

The Asparagine-linked Oligosaccharides on Human and Porcine Plasma Vitronectins

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Abstract

The structures of asparagine-linked oligosaccharides present on plasma vitronectins of human (hVN) and porcine (pVN) are reported. Oligosaccharides were released from vitronectins by *N*-glycanase digestion before and after desialylation. The reducing ends of the oligosaccharides were tagged with 2-aminopyridine.

Pyridylamino (PA)-derivatives of asialooligosaccharides were fractionated and analyzed by two-dimensional sugar mapping method (N. Tomiya et al. *Anal. Biochem.*, 171, 73-90 (1988)) using reversed-phase and amide-adsorption high performance liquid chromatography (HPLC) in combination with exoglycosidase digestion. More than 70% of the total PA-asialooligosaccharides of both vitronectins were biantennary lactosamine structures. Structures of the major oligosaccharides were confirmed by 400 MHz ^1H NMR spectroscopy.

PA-sialooligosaccharides were fractionated by anion-exchange and reversed-phase HPLC. Ten and nine major PA-sialooligosaccharides were isolated from hVN and pVN, respectively, and after desialylation their asialo-forms were assigned to PA-asialooligosaccharides by the two-dimensional sugar mapping method. Using desialylation with *Salmonella* sialidase, which has a kinetic preference for sialyl $\alpha 2 \rightarrow 3$ linkages, in combination with acid-desialylation, the linkage and location of sialic acids were presumed by comparing the elution position on HPLC with those of standard PA-sialooligosaccharides and standard derivatives obtained by exoglycosidase digestion.

The oligosaccharides of hVN and pVN differed in extent of sialylation; 8% mono-, 70% di- and 22% tri-sialylated for hVN; 7% mono-, 89% di- and 4% tri-sialylated for pVN. Sialic acid was linked dominantly by $\alpha 2 \rightarrow 6$ linkage, although $\alpha 2 \rightarrow 3$ linkage was also present on both vitronectins. The most remarkable difference between hVN and pVN is the amount of fucose linked to the innermost *N*-acetylglucosamine by $\alpha 1 \rightarrow 6$ linkage.

Introduction

Vitronectin (VN) is a cell-adhesive glycoprotein existing abundantly in plasma and in connective tissues. VN participates in cell-substrate adhesion by the binding to integrin through Arg-Gly-Asp (RGD) peptide [1]. It also modulates the immune system by the binding to complement C5b-7 complex [2] and the coagulation system by the binding to thrombin-antithrombin III complex [3-6]. VN can bind to various biomolecules including heparin [7], collagen [8,9] and plasminogen activator inhibitor-1 [7,10,11] and plays multifunctional roles through these interactions. Progress has been made in mapping of each binding site on the molecule [1,9,12-14]. To play a highly organized function *in vivo*, VN must have a regulatory factor or factors inside and/or outside the molecule, although they are not yet clarified.

Previously we have purified six animal plasma VNs and demonstrated that marked diversities are present in their molecular weights, immunoreactivity and glycosylation, while essential biological activities including cell-spreading activity, heparin- and collagen-binding activities are well conserved among all species [15]. All six VN had 10-20% (w/w) carbohydrate contents and partial characterization by lectin binding assay indicated that *N*-linked oligosaccharides are present on all species, while the amount of *O*-linked oligosaccharides varied by species. Recently, the diversity of the molecular weights was extensively studied for 14 animal VNs, and uniformity of the core polypeptide has been shown [16]. Other recent works suggested that the complete primary structure of human [12,13], rabbit [17] and mouse [18] deduced from cDNA sequences have similar size of 52 kDa (456-459 amino acids) and highly homologous sequence throughout the molecule including complete conservation of three potential *N*-glycosylation sites of human VN with one additional potential site in mouse. Despite extensive efforts to elucidate the biological role and structure-function relationship of VN, however, structural refinement of VN oligosaccharides has remained unknown.

In this study, detailed structural analysis for *N*-linked oligosaccharides on human and porcine plasma VNs are reported. The entire population of sialylated *N*-linked oligosaccharides released by *N*-glycanase digestion were pyridylaminated (PA) and separated by high-performance liquid chromatography (HPLC). By a novel approach using *Salmonella* sialidase with virus-typical kinetic preference for sialyl $\alpha 2 \rightarrow 3$ linkages as a probe and a successful docking with two-dimensional (2-D) sugar mapping method, the linkage and location of sialic acids were precisely defined for each oligosaccharide. This is the first report describing the carbohydrate structures

of vitronectin.

