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Preparation and partial characterization of monoclonal antibodies specific for the nascent non-triple helical form of the type IV collagen alpha 1 chain

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ABSTRACT

This report describes the preparation and partial characterization of monoclonal antibodies that are reactive specifically with the nascently produced non-triple helical form of the type IV collagen α1 chain, designated as NTH α1(IV). These antibodies were nonreactive with the α1 chain of the type IV collagen in the triple-helical conformation. Three antibodies, #141, #179 and #370, with different epitopes in NTH α1(IV) were found to be reactive with the nascent polypeptide secreted from human normal cells and a human carcinoma cell line. The antibodies with different epitopes may provide a key method for elucidating the physiological function and tissue distribution of NTH α1(IV), which is distinct from the chain derived from triple-helical type IV collagen.

1. Introduction

Type IV collagen, a major protein in the basement membrane, forms a flat mesh-like network structure, and contributes to the stability and importantly the mechanical strength of the basement membrane [1]. There are six kinds of polypeptide chains from α1(IV) to α6(IV) grouped in the type IV collagen family, which form three kinds of triple helical structures that are composed of different chain arrangements: α1α1α2, α3α4α5α and α5α5α6. The α1α1α2 type IV collagen is found in most basement membranes. Each type IV collagen α chain consists of a collagenous region called the triple helical domain and a globular region called the noncollagenous domain 1 (NC1). Type IV collagen molecules in the triple-helical form are stabilized by interchain disulfide bonds present in the N-terminal part of the triple helical domain, called the 7S domain, whereas the NC1 domain on each chain contains intrachain disulfide bonds [2].

During angiogenic processes, endothelial cells and/or other cells including pericytes secrete type IV collagen, which form the outer basement membrane of vessel walls. Tumor cells secrete metalloproteases that digest components of the basement membrane, including type IV collagen and/or metastasize into other tissues [3]. Some of the peptides derived from type IV collagen are reported to promote expansion and angiogenesis of tumor tissues, affecting tumor cell adhesion and metastasis [4]. Arresten and canstatin, comprising the NC1 domains of α1(IV) and α2(IV) chains, respectively, are reported inhibitors of angiogenesis and tumor growth [5,6]. Further studies are needed to clarify the mechanisms of how anti-angiogenic peptides are generated in vivo from type IV collagen and how they are involved in angiogenic processes.

In general, type IV collagen gene products are triple helical molecules that are secreted from cells and deposited into basement membranes. We have reported the existence of a non-triple helical form of type IV collagen α1 and α2 chains in cultured media as well as human placenta extracts as type IV collagen gene products with essential differences in chemical structure, or reduced post-translational hydroxylation and more glycosylation [7,8]. These polypeptides are designated as NTH α1(IV) and NTH α2(IV), respectively, or collectively NTH α(IV)s hereafter. Furthermore, ascorbate depletion in cultured cells increased the secretion of NTH α(IV)s and inversely decreased that of triple helical molecules. Since the level of prolyl hydroxylation in NTH α1(IV) was approximately one fifth of that in triple helical molecule, it is thought that insufficient hydroxylation destabilizes triple helix formation [9]. We have found, however, that the non-triple helical polypeptides of type IV collagen were able to escape the “quality control system” of collagen that has been proposed from studies for type I collagen biosynthesis [10–12]. NTH α(IV)s bind

Abbreviations: CNT, control; FCS, Fetal Calf Serum; IP, immunoprecipitation; 2ME, 2-mercaptoethanol; NTH α1(IV), non-triple helical form of the type IV collagen α1 chain; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; WB, western blotting

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mushroom lectin (*Agaricus bisporus* agglutinin, ABA), which recognizes Galβ1-3GalNAC, indicating that NTH α(IV)s are glycated with O-linked oligosaccharides. However, ABA did not bind to chains derived from triple-helical type IV collagen [8], indicating that NTH α(IV)s with reduced hydroxylation and additional glycosylation are chemically distinct from polypeptides in the triple helical molecule.

Since NTH α(IV)s contain NC1 domains that promote and inhibit vessel growth, one of our interests is in understanding the possible roles NTH α(IV)s during angiogenic processes. A recent immunohistological study showed unique localization of NTH α1(IV) in the neovascular tip of a rabbit angiogenic model, suggesting physiological roles in relation to the dynamics of the vascular system [13].

