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Separation of the Subtypes of Type V Collagen Molecules, $[\alpha1(V)]_2\alpha2(V)$ and $\alpha1(V)\alpha2(V)\alpha3(V)$, by Chain Composition-Dependent Affinity for Heparin: Single $\alpha1(V)$ Chain Shows Intermediate Heparin Affinity between Those of the Type V Collagen Subtypes Composed of $[\alpha1(V)]_2\alpha2(V)$ and of $\alpha1(V)\alpha2(V)\alpha3(V)$[1]

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The heparin affinities of heat-treated type V collagen α-chains and the triple-helical molecules were evaluated in terms of the NaCl concentration required for prevention of binding to a heparin-Sepharose column. After heat treatment, $\alpha1(V)$ chain required approximately two-fold higher NaCl concentration to pass through the column than the other two chains, $\alpha2(V)$ and $\alpha3(V)$. Thus, the heparin affinity of $\alpha1(V)$ may be approximately two-fold higher than those of the other $\alpha(V)$-chains. The type V collagen molecules in triple-helical conformation were separated into two fractions at 170 mM NaCl in 20 mM phosphate buffer, pH 7.2, containing 2 M urea; bound and non-bound. The ratio of the three α-chains, $\alpha1(V):\alpha2(V):\alpha3(V)$ was 2 : 1 : 0 and 1 : 1 : 1 in the bound and flow-through fractions, respectively, on analysis by SDS-PAGE. The differential affinity of the two fractions could be accounted for by the number of $\alpha1(V)$ chains in the triple-helical molecule, if these fractions contained triple-helical subtypes with the chain compositions of $[\alpha1(V)]_2\alpha2(V)$ and $\alpha1(V)\alpha2(V)\alpha3(V)$, respectively. From the comparison of the NaCl concentration required for prevention of the binding, $[\alpha1(V)]_2\alpha2(V)$ had about two-fold higher affinity than $\alpha1(V)\alpha2(V)\alpha3(V)$, and the separated $\alpha1(V)$ chain showed an intermediate affinity. A possible explanation for difference in heparin affinity among the subtypes of molecules and the separated α-chains is that the heparin affinity of type V collagen molecule is governed by the number of $\alpha1(V)$ chains contained in the molecule and that steric restraint in a triple-helical conformation weakens the binding of $\alpha1(V)$ chain to heparin.

Key words: affinity chromatography, heparin binding, heparin column, triple-helical structure, type V collagen.

Type V collagen is a member of the fibrillar collagen family. The characteristic structure and histochemical localization of the fibrillar aggregates raise the possibility that the type V collagen is involved in connecting basement membranes and collagen fibers (*1-3*). Immunohistochemical studies have demonstrated that the type V collagen is located on collagen fibrils with a D-periodic banding pattern in cornea (*4, 5*) and in spleen (*6*). Recently, targeted mutation in *col5a2* gene revealed a regulatory role for the type V collagen during matrix assembly (*7*).

The type V collagen was initially considered to consist of $\alpha1(V)$ and $\alpha2(V)$ chains (*8*). A third chain, clearly distinguishable from the other chains in electrophoretic mobility as well as in peptide mapping after enzymatic and CNBr cleavages, was found to be copurified with $\alpha1(V)$ and $\alpha2(V)$ chains during the isolation of type V collagen from human placenta (*9*). It was originally called αC chain (*9*) and later $\alpha3(V)$ chain (*10*). Some tissues, such as placenta (*9, 11*), skin (*12*), and synovial membrane (*12*), contain a small amount of $\alpha3(V)$ chain relative to $\alpha1(V)$ and $\alpha2(V)$ chains. The molecular organization of the type V collagen, in terms of $\alpha1(V)$, $\alpha2(V)$, and $\alpha3(V)$ chain compositions, still remains to be elucidated (*3*). Some reports have suggested that the two subtypes with the chain compositions of $[\alpha1(V)]_2\alpha2(V)$ and $\alpha1(V)\alpha2(V)\alpha3(V)$ are major components, at least in placenta (*13, 14*), while others have proposed that other subtypes with chain compositions such as $[\alpha1(V)]_3$ (*15-17*) and $[\alpha3(V)]_3$ (*11*) exist in different sources.

