6, 8, and 10), whereas it did not react with α(IV) chains derived from the triple-helical type IV collagen molecules secreted by any of the cell types (Fig. 2B, lanes 1, 3, 5, 7, and 9). The results indicate that ABA reactivity is exclusively correlated to the non-helical form of the α1(IV) chain regardless of cell type.

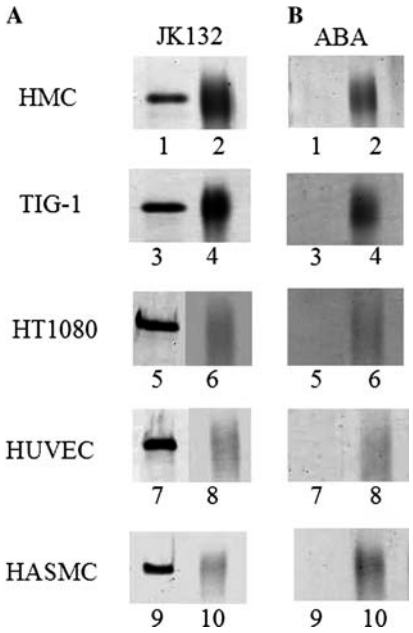

Fig. 2. Cell type-independent ABA reactivity of the non-helical α1(IV) chain. The triple-helical type IV collagen molecule (odd-numbered lanes) and the non-helical α1(IV) chain (even-numbered lanes) were purified with antibody-coupled columns from the conditioned media of different cells cultured with and without 2 mM asc 2-p, respectively. Cell types examined were as follows: HMC (lanes 1 and 2), TIG-1 (lanes 3 and 4), HT1080 (lanes 5 and 6), HUVEC (lanes 7 and 8), and HASMC (lanes 9 and 10). The purified polypeptides were subjected to blotting analysis with JK132 (A) and ABA (B) under reducing conditions. (SDS–PAGE was performed using 4.5% acrylamide for the separating gel).

The non-helical α1(IV) chain from human placenta

The human placenta tissue homogenate suspension in Tris-buffer with 200 mM NaCl and 5 mM EDTA was spun down. The supernatant fraction was applied to the JK132-coupled column. The bound fraction was subjected to reactions with JK132 and lectin ABA. A 180-kDa band in the bound fraction showed the same molecular weight as the non-helical α1(IV) chain purified from the HMC-conditioned media (Fig. 3A, lanes 1 and 2), and had positive reactions with both the monoclonal antibody JK132 and ABA (Figs. 3A and B, lane 2). The superimposed reactivity of both JK132 and ABA indicates that the smeared band at 180 kDa was derived from the non-helical α1(IV) chain produced and sustained in the human placental tissue.

Characterization of the non-helical α1(IV) chain recovered from the affinity column with EDTA-free elution buffer

Affinity chromatography of the JK132-coupled column with the EDTA-free buffer yielded peptides with sizes of 23 and 19 kDa corresponding to the NC1 domain in size, together with bands of 180 and 90 kDa

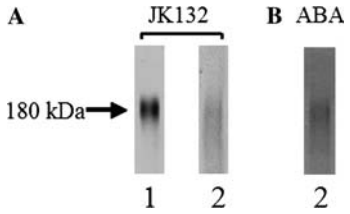

Fig. 3. The α1(IV) chain isolated from the human placenta. The placenta homogenate was suspended in a 50 mM Tris–HCl buffer (pH 7.5), containing 1% NP-40, 200 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1 μM pepstatin A, and 0.5 mg/ml sodium azide with stirring for 48 h at 4 °C. The placenta tissue suspension was centrifuged and the supernatant was applied to JK132-coupled column. The JK132-bound fraction (lane 2) in the human placenta suspension was subjected to blotting with JK132 (A) and ABA (B) under non-reducing conditions. The isolated polypeptide(s) migrated as a 180-kDa band similar in size, but with more extensive smearing in comparison with the non-helical α1(IV) chain purified from the HMC-conditioned medium (lane 1). SDS–PAGE was performed using 4.5% acrylamide for the separating gel.

in the bound fraction (Fig. 4A). Since affinity chromatography of the JK132-coupled column with the EDTA-containing buffer yielded only the 180-kDa non-helical α1(IV) chain, divalent cation-dependent enzymes, presumably bound with the non-helical α1(VI) chain in the column, might have been activated and might have cleaved the non-helical α1(IV) chain during or after the purification step using immuno-affinity column chromatography. The 23- and 19-kDa bands showed apparent inhibition to the activated pro-MMP-9 (Fig. 4B). The 23-kDa band with less intensity in CBB staining showed a higher inhibition than the 19-kDa band. In addition, two bands corresponding to a 180-kDa band

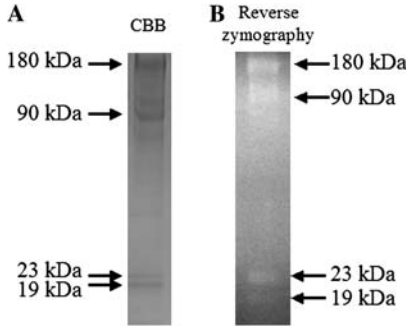

Fig. 4. TIMP-like activity of the non-helical α1(IV) chain. The whole procedure of affinity chromatography using the JK132-coupled column was done with an EDTA-free buffer. The eluted fraction contained degraded polypeptides with sizes of 23 and 19 kDa corresponding to NC1 domains, together with the 180-kDa full-length non-helical α1(IV) chain and a fragment of about 90-kDa in CBB staining (A). The eluted fraction was applied to reverse zymography using MMP-9. NC1 domain-sized polypeptides, as well as the 180-kDa full-length non-helical α1(IV) chain and the 90-kDa fragment, showed TIMP-like activity (B). (SDS–PAGE for CBB staining was performed using 4-20% gradient gel. SDS–PAGE for reverse zymography was performed using 12.5% acrylamide for the separating gel.)