In this report, the preparation of three kinds of mouse monoclonal antibodies (#141, #179 and #370) that can recognize only NTH α1(IV) at different recognition sites, but not the triple helical molecule, are presented. These antibodies will facilitate investigation of the biological roles of NTH α1(IV).

2. Materials and methods

2.1. Cell culture and preparation of medium containing NTH α1(IV)

The human hepatocellular carcinoma cell line (HLF; Riken Cell Bank, Japan) was cultured in RPMI 1640 (Mediatech, Manassas, VA, USA) with 10% FCS (Tissue Culture Biologicals, Tulare, CA, USA) under 5% CO_2 at 37 °C. After reaching confluence, the culture medium was changed to RPMI 1640 without FCS. The conditioned media cultured for 5–7 days were used for NTH α1(IV) preparation.

2.2. Antibodies and type IV collagens

Anti-type IV collagen rabbit polyclonal antibody, Ab6586, was purchased from Abcam (Tokyo, Japan). Mouse monoclonal antibody, JK132, was prepared using human placenta collagen IV as the antigen [14] and recognizes only a specific sequence of the human α1 (IV) chain of the helical domain in a nonhelical conformation [15]. Thus, as JK132 only binds NTH α1(IV) but not triple-helical type IV collagen, affinity chromatography with immobilized JK132 was used to purify NTH α1(IV) specifically [9].

Bovine type IV collagen with the intact NC1 domain was extracted from bovine lens capsules with acetic acid without enzymatic treatment [16,17]. Pepsin-treated human placenta type IV collagen was purchased from Sigma-Aldrich (Tokyo, Japan).

2.3. Preparation and analysis of purified NTH α1(IV)

The cultured medium of HLF cells was used for purification of NTH α1(IV) using an affinity chromatography column that contained JK132 immobilized to HiTrap NHS-activated HP (GE Healthcare, Tokyo, Japan). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using XV Pantera MP Systems (DRC, Tokyo, Japan) according to Laemmli [18]. Briefly, samples were mixed with SDS sample buffer to a final concentration of 2% SDS, 10% glycerol and 0.0625 M Tris-HCl (pH 6.8). The samples were boiled at 90 °C for 5 min and subjected to electrophoresis. After electrophoresis, gels were silver stained with the Silver Stain Kit II (Wako chemicals, Tokyo, Japan). Amino acid analysis and mass spectrometry of purified NTH α1(IV) were conducted by the Nippi Research Institute of Biomatrix (Ibaraki, Japan).

2.4. Immunoprecipitation and western blotting

Immunoprecipitation was carried out as follows. Briefly, after incubation of mouse monoclonal antibodies with the supernatant of HLF cultured medium, which contains both NHT α1(IV) and type IV collagen (antigens 1 and 2 in Table 2), the complexes were incubated with anti-mouse IgG goat antibody conjugated to Dynabeads (Veritas, Tokyo, Japan) and this was followed by precipitation with magnets. Precipitated samples were analyzed by SDS-PAGE. For antigens 1 and 2, the anti-collagen type IV polyclonal antibody (Ab6586) was used for detection of type IV collagen by western blotting, which was performed according to the manufacturer's manual. Briefly, separated proteins on SDS-PAGE gels were blotted onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA), blocked with 5% (weight/volume) skim milk, incubated with antibodies and washed with a Tris-buffered saline-Tween solution (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% Tween20). The bound antibodies were detected by horseradish peroxidase-labeled anti-mouse or rabbit IgG antibody and developed by an enhanced chemiluminescent substrate and analyzed.

2.5. Sandwich enzyme-linked immunoabsorbent assay (ELISA)

Sandwich ELISA were carried as follows. Ninety-six-well microtiter plates (Maxisorp, Nunc, Rochester, NY, USA) were coated with 100 μL/well of the capture antibody (5 μg/mL in phosphate-buffered saline (PBS)) overnight at 4 °C and then blocked with 200 μL/well of 1% bovine serum albumin in PBS-Tween20 (0.05%) for 1 h at 37 °C. Thereafter, 50 μL of the HLF cultured medium was added, followed by incubation for 1 h at 37 °C. As a third step, 50 μL/well of the detection antibody–horseradish peroxidase (0.2 μg/mL) was added and incubated for 1 h at room temperature in the dark. Between one incubation step and the next, the plates were washed four times with PBS-Tween20 (0.05%). Finally, 100 μL/well of the substrate (tetramethylbenzidine) solution was added. The reaction was stopped after 5 min with 100 μL 0.5 M H_2SO_4. The optical densities were measured at 450 nm with an ELISA plate reader (Biotrak visible plate reader, Biorad, Hercules, CA, USA).