The strong affinity for heparin of the type V collagen subtype with the chain composition of $[\alpha1(V)]_2\alpha2(V)$ has been ascribed to the presence of $\alpha1(V)$ chain, that strongly binds to heparin, since $\alpha2(V)$, $\alpha1(I)$, and $\alpha2(I)$ chains did not bind to heparin (*18, 19*). Thus, the triple-helical type V collagen subtype which would lack the $\alpha1(V)$ chain should

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Abbreviations: PBS, Ca^{2+} and Mg^{2+}-free Dulbecco's phosphate-buffered saline; PB/U, 20 mM Na_2HPO_4-NaH_2PO_4, pH 7.2, containing 2 M urea.

show weak binding to heparin, and should be separable on heparin chromatography. The previous studies thus have led us to examine the heparin affinities of α3(V) chain and the molecular species containing the α3(V) chain. The heparin affinity of α3(V) chain has not previously been examined. In the one-third of the collagenous domain of α3(V) chain for which the amino acid sequence has been determined (*20*), a sequence homologous to the putative heparin binding domain of α1(V) chain is not present. If the heparin affinities of the three chains are different, different subtypes of type V collagen molecules should be resolvable by heparin column chromatography, depending on the α-chain composition. In this study, we compared the interactions of the separated α-chains and the subtypes of triple-helical type V collagen molecules.

MATERIALS AND METHODS

Preparation of Collagen—The preparation procedure was performed at 4°C. Pepsin solubilized type V collagen was prepared from human placenta as described by Miller and Rhodes (*21*). Briefly, human placenta homogenate, 20 g wet weight, was suspended in 100 ml of 0.5 M acetic acid containing 50 mg of pepsin. Type V collagen was isolated by salt fractionation in both acidic and neutral solutions.

Heparin Column Chromatography—Heparin-Sepharose column chromatography was performed as follows. An appropriate amount of the stored collagen solution in 0.15 M acetic acid was rapidly neutralized with cold 2 M Tris containing 1 M urea and 0.5 M NaCl, and then dialyzed against 200 volumes of the buffer in which the heparin chromatography was to be performed at 4°C. The chromatographic buffer consisted of 20 mM Na_2HPO_4-NaH_2PO_4, pH 7.2, containing 2 M urea (referred to as PB/U). Urea solution was deionized through a mixed resin of anion and cation exchangers just before use. The dialysis was continued for about 8 h with two changes of the external solution, which had the same composition as that used for the chromatography. After ultracentrifugation of the dialysate at 250,000 $\times g$ for 20 min at 4°C, the supernatant was applied to the column at 25°C. The chromatographic column used had a size of 0.5 $\times$ 5 cm or 0.5 $\times$ 2.5 cm with a volume of 1 ml or 0.5 ml. Absorbance at 220 nm was recorded with a Beckman UV 68 spectrophotometer. No significant change in the absorbance was observed between before and after heat treatment of the sample at 50°C for 10 min.

Purification of Type V Collagen by Heparin Column Chromatography—The salt-fractionated type V collagen preparation contained small polypeptides that migrated at the front of 5% gel, as well as high-molecular-weight polypeptides, which disappeared upon reduction with dithiothreitol, at the top of the gel on SDS-PAGE. About 50 ml of the salt-fractionated type V collagen preparation (0.3 mg/ml) dissolved in PB/U containing 100 mM NaCl was applied to a heparin column (1.1 cm $\times$ 20 cm; 20 ml). The column was washed with 200 ml of buffer, the bound protein was eluted with 2 M NaCl. Both the small peptides and the high-molecular-weight components were essentially recovered in the flow-through fractions. A small amount of high-molecular-weight components was occasionally contaminated the heparin-bound fraction for unknown reasons. In the present study, the type V collagen with little contamination, as judged from SDS-PAGE, was used. The α-chains of type V collagen were resolved into α1(V), α2(V), and α3(V) on SDS-PAGE. The collagen solution thus purified was dialyzed against 0.15 M acetic acid at 4°C and stored at 4°C until used.

