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Improved chromatographic purification of human and bovine type V collagen sub-molecular species and their subunit chains from conventional crude preparations Application to cell-substratum adhesion assay for human umbilical vein endothelial cell

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Abstract

Two human type V collagen sub-molecular species, designated $[\alpha1(V)]_2\alpha2(V)$ and $\alpha1(V)\alpha2(V)\alpha3(V)$, were purified chromatographically from a commercially available preparation, in which cystine-rich collagenous contaminants were contained, with a column packed with Fractogel EMD SO_3^-. From bovine crude preparations, the $[\alpha1(V)]_2\alpha2(V)$ form free from the collagenous contaminants was purified. Type V collagen subunit chains were isolated from each type V collagen molecule by anion-exchange HPLC with a Bakerbond PEI Scout column. The highly purified human type V collagen molecules and their subunit chains were used to examine the inhibitory effect on human umbilical vein endothelial cell proliferation. It was confirmed that the $\alpha1(V)$ chain has inhibitory activity and it was found that the inhibitory effect of the $[\alpha1(V)]_2\alpha2(V)$ form is stronger than that of the $\alpha1(V)\alpha2(V)\alpha3(V)$ form and that the $\alpha3(V)$ chain has no inhibitory activity.

1. Introduction

Type V collagen was first isolated from pepsin digest of human placenta by salt fractionation by using NaCl [1]. Type V collagen is classified as a fibril-forming collagen with type I, II, III and XI collagens [2]. Mammalian type V collagen has three distinct subunit chains designated $\alpha1(V)$, $\alpha2(V)$ and $\alpha3(V)$. These subunit chains assemble into at least two distinct molecular forms (sub-molecular species), $\alpha1(V)\alpha2(V)\alpha3(V)$ and $[\alpha1(V)]_2\alpha2(V)$ [2]. Recently, it has been demonstrated that type V collagen specifically inhibits cell-substratum adhesion of vein endothelial cell and other cell types [3–10] and also binds biologically active materials such as thrombospondine [11], heparin [12], insulin [13], SPARC (secreted protein acidic and rich in cysteine) [14] and macrophage colony-stimulating factor [15]. However, the biological significance of molecular and subunit diversities of type V collagen remains unclear. To solve these problems, some simple

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isolation methods for type V collagen sub-molecular species and their subunit chains are necessary. This paper describes an improved chromatographic purification of two molecular forms of type V collagen and their subunit chains from crude type V collagen preparations. We also demonstrate that conventional crude preparations, including commercially available ones, contain significant amount of cystine-rich collagenous contaminants. The highly purified human type V collagen molecules and subunit chains were used to examine the inhibitory effect on human vein endothelial cell proliferation.

2. Experimental

2.1. Materials

Human and bovine placenta type V collagen preparations were obtained from Sigma (St. Louis, MO, USA) and Koken (Tokyo, Japan). Type V collagen was also prepared from bovine tendon according to the method of Nyibizi et al. [16] with slight modifications [17]. They were used as crude type V collagen preparations. Calf skin acid-soluble type I collagen was purchased from Koken (Tokyo, Japan). Trypsinized human umbilical vein endothelial cell (HUVEC) was a kind gift from Dr. H. Hoshi (Bio Science Lab., Yamagata, Japan). Human acidic fibroblast growth factor (aFGF) and human endothelial growth factor (hEGF) were obtained from Bio Science Lab.

Urea (biochemical grade) was obtained from Wako (Osaka, Japan) and was purified by passage through a column packed with Amberlite IRA-410 (Rohm and Haas, Philadelphia, PA, USA). Acetonitrile (HPLC grade) and trifluoroacetic acid (TFA) were obtained from Nacalai Tesque (Kyoto, Japan) and Merck (Darmstadt, Germany). Protein molecular mass marker was obtained from TEFCO (Nagano, Japan).

2.2. Chromatographic resins, columns and apparatus

A Superspher RP-18 column (250 × 4 mm I.D.) and Fractogel EMD SO_3^- 650(S) were obtained from Merck. A C10/10 column with a flow adaptor was obtained from Pharmacia–LKB Biotechnology (Uppsala, Sweden). Bakerbond PEI and Butyl Scout columns were purchased from J.T. Baker (Phillipsburg, NJ, USA). An Econo-Pack 10DG column was obtained from Bio-Rad (Richmond, CA, USA).