Experimental Procedures

Materials

Plasma VNs were purified from human and porcine sera as previously described [15,19]. *N*-Glycanase (*Flavobacterium meningosepticum*), β -*N*-acetylhexosaminidase (jack bean), and β -galactosidase (*Charonia lampas*) were purchased from Seikagaku Kogyo., Tokyo, Japan, pepsin, β -*N*-acetylhexosaminidase (jack bean) and β -galactosidase (jack bean) from Sigma Chem. Co., St. Louis, MO, USA, and α -L-fucosidase (beef kidney) from Boehringer Mannheim, Germany. Sialidase (*Salmonella typhimurium* LT2) was a gift from Takara Shuzo Co., Kyoto, Japan. Standard pyridylamino(PA)-oligosaccharides were purchased from Takara Shuzo Co.: Neu5Aca2 \rightarrow 6Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 6(Neu5Aca2 \rightarrow 6Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4)(Neu5Aca2 \rightarrow 6Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2)Man α 1 \rightarrow 3Man β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4GlcNAc-PA (666T), Neu5Aca2 \rightarrow 6Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 6(Neu5Aca2 \rightarrow 6Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 3)Man β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4GlcNAc-PA (66B), Neu5Aca2 \rightarrow 6Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 6(Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 3)Man β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4GlcNAc-PA (60B), and Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 6(Neu5Aca2 \rightarrow 6Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 3)Man β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4GlcNAc-PA (06B). Standard PA-oligosaccharide containing α 2 \rightarrow 3 linked Neu5Ac was a gift of Prof. S. Hase, Osaka Univ. Standard PA-glucose oligomers were prepared from the acid hydrolysate of dextran [20] or purchased from Takara Shuzo Co. Borane-dimethylamine complex was purchased from Aldrich, Milwaukee, WI, USA. A TSK GEL DEAE-5PW column (0.75 \times 7.5 cm) and an Amide-80 column (0.46 \times 25 cm) were purchased from Tosoh Co., Tokyo, Japan, a Cosmosil 5C18-P column (0.46 \times 15 cm) from Nacalai Tesque Inc., Kyoto, Japan, and a Shim-pack CLC-ODS column (0.6 \times 15 cm) from Shimadzu Co., Kyoto, Japan.

Preparation of PA-asialooligosaccharides from VNs

Each VN (10 mg) was desialylated by mild acid hydrolysis (pH 2.2) at 90°C for 1 h and digested with pepsin (0.04%, w/v) at 37°C for 4 days, then neutralized and dried *in vacuo*. The resultant glycopeptides were dissolved in 0.1 M sodium phosphate buffer (pH 8.6)-20 mM EDTA-1% 2-mercaptoethanol and treated with 2U of *N*-glycanase at 37°C for 24 h. The released oligosaccharides were pyridylaminated by the same procedure as sialooligosaccharides described later.

Isolation and analysis of PA-asialooligosaccharides

PA-asialooligosaccharides were first separated with reversed-phase

HPLC using Shim-pack CLC-ODS column and each fraction was further separated with size-fractionation HPLC using TSK GEL Amide-80 column. The elution was performed as described by Tomiya et al. [21]. PA-oligosaccharides were detected by fluorescence using excitation and emission wavelengths of 320 nm and 400 nm, respectively. The structures of PA-oligosaccharides were assumed by the 2-D sugar mapping method using these two kinds of HPLC in combination with exoglycosidase digestion [21]. The values of elution positions (expressed as glucose oligomers) of intact and exoglycosidase digested PA-asialooligosaccharides were plotted on 2-D map and compared with the data of respective standard oligosaccharides. The identities of the elution position of the samples were further confirmed by co-injection of standard onto the both HPLC columns.

Preparation of PA-oligosaccharides from VNs

Human or porcine VN (5 mg) was denaturated by boiling for 3 min in the presence of 0.2% (w/v) SDS and 1% (w/v) 2-mercaptoethanol. To each sample was added 0.5 M sodium phosphate buffer (pH 8.6) and *n*-octyl- β -D-glucoside (final concentration to 20 mM and 2%(w/v), respectively). N-Glycanase (3U) was then added and the reaction mixture was incubated for 24h at 37°C. Then the mixture was boiled for 10 min, centrifuged and the supernatant was lyophilized.

The reducing ends of the oligosaccharides were pyridylaminated under conditions which sialyl linkages are not hydrolyzed using borane-dimethylamine complex as described by Kondo et al. [22]. Excess reagents were removed by toluene-methanol evaporation, and then gel-filtration on a Sephadex G-15 column.