Estimation of Heparin Affinities of Proteins—Since heparin is thought to interact with proteins essentially electrostatically, between the sulfate groups of heparin and the arginine and/or lysine residues of proteins, the difference in the heparin affinity was evaluated in terms of the NaCl concentration required for prevention of the binding to heparin. Thus, the affinity of a protein for heparin is expressed by the NaCl concentration which is sufficient to allow the protein to pass through the column. A predetermined amount (0.1 mg/ml; 0.5 ml) of solution containing type V collagen α-chain(s) or molecules was applied to a Sepharose CL6B column uncoupled with heparin. The column was washed with an adequate volume of the buffer to decrease the absorbance of the eluates to the baseline. The affinity of the α-chains or molecules of type V collagen for heparin was determined as follows. A sample dissolved in a certain concentration of NaCl was applied to a heparin-Sepharose CL6B column, then a predetermined volume of the buffer containing the same concentration of NaCl was applied to wash out nonspecifically bound polypeptides. Then, the bound fraction was eluted with 2 M NaCl. As a measure of heparin affinity the NaCl concentration was obtained at which, or above which, no significant absorbance was detected in the fraction eluted with 2 M NaCl. That is, the NaCl concentration required for prevention of the binding of the polypeptide(s) to heparin was regarded as representing the relative affinity of the polypeptide(s). In the cases of a mixed solution of two or more α-chains or the subtypes as well as the purified polypeptide, SDS-PAGE analysis was used to identify the kinds of the chain(s).

SDS-PAGE—SDS-PAGE was carried out in the buffer system of Laemmli (*22*) on a vertical slab gel (75 mm $\times$ 85 mm wide, 1 mm thick) at a constant current of 25 mA. Proteins were visualized by staining with Coomassie Brilliant Blue R-250.

Trypsin Treatment—Fifty μl of the collagen solution in PBS containing 2 M urea was incubated at 20°C for 10 min. Five μl (10 mg/ml) of trypsin in 1 mM HCl was added to the solution, which was then incubated at 20°C for 10 min. The enzymatic treatment was terminated by addition of 5 μl of trypsin inhibitor (20 mg/ml). Finally, the sample was heated to boiling on a heat block kept at 140°C, and 20 μl of a hot solution, incubated at 98°C, containing 4% SDS, 5 M urea, 40% glycerol, and 0.01% Bromophenol Blue, was rapidly added.

Reagent—The following chemicals were used: heparin-Sepharose CL6B (Pharmacia), urea (Nacalai Tesque), Ca^{2+} and Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS) (Nissui), pepsin (Worthington, porcine), trypsin (Wako Pure Chemical), and soybean trypsin inhibitor (Wako Pure Chemical). Other reagents were of the highest grade from commercial sources.

RESULTS

The Interactions of Heat-Treated Type V Collagen with Heparin—The type V collagen in PBS containing 2 M urea was thermally denatured at 50°C for 10 min, followed by

rapid cooling on ice and then warming at 25°C for 10 min. The heat-treated type V collagen was resolved into two fractions on heparin-Sepharose column chromatography (Fig. 1a). The bound fraction contained α1(V) chain (Fig. 1b). The flow-through fraction contained two α-chain-size components (Fig. 1b); a fast-migrating α2(V) chain and a slow-migrating α3(V) chain (*9, 11-14*). The two fractions were separately rechromatographed at different NaCl concentrations in phosphate buffer containing 2 M urea (PB/U) (Fig. 1c). The NaCl concentration in PB/U greatly influenced the affinity. When the bound fraction from the initial chromatography, containing only α1(V) chain, was applied to the second column, all the protein was bound to heparin at 100 mM NaCl and even at 150 mM, while at 200 mM NaCl, essentially no α1(V) chain bound to the column [Fig. 1c(i)]. On the other hand, proteins in the flow-through fraction from the first chromatography containing α2(V) and α3(V) chains did not bind to heparin at 100 mM NaCl in PB/U [Fig. 1c(ii)]. However, at 50 mM, a portion of α2(V) chain was bound to the column, while α3(V) chain was not bound (data not shown), implying that the affinity of α2(V) chain was slightly higher than that of the α3(V) chain. The two chains showed much weaker interaction with heparin than did α1(V) chain. The affinities of α2(V) and α3(V) chains with heparin were comparable to those of the α-chains of type I collagen, α1(I) and α2(I) chains, which passed through the heparin column in PB/U containing 100 mM NaCl (data not shown).