A Perista pump (SJ-1211) was obtained from ATTO (Tokyo, Japan). The HPLC apparatus consisted of a Shimadzu LC-9A pump, an FCV-9AL gradient valve unit, a DGU-2A degasser unit, an SPD-6AV variable-wavelength detector, a CR-6A integrator and Rheodyne Model 7125 sample injector with a 5-ml sample loop. All were obtained from Shimadzu (Kyoto, Japan).

2.3. Fractionation of crude type V collagen preparations

Human and bovine crude type V collagen preparations were dissolved in 0.04 *M* Tris–HCl buffer (pH 8.2) containing 2 *M* urea and 0.05 *M* NaCl and then applied to a column (C10/10) packed with Fractogel EMD SO_3^- 650(S) equilibrated with the same buffer. Elution was performed with a 300-ml linear gradient of NaCl (0.05–1.0 *M* NaCl in the buffer) at 2 ml/min delivered with a Perista pump.

2.4. Isolation of type V collagen subunits

Aliquots containing each type V collagen molecule were applied to an Econo-Pack 10DG column equilibrated with 0.02 *M* Tris–HCl buffer (pH 7.0) containing 2 *M* urea to exchange the buffer. Type V collagen molecules eluted from the column were heated at 50°C for 5 min to resolve them into subunit chains. The subunit chains were separated by HP anion-exchange chromatography with a Bakerbond PEI Scout column equilibrated with the same buffer. Elution was performed with a linear gradient of NaCl from 0 to 0.35 *M* in the buffer over 20 min at 1 ml/min.

2.5. Reversed-phase HPLC

To remove non-volatile buffer and salts from the protein, reversed-phase HPLC with a Baker-

bond Butyl Scout column was performed. The sample was applied to the column equilibrated with 10% (v/v) acetonitrile containing 0.1% TFA. After washing the column with the initial solvent, protein was eluted by increasing the acetonitrile concentration from 10 to 80% over 10 min at 1 ml/min.

2.6. Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [18]. Proteins were developed with Commassie Brilliant Blue R-250. Collagen and its derivatives were stained metachromatically with the dye [19,20]. In some instances, the staining intensity of each band was estimated by using a Chromato Scanner CS-930 (Shimadzu).

2.7. Amino acid analyses

Amino acid analyses were performed according to the method of Bidlingmeyer et al. [21] with a slight modification [22]. Phenylthiocarbamylamino acids were resolved by using a Superspher RP-18 column with binary gradient elution at 0.8 ml/min as described previously [22]. Cysteine and cystine were determined as cysteic acid. Formic acid (97%) was mixed with hydrogen peroxide (30%) (19:1, v/v) and left for 1 h at room temperature. A 10-μl volume of the resultant performic acid was reacted with protein for 30 min. After removal of the performic acid under vacuum, the oxidized sample was hydrolysed with HCl vapour at 150°C for 1 h according to the method of Bidlingmeyer et al. [21]. The resultant cysteic acid was determined by the procedure described above.

2.8. Cell culture

Calf type I collagen, human type V collagen molecules and their subunit chains were dissolved in ethanol– water (50:50) the pH of which was adjusted to 3.0 by adding HCl. The protein concentration was determined by amino acid analysis. A 50-μl volume of the collagen solutions was poured into a 24-well culture plate and then air-dried. Trypsinized HUVEC were suspended in MCDB medium supplemented with 10 μg/ml of heparin, 5 ng/ml of aFGF, 10 μg/ml of hEGF and 2% foetal bovine serum. The HUVEC (5×10^4 cells/well) were cultured on the collagen molecule- or subunit chain-coated plate. After 1 and 4 days of cultivation, attached cell numbers were counted with a Coulter Counter.