corresponding to the full-length of the non-helical α1(IV) chain and a band of about 90 kDa, presumably a fragment from the non-helical α1(IV) chain, also showed apparent inhibition as detected by the reverse zymography (Fig. 4B).

Discussion

Our previous reports indicate that secretory forms of type IV collagen gene products depend on the ascorbate level in the culture medium. Two conformational isomers of type IV collagen polypeptides are produced by the cells reversibly: the non-helical α(IV) chains in the absence of ascorbate, whereas the triple-helical type IV collagen molecule in the sufficient concentration of ascorbate [10]. The results suggest a possibility that the non-helical α(IV) chains are produced and sustained as gelatin form in the tissue. However, proof for the existence of the non-helical α1(IV) chain in tissue encounters with methodological difficulties, since extracting it from tissue requires several steps that could change the triple-helical conformation of type IV collagen to yield partially denatured or non-helical conformation. In the present report, since lectin ABA binding was found to be a decisive method to identify the non-helical α1(IV) chain from the experiments with cell culture, the reaction with lectin ABA was applied to the isolated α1(IV) chain from human placental tissue human placenta.

The triple-helical structure of type IV collagen was always correlated with the interchain disulfide bonds between the α(IV) chains [9]. Furthermore, the triple-helical structure was always reciprocally correlated with ABA reactivity (Fig. 1Aa and b). Any intermediate form of α(IV) chain, such as ABA-reactive triple-helical type IV collagen molecules or non-ABA-reactive non-helical α(IV) chains, has never been observed, even after culturing cells alternately with or without ascorbate (Figs. 1B and C). This indicates that both the biosynthesis and secretion of the two conformational isomers are strictly regulated.

Thus, the positive reaction with ABA provides a decisive evidence that α(IV) chains are produced and secreted in a non-helical form, since it distinguishes between the two conformational isomers. Combinatorial use of JK132 and ABA provides good evidence for the tissue existence of the non-helical α1(IV) chain regardless of the manipulations involved in any isolation procedures that cannot totally exclude partial degradation or denaturation.

ABA recognizes the O-glycan structure Galβ1-3GalNAc [17]. One concern about using ABA to distinguish between the two conformational isomers was polymorphism of the oligosaccharide structure among the non-helical α(IV) chains. However, this concern is most unlikely, since the reactivity of ABA to the non-helical α(IV) chains was always observed regardless of the cell type (Fig. 2).The ABA-reactive 180-kDa polypeptide isolated and purified on JK132-coupled column from human placenta was concluded to be the α1(IV) chain produced and sustained in a non-helical form (Fig. 3). This is the first finding that a gelatin form of protein exists in the human body.

What then would be a biological significance of the non-helical α(IV) chains existing in tissues? The NC1 domains of the basement membrane collagens, type IV, type VIII, type XV, and type XVIII, are reported to have anti-angiogenetic activity and/or TIMP-like activity [18–22]. The inhibitory activity against the activated proMMP-9 was found for the 23 and 19-kDa NC1 domain size polypeptides (Fig. 4B), derived from the non-helical form (naturally gelatin form) of α1(IV) chain, suggesting that non-helical α1(IV) chain existing in the placenta might be involved in the regulation of pro-MMP-9 activity in terms of the localization through binding or inhibition against the activated enzyme.

The monoclonal antibody H11 reacts with the α1(IV) chain in the triple-helical but not with the α1(IV) chain in the non-helical form (Fig. 1Ab and c). The reciprocal reactions of ABA and H11 with α1(IV) chain can be most simply explained by the O-glycosylation of the H11 epitope sequence, KKPTPSTL [13], in the non-helical α1(IV) chain since proline residues are found with considerable frequency around O-glycosylation sites [16]. In contrast to the non-reactivity of H11 to α1(IV) chain, H21 reacts with α2(IV) chains in both triple-helical and non-helical forms [10]. This difference in the reactivities of H11 and H21 to the non-helical α1(IV) and α2(IV) chains might be explained by the presence or absence, respectively, of O-glycan in their epitopes.

α1(IV) and α2(IV) chains are able to escape from the quality control system and are secreted even when they do not take a triple-helical structure [9,10]. All the secreted non-helical α(IV) chains were always ABA-reactive. ABA-non-reactive non-helical α(IV) chains were never observed (Fig. 1B), suggesting that the formation and/or structure of ABA-reactive O-glycan might be involved in the secretory regulation of the protein as Huet et al. [23] proposed.

In this report, we have presented strong evidence for the existence of the non-helical α1(IV) chain in vivo. Further study on the non-helical α(IV) chains in terms of their biosynthetic regulation and interaction with other proteins might bring better understanding of the dynamic regulation of the structure and function of type IV collagen.

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