The Interactions of Triple-Helical Type V Collagen with Heparin—The type V collagen in a native conformation was dissolved in PB/U at three different NaCl concentrations and applied to the heparin column. The amount of the bound fraction decreased with increasing concentration of NaCl in PB/U [Fig. 2a(i)]. At 100 mM NaCl, where separated α1(V) chain bound, while separated α2(V) and α3(V) chains did not, all the proteins were recovered in the bound fraction. At 150 mM NaCl, a significant amount of the sample passed through the column. The SDS-PAGE analysis showed that α1(V), α2(V), and α3(V) chains were present in the flow-through fraction in almost equal amounts [Fig. 2a(ii)]. The bound fraction contained only a small amount of α3(V) chain at 150 mM NaCl. At 200 mM NaCl, a condition where none of the separated three kinds of α-chain could bind to the heparin column, more than half of the type V collagen fraction applied was recovered as a bound fraction [Fig. 2a(i)]. The bound fraction at 200 mM NaCl contained no α3(V) chain [Fig. 2a(ii)]. The amount of α1(V) chain was approximately twice that of α2(V) chain

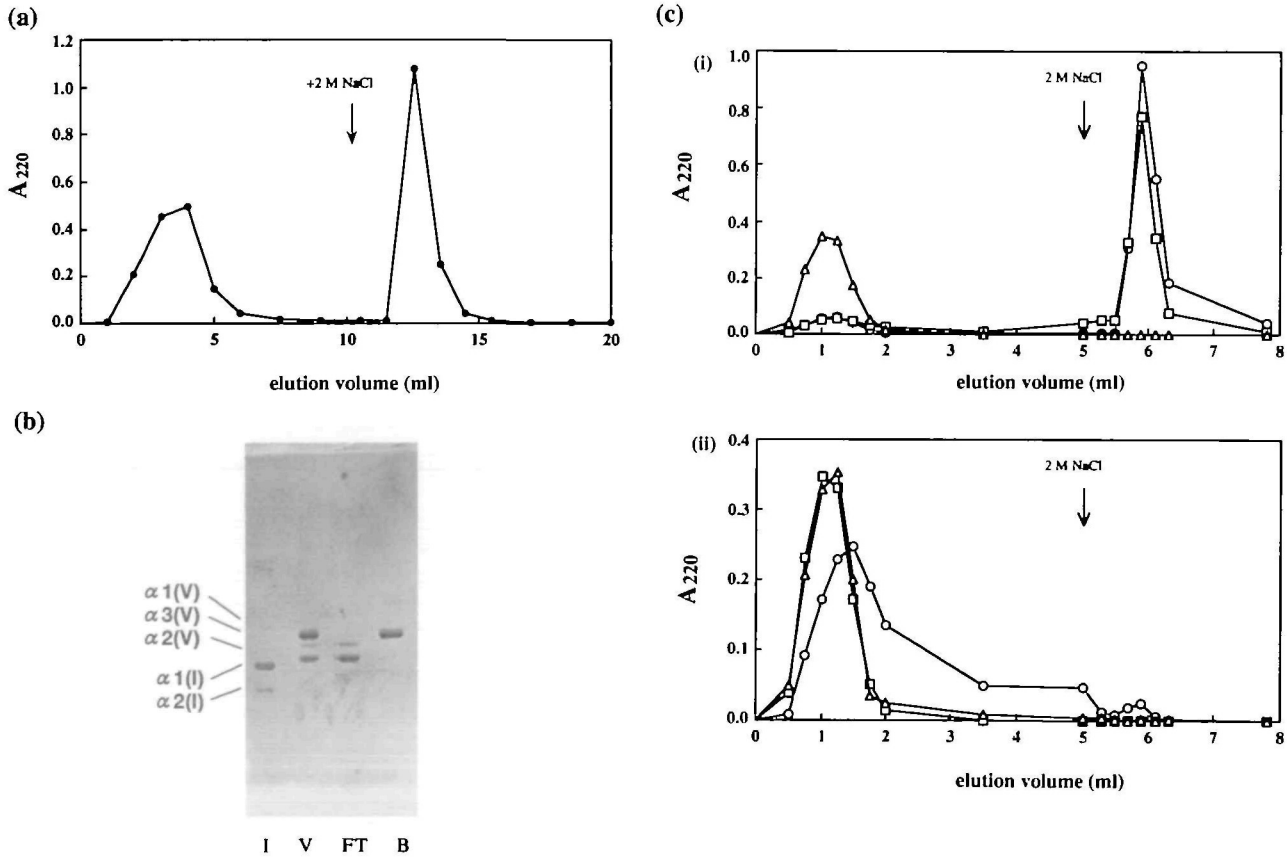

Fig. 1. **Heparin column chromatography of heat-treated type V collagen.** Heat-treated type V collagen (0.5 mg/ml, 0.5 ml) in PBS containing 2 M urea was applied to a 1.0 ml column. The column was washed with 10 ml buffer, and the bound protein was eluted with 2 M NaCl. (a) The elution pattern on heparin column chromatography. Ordinate: protein concentration in A_{220}, abscissa: elution volume. (b) Band patterns of the flow-through fraction (FT) and bound fraction (B) on SDS-PAGE. Type I collagen from chick embryo (I) and the starting material of type V collagen (V) are shown for reference. (c) The separated type V collagen α-chains dissolved in PB/U containing different NaCl concentrations, 100 mM (○), 150 mM (□), and 200 mM (△), were applied to the heparin column. The column was washed with the buffer, and the bound fractions were eluted with 2 M NaCl. (i) The bound fraction, and (ii) the flow-through fraction.