3. Results

3.1. Purification of type V collagen molecules

Human (Sigma) and bovine placenta (Koken) and bovine tendon crude type V collagen preparations were further fractionated into each submolecular species by Fractogel EMD SO_3^- 650(S) column chromatography. With the human crude type V collagen preparation, four major peaks appeared (fractions A–D in Fig. 1). As shown in Fig. 2, human type V collagen molecules, $\alpha1(V)\alpha2(V)\alpha3(V)$ and $[\alpha1(V)]_2\alpha2(V)$, were recovered in the third and fourth peaks (fractions C and D), respectively. Bovine type V collagen in both preparations consisted predominantly of $[\alpha1(V)]_2\alpha2(V)$ form and it was eluted from the column after elution of collagenous contaminant (lanes E and F in Fig. 2).

3.2. Partial characterization of collagenous contaminants in crude type V collagen preparations

SDS-PAGE revealed that the first and second peaks from the human crude type V collagen preparation (fractions A and B in Fig. 1) contained bands stained collagen-methachromatically [19,20] (lanes A and B in Fig. 2). Fraction A contained two collagenous bands, which had a mobility corresponding to a molecular mass of 78 000 of globular protein and retained on the top of the separation gel, respectively (lane A, triangles). Reduction with 2-mercaptoethanol resulted in the occurence of several bands in a range of molecular mass from 28 000 to aggregate which was retained on the top of the separation gel (lane rA). In fraction B, a col-

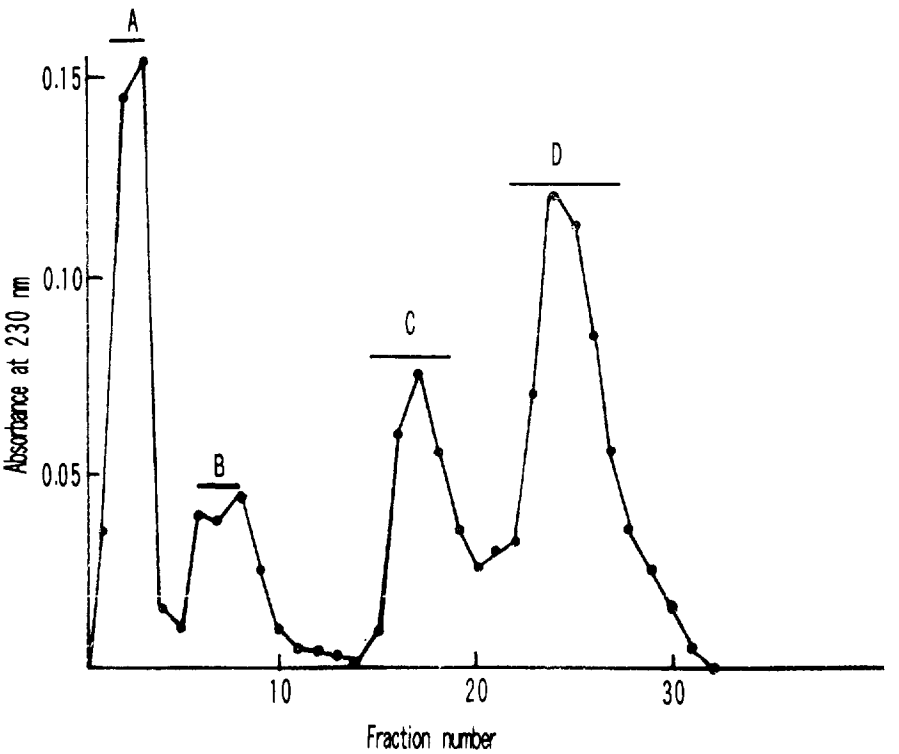

Fig. 1. Fractionation of human crude type V collagen preparation by Fractogel EMD SO_3^- 650(S) column chromatography. See Experimental for elution conditions. Fractions marked A–D were collected.