Isolation and analysis of PA-sialooligosaccharides

PA-sialooligosaccharides were first separated with anion-exchange HPLC (DEAE-5PW column) by the number of sialic acids. The elution was performed as described by Yamamoto et al. [23]. Then each fraction was further separated with reversed-phase HPLC on a Cosmosil 5C18-P column. Solvent A (0.1 M acetic acid) and solvent B (solvent A containing 0.5% 1-butanol) were used. The column was equilibrated with a mixture of solvents A and B at the ratio of 95:5 at 23°C. After injection of a sample, the ratio of solvent B was increased linearly to 100% in 55 min at a flow rate of 1.5 ml/min at 23°C.

Exoglycosidase digestion and hydrolysis of sialic acid

β -Galactosidase, β -N-acetylhexosaminidase and α -L-fucosidase digestions were done as described by Tomiya et al. [21].

To examine the presence of $\alpha 2 \rightarrow 3$ linked sialic acid, a PA-oligosaccharide (20 pmol) was digested with *Salmonella* sialidase (3 mU), which has kinetic preference for sialyl $\alpha 2 \rightarrow 3$ linkage [24], in 5 μ l of 0.1 M sodium acetate buffer, pH 5.5 for 16 min (for PA-mono- and di-sialo oligosaccharide) or 45 min (for PA-trisialooligosaccharide) at 37°C. The reaction was stopped by boiling for 3 min, and injected onto the DEAE-5PW column to see the elution position.

To remove all sialic acid residues, acid hydrolysis was performed in 20 μ l of 0.01M HCl at 90°C for 1 h.

¹H-NMR measurements

The purified PA-asialooligosaccharide fraction was desalted by gel-filtration on a Sephadex G-15 column. Sample was evaporated and dissolved in D₂O, then repeatedly exchanged in D₂O with intermediate lyophilization. ¹H-NMR spectroscopic analysis was performed on a JEOL GX-400 spectrometer operating at 400MHz in the Fourier transform mode at a probe temperature of 30°C or 65°C. Chemical shifts are expressed from internal 3-(trimethylsilyl)propionic-2,2,3,3-d-acid, sodium salt but were actually measured with internal acetone: $\delta = 2.22$ ppm, with an accuracy of 0.002 ppm.

Carbohydrate analysis

The reducing end PA-sugar was analyzed after hydrolysis of PA-oligosaccharide sample (100–200 pmol) with 2.5 M TFA at 100°C for 6 h. The hydrolysate was acetylated and analyzed as described by Suzuki et al. [25].

For sialic acid analysis, the PA-oligosaccharide (50–100 pmol) was hydrolyzed with 0.01 M HCl at 90°C for 1 h. The hydrolysate was evaporated *in vacuo* and dissolved in a suitable volume of distilled water. Sialic acid analysis was performed as described previously [15].

Results

Isolation of PA-asialooligosaccharides of VNs

Asialooligosaccharides were released by sequential digestion of VNs with pepsin and N-glycanase. The yield of PA-asialooligosaccharides was 76–100% on the basis of the sugar content of VNs and quantitative assessment of the fluorescence intensity of PA-asialooligosaccharides.

PA-asialooligosaccharides prepared from hVN were separated into 7 peaks by reversed-phase HPLC as shown in Fig. 1(A). Each fraction was then applied onto size fractionation HPLC and the elution positions are

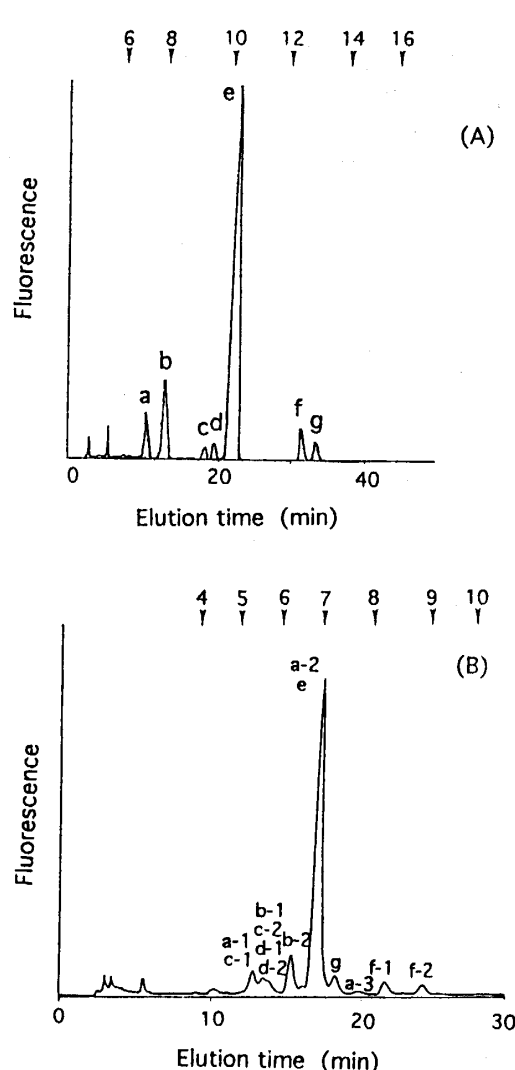


Fig. 1.