2.6. Monoclonal antibodies screening

Monoclonal antibodies were produced by Bio Matrix Research Inc. (Chiba, Japan) using its proprietary technology CELIXSYS™. Briefly, purified NTH α1(IV) was injected with Freund's adjuvant into three mice using three different conditions. After detection of the anti-NTH α1(IV) serum, the spleens were extracted for preparation of B-cells. B-cells were fused with myeloma cells to produce hybridoma cells. Hybridomas that produced an antibody reacting with NTH α1(IV), but not with bovine type IV collagen, were prepared. Antibodies produced by hybridoma cells were subjected to further selection in order to obtain antibodies that have higher affinity than JK132. Competitive sandwich ELISA was carried out for the selected antibodies to detect the steric relations of the epitopes.

Isotyping of selected antibodies were performed by sandwich ELISA using anti-mouse IgG goat polyclonal antibody (DAKO, Tokyo, Japan) as a capture antibody and anti-mouse isotype specific antibodies as detection antibodies (HRP Conjugated Goat anti-Mouse Detection Antibodies for IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM, Bethyl Laboratories, Inc, Montgomery, TX).

3. Results and discussion

3.1. Composition of amino acids of purified single chain collagen type IV NTHα1 (IV)

Purified NTH α1(IV) showed a main band in the SDS-PAGE analysis and corresponded to a protein molecular weight of 180 kDa, which matched the band detected with the JK132 monoclonal antibody by western blotting (Fig. 1). The triple helical molecule of type IV collagen is a heterotrimer consisting of two α1(IV) chains and one α2(IV) chain, and appears on an SDS-PAGE under nonreducing condition as a protein of ~500 kDa, because the three chains are interconnected by disulfide bonds. As shown in Fig. 1, a 500 kDa band

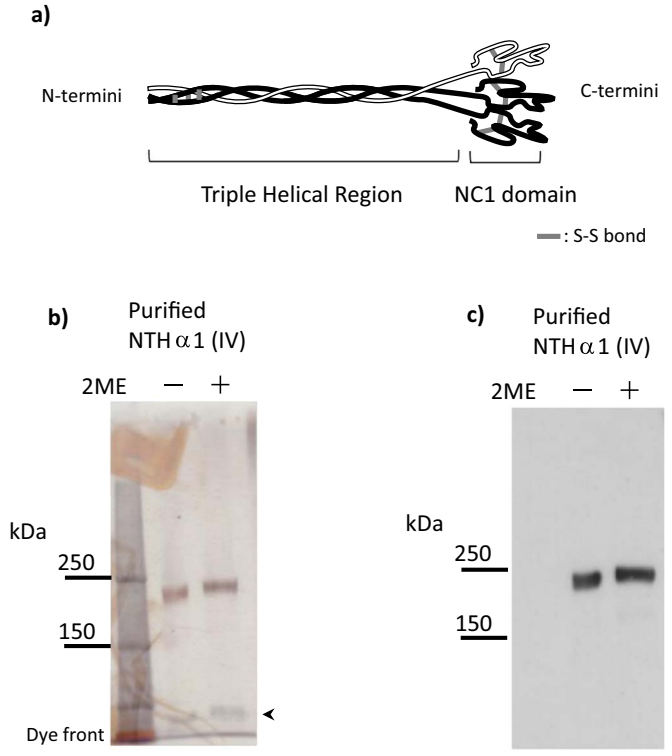

Fig. 1. Electrophoresis and western blotting analysis of purified NTH α1(IV). Purified protein by the affinity column method using JK132 as a ligand was analyzed by electrophoresis and western blotting. (**a**) A diagram of type IV collagen molecule. The molecule is divided to the triple helical region in N-terminal part and NC1 domain 1 in C-terminal part. Inter chain disulfide bondings near N-termini and intra chain disulfide bondings in NC1 domain are shown in the figure. (**b**) Silver staining of the purified NTH α1(IV) after electrophoresis. Affinity purified NTH α1(IV) was electrophoresed in 4.5% PAGE gel and stained with silver (Wako pure chemical industries, ltd., Tokyo, Japan) according to a manufacturer's manual. A single 180 kDa band was stained. (**c**) Western blot analysis was performed with JK132. The electrophoresis conditions were same as those of the purified NTH α1(IV). Only one single band was detected at the same molecular size of the silver stained band.