lagenous band with a mobility corresponding to calf α1(I) chain was observed under non-reducing conditions. After the reduction, a band with a mobility corresponding to a molecular mass of 45 000 appeared (lane rB). These results indicate that these collagenous components have disulfide bonds in and between molecules. In the purified type V collagen fractions (lanes C and D), faint bands whose mobilities were less than those of α chains of type V collagen were present. As the reduction with 2-mercaptoethanol did not affect the mobility of these faint bands (data not shown), we identified them as polymerized type V collagen α chains, which have been observed in mammalian type V collagen preparations [1,16]. The collagenous components in fractions A and B were eluted in a single peak in reversed-phase HPLC (data not shown). The amino acid composition of both components is shown in Table 1 along with that of human type V collagen subunit chains. They show a significantly different composition to type V collagen subunits, especially having a lower level of glycine and a higher level of cysteine (cystine). Such a high level of cystine in collagen preparation has not previously been described. The contents of the collagenous contaminants in fractions A and B were estimated to be approximately 1.0 and 0.3% in human type V collagen preparation (Sigma) on the basis of amino acid analysis.

A similar collagenous contaminant was also contained in the bovine preparation (lane E in Fig. 2). The contaminant content in the bovine preparation (Koken) was roughly estimated to be more than 20% on the basis of the intensity of the stained bands. We tried to remove these contaminants from type V collagen by NaCl and ammonium sulfate fractionations according to methods reported previously [1,16,17]. How-