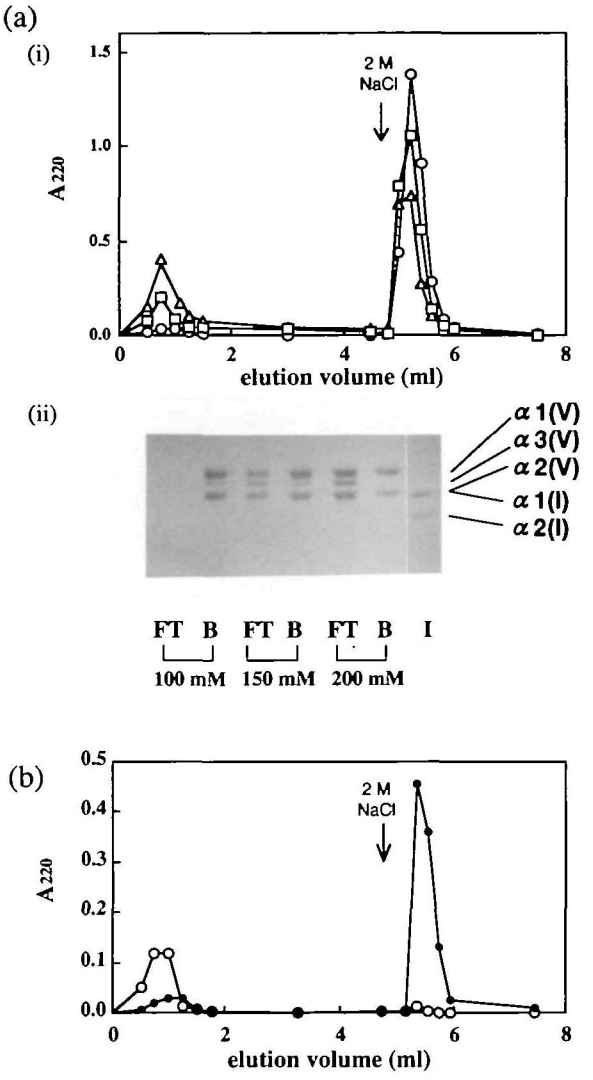

Fig. 2. **Heparin column chromatography of type V collagen in native conformation at different NaCl concentrations.** (a) A sample (0.1 mg/ml) in a volume of 0.5 ml was applied to a 0.5 ml column. Nonspecifically bound materials were eluted with 5 ml of buffer, then the bound fractions were eluted with 2 M NaCl. (i) Chromatographic profiles at NaCl concentrations of ○: 100 mM, □: 150 mM, and △: 200 mM. (ii) SDS-PAGE of the fractions. FT: flow-through fraction, B: bound fraction, I: type I collagen. (b) Two fractions resolved on the heparin column at 200 mM NaCl were rechromatographed. The flow-through fraction and the bound fraction were rechromatographed in the same buffer (PB/U containing 200 mM NaCl). The flow-through fraction (○) and the bound fraction (●) on the first chromatography.

in the bound fraction. Rechromatography of the bound fraction at 200 mM NaCl showed a similar binding profile to the first chromatography, while the flow-through fraction passed through the column in the second run, implying that the fractions were composed of different molecular species (Fig. 2b). The triple-helical conformation of the fractions was confirmed by the resistance to trypsin digestion (Fig. 3).