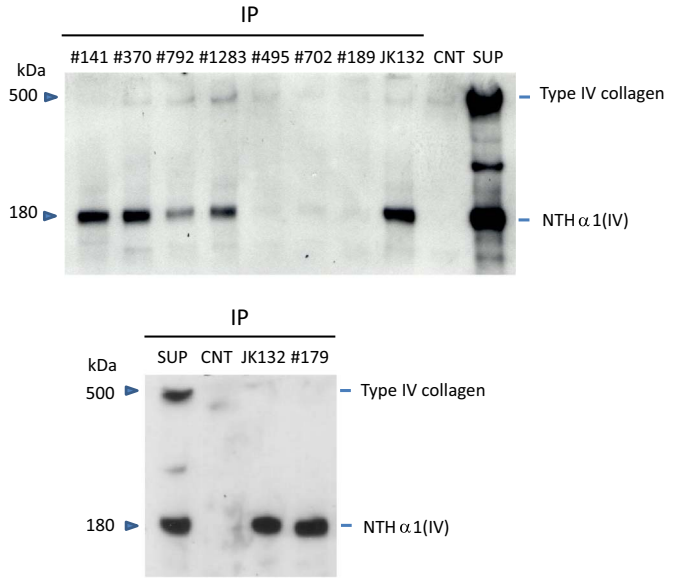

Fig. 2. Screening of antibodies by immunoprecipitation and western blotting. Three micrograms of each antibody designated in the figure was incubated with the supernatant of HLF cell cultured medium and the antibody was precipitated with anti-mouse IgG antibodies. The precipitates were subjected to western blotting under nonreducing condition. A polyclonal antibody for human type IV collagen, Ab6586, was used to detect NTH α1(IV) and type IV collagen polypeptides. A band migrating at 180 kDa on the blot is NTH α1(IV). A band migrating at ~500 kDa corresponds to a trimer of three α chains of the type IV collagen molecule, which are connected by interchain disulfide bondings. IP, immunoprecipitation; SUP, supernatant; CTN, control. (**a**) In the first screening, immunoprecipitates of seven prepared antibodies #141, #370, #792, #1283, #495, #702 and #189, were tested for the samples developed on 4.5% SDS-PAGE under nonreducing conditions. Three antibodies, #141, #370 and #1283, were selected for further analysis by sandwich ELISA to distinguish overlapping epitopes. (**b**) In the second screening, only #179 was selected because of its unique binding characteristics, as described in Table 2.

was not detected by electrophoresis under nonreducing conditions. A faint band also was detected just above dye front on SDS-PAGE shown by an arrow head in Fig. 1b, but not with the western blotting.

Amino acid analysis of the NTH α1(IV) preparation showed a high content of glycine and proline amino acids, which are typical for collagenous proteins (Supplemental Table 1). Comparison of the amino acid composition of purified NTH α1(IV) with that of type IV collagen from bovine lens capsule indicates that the ratios of hydroxyproline and hydroxylysine of triple helical collagen from bovine lens capsule are 59% and 74%, respectively, while those of NTH α1(IV) were 9% and 24%, respectively. The results are consistent with those reported by Yoshikawa *et al.*[9] and suggest that NTH α1(IV) and type IV collagen are chemically different proteins. Mass spectrometric analysis of the preparation identified several peptides including the type IV collagen α1 chain sequence although the analysis covered only limited parts (Supplemental Figure S1). This purified NTH α1(IV) preparation was used as the antigen for the production of monoclonal antibodies.

3.2. Selection of antibodies

In total, 49 of the hybridoma clones were established and the supernatant of each clone was used for monoclonal antibody selection. In order to select antibodies specific for NTH α1(IV), we performed immunoprecipitation followed by western blotting using the cultured medium of HLF cells containing both NTH α1(IV) and type IV collagen (Fig. 2). In the first screening we performed immunoprecipitation to analyze seven clones (Fig. 2a). Four clones, namely #141, #370, #792 and #1283, were positive. Since the antibody #792 showed only a weak signal, only the other three clones that showed strong signals were selected (Fig. 2a). Next, the steric relationship of the epitopes among #141, #370, #1283 and JK132 were investigated by sandwich ELISA. In sandwich ELISA, a shorter distance between epitopes on the antigen for each pair of capture and detection antibodies leads to a weaker signal because the monoclonal antibodies compete with each other. In the case of #141 and #1283, the signals showed ca. 0.2, which are the same value when using a combination of only one monoclonal antibody , 0.21 for #141 and 0.14 for #1283(Table 1a). This result suggests that the binding of #141 to NTH α1 (IV) sterically hinder #1283 binding and vice versa. The two antibodies may compete for the same epitope. The combination of #141 with other antibodies showed stronger signals than the combination with #1283. We selected #141 and stopped further analysis of #1283.