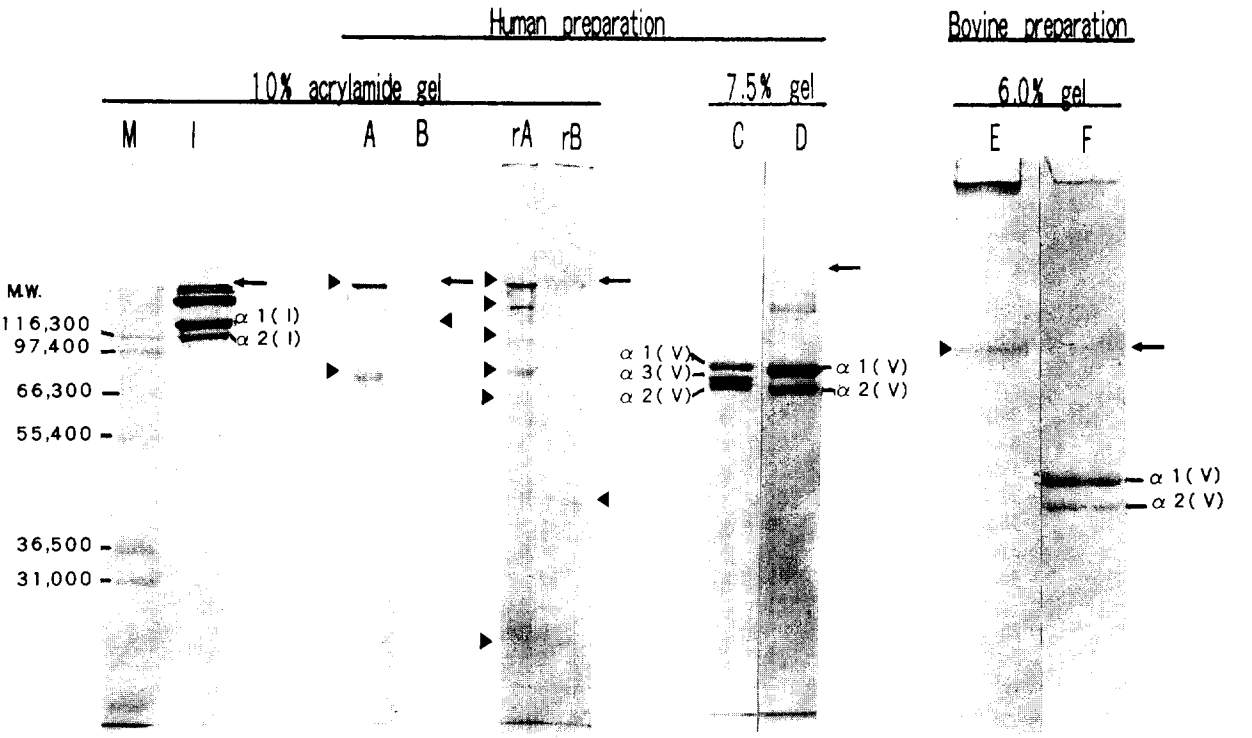

Fig. 2. SDS-PAGE patterns of type V collagen molecules and contaminants in human and bovine crude type V collagen preparations. Lanes A–D, fractions A–D in Fig. 1; rA and rB, fractions A and B after reduction with 2-mercaptoethanol; lane E, collagenous contaminants in the bovine preparation; lane F, bovine type V collagen $[\alpha 1(V)]_2\alpha 2(V)$ molecule. M, protein molecular mass marker, I, calf skin type I collagen. Arrows indicate the top of the separation gel and triangles indicate contaminant band stained methachromatically for collagen.

ever, all our efforts were unsuccessful. Hence we conclude that it is difficult to remove them by conventional methods.

3.3. Separation of type V collagen subunit chains

As shown in Fig. 3, simultaneous separations of all subunit chains from human type V collagen molecules were achieved by using anion-exchange HPLC (Bakerbond PEI). The polymerized chains were eluted in the tailing shoulder peak of the $\alpha 1(V)$ chain. All chains were eluted in a single protein peak by reversed-phase HPLC (data not shown). As shown in Table 1, the purified α chains showed typical compositional features as reported previously [23]. Bovine $\alpha 1(V)$ and $\alpha 2(V)$ chains were also separated under the same conditions (data not shown).

3.4. Inhibitory effect of type V collagen molecules and subunit chains on proliferation of HUVEC

After cultivation for 1 day, the number of cells attached to the plate coated with 100 μg of $\alpha 1(V)$ chain was significantly lower than the others (Fig. 4). After 4 days, only a small number of cells were attached to the $\alpha 1(V)$-coated well, whereas $\alpha 2(V)$ and $\alpha 3(V)$ chains, and also type I collagen, somewhat enhanced the number of attached cells with respect to the uncoated plate. These results indicate that only the $\alpha 1(V)$ chain has inhibitory activity against

Table 1
Amino acid composition of human type V collagen subunit chains and collagenous contaminants in fractions A and B from human crude type V collagen preparation (residues per 1000 residues)

Amino acid	Fraction A	Fraction B	α1(V)	α2(V)	α3(V)
Cya[a]	73.0	12.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Asx	81.4	52.5	48.0 ± 0.7	49.0 ± 0.2	41.6 ± 4.0
Glx	91.7	104.5	96.3 ± 1.4	84.1 ± 1.1	89.6 ± 1.0
Hyp[b]	55.0	72.3	109.2 ± 1.8	111.3 ± 0.1	98.5 ± 6.2
Ser	55.2	82.8	22.1 ± 0.7	34.7 ± 0.6	35.1 ± 1.5
Gly	213.1	287.1	345.7 ± 2.4	347.3 ± 2.7	353.9 ± 7.5
His	13.4	13.2	6.1 ± 0.2	9.2 ± 0.2	12.7 ± 0.2
Arg	41.9	41.2	38.2 ± 0.5	50.4 ± 0.0	35.8 ± 6.2
Thr	42.3	30.3	23.8 ± 0.4	32.2 ± 0.3	24.6 ± 0.9
Ala	39.2	55.3	36.8 ± 0.3	51.3 ± 0.2	45.2 ± 1.9
Pro	82.7	79.0	125.3 ± 0.7	104.3 ± 1.1	100.8 ± 1.2
Tyr	13.0	0.0	1.3 ± 0.2	0.5 ± 0.6	1.9 ± 0.3
Val	31.2	25.5	17.0 ± 0.5	25.8 ± 0.2	24.8 ± 1.1
Met	15.6	0.0	7.5 ± 0.1	11.2 ± 0.0	7.1 ± 1.6
Ile	31.6	26.0	16.9 ± 0.3	12.5 ± 0.4	15.9 ± 0.6
Leu	46.3	43.5	35.8 ± 0.4	30.9 ± 0.8	44.7 ± 1.6
Hyl[c]	22.4	21.8	44.2 ± 1.1	22.4 ± 0.4	37.1 ± 5.0
Phe	26.7	25.1	10.2 ± 0.1	9.6 ± 0.2	10.1 ± 0.5
Lys	24.0	25.5	15.6 ± 0.2	13.9 ± 0.8	20.1 ± 0.3

Values for type V collagen subunit chains represent means ± S.D. ($n = 3$).
[a] Cysteic acid.
[b] Hydroxyproline.
[c] Hydroxylysine.