To compare the heparin affinity of type V collagen α-chains and the subtypes of type V collagen molecules, the same amount of proteins or polypeptides (0.1 mg/ml, 0.5 ml) were applied to the column in 0.5 ml of PB/U containing different concentrations of NaCl. The NaCl concentration at which, or above which, neither significant absorbance nor any staining band in the gel of the bound fraction was detected was determined. Two samples of α-chains, the two samples, one with α1(V) chain, and the other with α2(V) and α3(V) chains, were used. In addition, unfractionated native type V collagen sample was used. For weakly interacting materials, or the α3(V) chain and the subtype containing α3(V) chain, SDS-PAGE analysis was used for the examination. The NaCl concentrations employed were 25, 50, 75, 100, 125, 150, 175, and 200 mM for the separated α-chains, and 100, 150, 160, 170, 180, 190, 200, 250, 300, 325, 350, and 400 mM for the native molecules. The results are summarized in Table I. In the present experiment, the amount of α1(V) chain corresponding to $A_{220}=0.02$ was the least that could be detected on the gel with Coomassie Brilliant Blue R-250 protein staining.

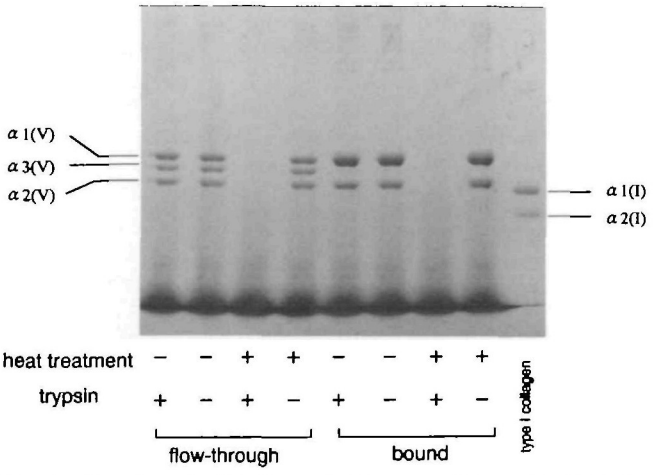

Fig. 3. **Treatment of the type V collagen with trypsin.** Both the flow-through and the bound fractions at 200 mM NaCl in PB/U were incubated with or without trypsin before or after heat-treatment. Type I collagen is shown for reference.