In the second screening, antibody #179 was selected among 42 clones analyzed. The antibody was compared to 3 antibodies from the first screening which had been well characterized prior to the second screening. As shown in Table 1b, combinations of only one monoclonal antibody gave weaker signals of ca. 0.2 but all pairs of different antibodies gave stronger ones of more than 1.0. Since the relationship of signal intensities were consistent between the two experiments except for a combination of #141 and #1283, a cutoff value for the second experiment was set to 1.0 although it was less than 2.0 for the first experiment probably due to experimental errors. The result clearly showed that #179 has a different epitope. In total, three novel anti-NTH α1(IV) antibodies, #141, #179 and #370, were further characterized.

Table 1
Sandwich ELISA to distinguish binding sites of antibodies.

(a) Sandwich ELISA for the first screening of antibodies.

	Clone#	HRP-labeled detection antibodies (0.2 μg/mL)				
		JK132	#141	#1283	#370	Buffer
Capture antibodies (5 μg/mL)	JK132	0.27	3.35	2.66	3.32	0.04
	#141	3.28	0.21	0.16	2.85	0.04
	#1283	3.44	0.19	0.14	2.86	0.04
	#370	3.65	3.65	3.04	0.19	0.04
	Buffer	0.09	0.13	0.06	0.09	0.04

(b) Sandwich ELISA for #179 to compare with selected antibodies from the above.

	Clone#	HRP-labeled detection antibodies (0.2 μg/mL)		
		JK132	#141	#370
Capture antibodies (5 μg/mL)	JK132	0.27	1.84	2.06
	#141	1.35	0.15	1.22
	#370	1.12	1.13	0.11
	#179	1.06	1.49	1.53

3.3. Characterization of #141, #179 and #370 antibodies

In this study we selected three monoclonal antibodies that do not react with the triple-helical form of type IV collagen, but only with the nascent form of NTH α1(IV): #141, #179 and #370. Monoclonal antibodies #141, #179 and # 370 were assessed as the IgG class with the subclasses IgG2b, IgG1 and IgG1, respectively. In order to characterize the epitopes of these monoclonal antibodies, reactivities to type IV collagen α1 chains that are different in structure and conformation were analyzed by immunoprecipitation and western blotting. The results are summarized in Table 2.

Reactions of #141 against these α1 chains (cultured medium of HLF) are the same as those of JK132 as shown in the table. Both react with SDS denatured NTH α1(IV), the denatured form of pepsin digested collagen type IV, reduced NTH α1(IV) and the native form of NTH α1(IV). As shown in Table 2, #141 recognizes all antigens except antigen 1 or the triple-helical form of type IV collagen. As #141 recognizes the denatured form of pepsin-digested type IV collagen, the epitope of the antibody is postulated to be within the collagenous domain. Triple helix structure in collagen molecules is resistant to protease digestion but non-collagenous domains are sensitive to the digestion. In type IV collagen, NC1 domain is cleaved even under a mild condition of pepsin digestion and the triple helical region is partially sensitive to the protease because there are more than 20 interruptions in collagenous sequences [19,20]. The pepsin-digested type IV collagen preparation lacks NC1 domain but contains collagen fragments with various size. Both of #141 and JK132 recognize epitopes with the triple helical region but the amino acid sequences of the epitopes are different as shown by the competitive sandwich ELISA in Table 1a. The epitope sequences for JK132 were reported as KGEPGLPGRGFPGFP [15] and those for #141 were PGIGTPLRG in NTH α1(IV) (for details, manuscript in preparation).

Antibody #179 reacted with native NTH α1(IV) as shown by immunoprecipitation. This antibody reacted with both reduced and nonreduced forms of NTH α1(IV). However, the antibody reacted with neither the SDS denatured form of native type IV collagen nor pepsin-treated type IV collagen. Antibody #179 reacts with antigens 2, 5 and 6 but not with antigens 1, 3 or 4 (Table 2). Antigens 3 and 4 are derived from triple-helical type IV collagen. Thus, antibody #179 recognizes specifically only the nascent form of NTH α1(IV).