HUVEC attachment and proliferation. As shown in Fig. 5, α1(V) chain and two type V collagen molecules inhibited HUVEC attachment and proliferation in a dose-dependent manner after cultivation for 4 days. More than 50% inhibition (IC_{50}) was observed at concentrations of 20, 20 and 80 μg/ml of α1(V) chain, $[\alpha1(V)]_2\alpha2(V)$ and α1(V)α2(V)α3(V) molecules, respectively.

4. Discussion

We found that a significant amount of cystine-rich collagenous contaminants, which have not been reported in type V collagen and other collagen preparations, is present in the conventional type V collagen preparations including commercially available ones. These preparations have been used to raise antibodies and to examine biological functions of type V collagen. In some cases, results obtained by using such preparations might be misinterpreted under the influence of these contaminants. However, the influence of these contaminants has not been considered, as their existence in type V collagen preparation has been overlooked. They can be removed from type V collagen molecules by strong cation-exchange chromatography as described here, whereas it is difficult to remove them by conventional salt fractionation [1,16,17].

Two type V collagen sub-molecular species, $[\alpha1(V)]_2\alpha2(V)$ and α1(V)α2(V)α3(V), have been separated by ammonium sulfate fractionation [16] or phosphocellulose (P-11, Whatman) chromatography [24]. Because of the poor selectivity and tediousness of these methods, alternative high-performance methods have been required. Recently, a high-performance anion exchanger (Mono Q HR 5/5, Pharmacia–LKB)