TABLE I. **Heparin binding of type V collagen α-chains and the subtypes of type V collagen molecules at different NaCl concentrations and the affinities expressed in terms of the NaCl concentration required for prevention of the binding.**

	Bound or flow-through at NaCl concentration of			NaCl concentration required for prevention of the binding (mM)
	100 mM	150 mM	200 mM	
α1(V) chain	Bound	Bound	Flow-through	200
α2(V) chain	Flow-through	Flow-through	Flow-through	100
α3(V) chain	Flow-through	Flow-through	Flow-through	50
[α1(V)α2(V)α3(V)] subtype	Bound	Partially bound mostly flow-through	Flow-through	170
$[\alpha 1(V)]_2\alpha 2(V)$ subtype	Bound	Bound	Bound	350

DISCUSSION

The results indicate that at least two subtypes of type V collagen molecules with different affinity for heparin, one with $\alpha 3$(V) chain and the other without $\alpha 3$(V) chain, exist in human placenta (Fig. 2a). The flow-through fraction at 200 mM NaCl in PB/U contained type V collagen $\alpha 1$(V), $\alpha 2$(V), and $\alpha 3$(V) chains in 1 : 1 : 1 ratio, while the bound fraction showed the $\alpha 1$(V), $\alpha 2$(V), and $\alpha 3$(V) chain composition of 2 : 1 : 0. Previous reports on separation of the subtypes of type V collagen molecules from human placenta have suggested the existence of two molecular subtypes of type V collagen; one with a chain composition of $[\alpha 1(V)]_2\alpha 2(V)$, and the other, $\alpha 1(V)\alpha 2(V)\alpha 3(V)$ (*13, 14*). In our study, the subtype of $[\alpha 1(V)]_2\alpha 2(V)$ bound to the heparin column, while the subtype of $\alpha 1(V)\alpha 2(V)\alpha 3(V)$ passed through on the assumption that the type V collagen fraction contained only two subtypes. Since the affinity of separated $\alpha 1$(V) chain was much higher than that of $\alpha 2$(V) and $\alpha 3$(V) chains (Fig. 1c), one obvious way to interpret the differential affinities of these two triple-helical subtypes of type V collagen molecules would be to consider that the affinity of the subtype depends on the number of $\alpha 1$(V) chains, one or two, in a triple-helical molecule. The following discussion is based on the assumption that the two subtypes of type V collagen molecules, with the chain compositions of $[\alpha 1(V)]_2\alpha 2(V)$ and $\alpha 1(V)\alpha 2(V)\alpha 3(V)$, are the main components of the type V collagen fraction.

In the present study, the heparin affinities of the proteins were evaluated in terms of the NaCl concentration required for prevention of binding to the heparin column. With this definition of heparin affinity, a protein that requires a higher NaCl concentration to prevent binding is defined as having a higher affinity, since the electrostatic interaction involved decreases with increasing ionic strength of the solvent. The results showed that the affinity of separated $\alpha 1$(V) chain was intermediate between those of the two triple-helical subtypes, one with a single $\alpha 1$(V) chain and the other with two $\alpha 1$(V) chains, on heparin column chromatography (Figs. 1 and 2). Under a condition where separated $\alpha 1$(V) chain did not bind to the column, the subtype with two $\alpha 1$(V) chain, $[\alpha 1(V)]_2\alpha 2(V)$, did associate with heparin [Fig. 2a(ii)]. On the other hand, a portion of the subtype with one $\alpha 1$(V) chain, $\alpha 1(V)\alpha 2(V)\alpha 3(V)$, was recovered in the flow-through fraction under the condition where separated $\alpha 1$(V) chain bound [Fig. 2a(ii)]. The NaCl concentration required for prevention of the binding of the $\alpha 1(V)\alpha 2(V)\alpha 3(V)$ subtype to heparin was 170 mM, which is lower than that of separated $\alpha 1$(V) chain, 200 mM (Table I). The relative affinity of a single free $\alpha 1$(V) chain, A, is taken to be 200/170 in comparison with that of the subtype, which would be determined by the affinity of the chain in a triple-helical conformation. The conformational restraint may cause a reduction of the relative affinity of $\alpha 1$(V) to βA, where the value of β is less than 1, or would be close to 170/200 if the heparin affinity is governed by the number and conformational restraint of $\alpha 1$(V) chain, without a contribution from the other chains. If this assumption is extended to the subtype of $[\alpha 1(V)]_2\alpha 2(V)$, and if the value of β, 170/200, is used, $[\alpha 1(V)]_2\alpha 2(V)$ would be expected to have an affinity of 2βA or 1.7A. The NaCl concentration required for preventing the subtype with two $\alpha 1$(V) chains from binding was about 350 mM, suggesting that the relative affinity would be 350/200 or 1.75A, close to the above value.