Antibody #370 reacted with NTH α1(IV) and only scarcely with the triple helical molecule of type IV collagen, as shown by immunoprecipitation (Fig. 2), where a faint band at 500 kDa corresponding to type IV collagen was observed. Since the intensity of the band was very weak and immunoprecipitation with the other antibodies showed essentially the same faint band, we assumed that the band was due to weak

Table 2
Characters of antibodies.

No.	Antigen	Conformation of antigen	Methods	Reactivity			
				JK132	#141	#179	#370
1	Native type IV collagen		IP	–	–	–	–
2	Native NTHα1(IV)		IP	+	+	+	+
3	Unfolded type IV collagen		WB (non-reduced)	+	+	–	+
4	Pepsin-digested type IV collagen		WB (non-reduced)	+	+	–	–
5	Unfolded NTHα1(IV)		WB (non-reduced)	+	+	+	+
6	Reduced NTHα1(IV)		WB (reduced)	+	+	+	–

IP: immune-precipitation; WB: wectern blotting; ━ : type IV collagen α1 chain; ═ : type IV collagen α2 chain; ── : S-S bond

We selected three antibodies that recognize different sites of NTH α1(IV). Antibody #141 recognizes the helical sequence of collagen without the NC1 domain, whereas #370 recognizes the NC1 domain. Antibody #179 does not recognize the polypeptide derived from proteins secreted as type IV collagen, suggesting that the epitope contains modified residues of NTH α1 (IV). Antigens, except no. 4, are from the supernatant of culture media of HLF cells. Antigen no. 4 is from human placenta type IV collagen (Sigma-Aldrich, St Louis, MI, USA). In the IP method, antigens were detected with a polyclonal antibody after electrophoresis. Results from antigens 1 and 2 are shown in Fig. 2, and results from antigens 3–6 are shown in Fig. S2.

interactions of NTH α1(IV) with type IV collagen or nonspecific interactions. When NTH α1(IV) was reduced by 2-ME, no interaction with antibody #370 was observed, as shown in the Table 2 (see Fig. S2). This result is probably because cleavage of the disulfide bonds in the NC1 domain causes structural changes around the epitope. Antibody #370 does not bind to either pepsin digested type IV collagen or reduced NTH α1(IV) on the blot. However, #370 reacts with SDS-denatured NTH α1(IV), suggesting that NTH α1(IV) has a heat-labile region in a higher-order structure that can be returned to a nascent structure by a process involving SDS. These results indicate that #370 recognizes a higher order structure of the NC1 domain of type IV collagen.

In summary, three antibodies, #141, #179 and #370 are reactive with the nascent NTH α1(IV) and do not react with triple-helical type IV collagen molecule. As each antibody recognizes a different epitope of NTH α1(IV), we are now investigating the possible involvement of NTH α1(IV) in tissue remodeling, angiogenesis and structuring of the basement membrane with the use of these monoclonal antibodies. We don't know so much about the species specificity of our antibodies other than human but #370 reacted with rabbit and #179 did with mouse. Recent examination demonstrated that the tip of newly formed blood vessels contains only NTH α1(IV) [13]. The developed monoclonal antibodies may facilitate quantitative analysis of NTH α1(IV) in blood and interstitial fluid, and the relationship between NTH α1(IV) levels and disease states, including liver cirrhosis [21] and tumors.

In order to understand the molecular functions of NTH α1(IV), it is very important to study the proteins that interact with NTH α1(IV) in the body. Immunoprecipitation using our antibodies that recognize different epitopes on type IV collagen may provide clues that elucidate which proteins interact with the collagen or NC1 domains. As NTH α1(IV) is hypothesized to contain regions that promote or inhibit angiogenesis, its expression pattern—when and where it is expressed—should be of interest. The staining pattern with different antibodies may suggest the function of each domain containing the epitope of each antibody.

Proline is modified by prolylhydroxylase to hydroxyproline post-translationally to a level that is sufficient to ensure formation of stable collagen triple-helices. Hypohydroxylation causes the α chains to remain nonhelical and the chains are postulated to be blocked from being secreted, leading to these chains being digested inside a cell. However, since NTH α1(IV) is secreted in a significant amount by cells, the secretory pathway of NTH α1(IV) and triple-helical type IV collagen might be different as well as the regulatory mechanisms of the syntheses.

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Appendix A. Transparency document

Transparency data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.11.013.

Appendix B. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.11.013.

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