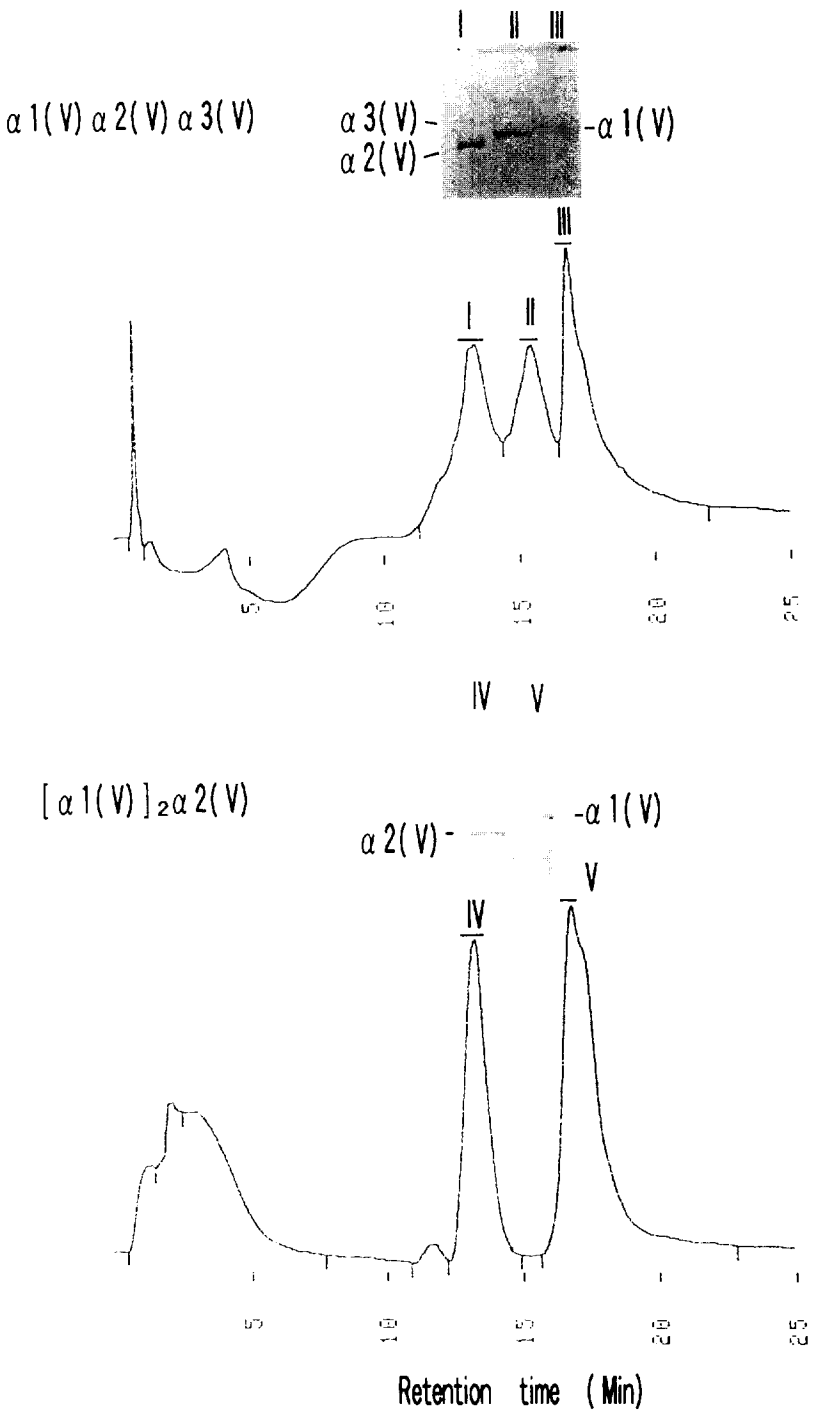

Fig. 3. Separation of subunit chain from human $\alpha 1(V)\alpha 2(V)\alpha 3(V)$ and $[\alpha 1(V)]_2\alpha 2(V)$ molecules by the Bakerbond PEI Scout column. Inset: SDS-PAGE patterns of the pooled fractions indicated with bars. Absorbance at 230 nm was monitored.

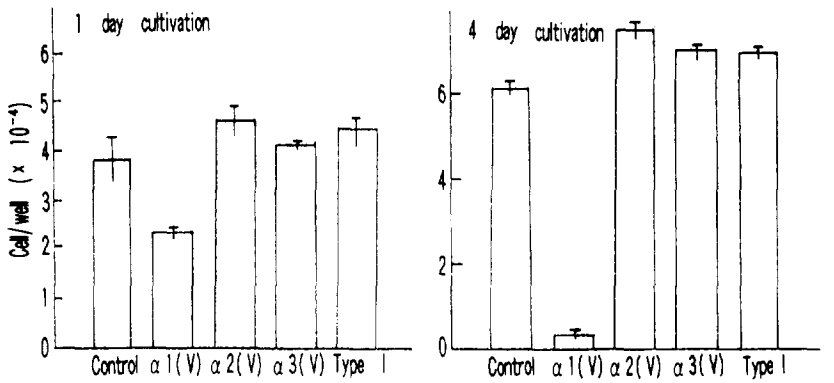

Fig. 4. Inhibition of cell-substratum adhesion of HUVEC by human type V collagen subunit chains. Dishes were coated with 100 μg of each subunit chain or type I collagen. The numbers of attached cells were counted after incubation at 37°C for 1 and 4 days. Control: uncoated dish.

was used for this purpose [25]. In this method, contaminants in the crude preparation are also adsorbed on the column and eluted with NaCl of increasing concentration. In the present method, all contaminants in the crude preparations were eluted before type V collagen and an excellent separation of two molecular forms of type V collagen was achieved. In preliminary experiments, sulfopropyl Toyopearl 650 (Tosoh, Tokyo, Japan) and Econo-Pac S cartridge (Bio-Rad) can be used for same purpose with modified elution conditions.