The high heparin affinity of type V collagen molecule with the chain composition of $[\alpha 1(V)]_2\alpha 2(V)$ compared with separated $\alpha 1$(V) chain can be explained by the parallel packing of the heparin-binding domains of the two $\alpha 1$(V) chains in the molecule. The triple-helical association of two $\alpha 1$(V) chains leads to twofold concentration of the basic residues on a region of the molecular surface. Hence, the negatively charged groups of heparin would bind to a cluster of the positively charged residues of the proteins. In contrast to the subtype of $[\alpha 1(V)]_2\alpha 2(V)$, the subtype of $\alpha 1(V)\alpha 2(V)\alpha 3(V)$ showed a lower affinity than that of the separated $\alpha 1$(V) chain. In addition to the conformational restraint of the $\alpha 1$(V) chain in the triple-helical association, resulting in confinement within a small range of the dihedral angles of the peptide bonds, the existence of $\alpha 3$(V) chain in place of one of $\alpha 1$(V) chains may cause a reduction in the affinity of the $\alpha 1$(V) chain. That is to say, it is possible that some negatively charged residue(s) on the $\alpha 3$(V) chain located sterically near the heparin binding site of $\alpha 1$(V) chain might interact with a basic residue, or bulky and/or hydrophobic amino acid residue(s) might weaken the interaction.

In this study, we obtained no evidence of the existence of subtypes other than $\alpha 1(V)\alpha 2(V)\alpha 3(V)$ and $[\alpha 1(V)]_2\alpha 2(V)$ in human placenta. If the above interpretation of the binding of type V collagen molecules is valid, gradient elution of the native type V collagen fraction from the column might yield trace amounts of other subtypes according to the number of $\alpha 1$(V) chain(s) in the molecule. However, neither a homotrimer subtype with the chain composition of $[\alpha 1(V)]_3$, as previously reported by two groups (*16, 17*), nor subtypes which lack $\alpha 1$(V) chain were recovered from human placental type V collagen fraction by heparin chromatography, as detected by SDS-PAGE (data not shown).

What would be a possible biological meaning for the existence of the subtypes in type V collagen molecules with differential heparin affinities? The $\alpha 3$(V) chain is present in tissues with a well-developed vascular system such as placenta. The lower affinity of the subtype of type V collagen molecule containing $\alpha 3$(V) chain might or might not be related to differential localization and function of the subtype. When PBS (which contains about 137 mM NaCl) containing 2 M urea was used as the solvent, the subtype $\alpha 1(V)\alpha 2(V)\alpha 3(V)$ partially bound to the column and partially passed through, while the subtype $[\alpha 1(V)]_2\alpha 2(V)$ bound (data not shown). The difference of approximately twofold in the NaCl concentration for elution of the two subtypes of the type V collagen molecules could imply a significant difference in the interacting modes of heparin or heparan sulfate with the different two subtypes of type V collagen *in vivo*. Since $[\alpha 1(V)]_2\alpha 2(V)$ bound to heparin at an ionic strength much higher than physiological, it is suggested that once heparin or heparan sulfate binds to $[\alpha 1(V)]_2\alpha 2(V)$, they will not readily dissociate. On the other hand, the interaction of heparin with $\alpha 1(V)\alpha 2(V)\alpha 3(V)$ could be switched on or off by a subtle environmental change. Our study shows that the two subtypes self-assembled *in vitro* to fibrils with different structures (Mizuno, Adachi, and Hayashi, unpublished results) under

a physiological condition. Fibrils of the two subtypes may also have different affinities for heparin.

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