Type V collagen subunit chains, α1(V), α2(V) and α3(V), were first isolated by a series of phosphocellulose (P-1, Whatman) and carboxymethylcellulose (CM-52, Whatman) column chromatographies [23]. However, this technique is tedious. Recently, a Mono Q HR 5/5 column was also used for this purpose [25]. In their method, re-chromatography with buffer of a

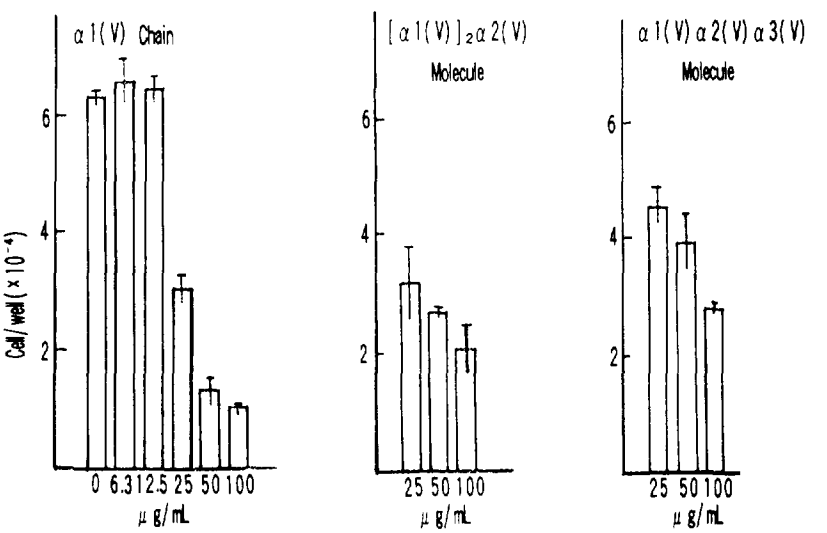

Fig. 5. Dose–response curve for the inhibitory effect with α1(V) chain and two type V collagen molecules. The numbers of attached cells were counted after incubation for 4 days.

different pH was required. In the present study, rapid and simultaneous separation of all three subunit chains was achieved.

Fukuda et al. [3] first found that human type V collagen inhibits HUVEC proliferation. Recently, Hashimoto et al. [4] demonstrated that $\alpha 1(V)$ chain is responsible for the inhibitory activity by using bovine type V collagen. However, in those studies, the inhibitory activity of $\alpha 3(V)$ chain and $\alpha 1(V)\alpha 2(V)\alpha 3(V)$ molecule was not examined. We confirmed their findings by using highly purified $\alpha 1(V)$ chain and found that $\alpha 3(V)$ chain has no inhibitory activity, whereas $\alpha 3(V)$ chain resembles $\alpha 1(V)$ chain in primary structure [25]. In addition, we found that the $[\alpha 1(V)]_2\alpha 2(V)$ form has a stronger inhibitory activity than the $\alpha 1(V)\alpha 2(V)\alpha 3(V)$ form at the same concentration. As the isolated $\alpha 1(V)$ chain, which lost its triple helical conformation by heat denaturation, has inhibitory activity, the primary structure of the $\alpha 1(V)$ chain is mainly responsible for the inhibitory activity. However, IC_{50} values of the $\alpha 1(V)$ chain in $[\alpha 1(V)]_2\alpha 2(V)$ and $\alpha 1(V)\alpha 2(V)\alpha 3(V)$ molecules can be calculated on the basis of subunit composition to be 13 and 25 μg/ml, respectively. These values are inconsistent with that of the isolated $\alpha 1(V)$ chain (20 μg/ml), although both $\alpha 2(V)$ and $\alpha 3(V)$ chains have an enhancing effect on HUVEC proliferation. These facts suggest that the three-dimensional organization of $\alpha 1(V)$ chain with other subunits might effect HUVEC proliferation to some extent. Together with these facts, the distribution of endothelial cells in organs might be regulated not only by diversity in collagen types but also in subunit composition of type V collagen. The present study enables one to examine the biological significance of the molecular diversity of type V collagen. If type V collagen molecules and the $\alpha 1(V)$ chain inhibit proliferation of human capillary endothelial cells and consequently suppress angiogenesis in vivo, there is a possibility that they could be used for therapy of tumours, rheumatoid arthritis, retinitis, and so on, in which extensive angiogenesis is observed. On the basis of the present results, further studies on the anti-angiogenesis effect by type V collagen and its derivatives are in progress.

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