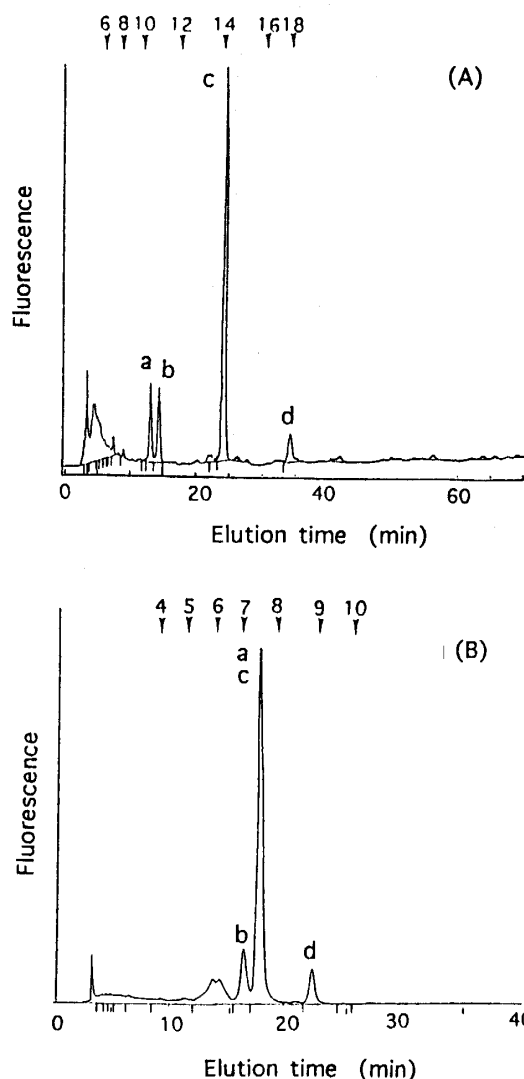


Fig. 2.

Fig. 1. HPLC of PA-asialooligosaccharides of hVN. Asialooligosaccharides obtained by *N*-glycanase digestion of the peptic peptides of hVN were pyridylaminated and fractionated by reversed-phase and size-fractionation HPLC as described in the text. The arrows indicate the elution positions of glucose oligomers expressed as the number of glucose units. (A) Reversed-phase HPLC of whole PA-asialooligosaccharides on CLC-ODS column. Fractions a-g were collected and further separated by size-fractionation HPLC. (B) Size-fractionation HPLC of whole PA-asialooligosaccharides on Amide-80 column. The elution position of each peak separated by reversed-phase HPLC is indicated in the chromatogram. Fractions a, b, c, d and f were separated into 2 or 3 peaks by size-fractionation HPLC. The symbols of the fractions correspond to those in Fig. 3.

Fig. 2. HPLC of PA-asialooligosaccharides of pVN. Asialooligosaccharides of pVN were pyridylaminated and fractionated by HPLC as described in the text. The arrows indicate the elution positions of glucose oligomers expressed as the number of glucose units. (A) Reversed-phase HPLC of whole PA-asialooligosaccharides on CLC-ODS column. Fractions a-d were collected and applied to size-fractionation HPLC. (B) Size-fractionation HPLC of whole PA-asialooligosaccharides on Amide-80 column. The elution position of each peak separated by reversed-phase HPLC is indicated in the chromatogram. The symbols of the fraction correspond to those in Fig. 4.

shown in Fig. 1(B). Major fraction (Fr.) e (73%) and Fr. g gave a single peak, respectively, on amide-silica HPLC, while fractions (Fr.s.) a, b, c, d and f were separated into 2 or 3 peaks.

Asialo PA-oligosaccharides of pVN were separated into 4 fractions by reversed-phase HPLC and each fraction gave a single peak on size fractionation HPLC as shown in Fig. 2(A) and 2(B).

Structure of PA-asialooligosaccharides of hVN

The structures of PA-asialooligosaccharides of hVN were identified by the 2-D sugar mapping method as described below. The values of elution positions (expressed as glucose oligomers) were plotted on the 2-D sugar map as shown in Fig. 3, and compared with those of respective standard oligosaccharides [21]. The numbers of standard PA-oligosaccharides corresponding to the elution position of each fraction from hVN are shown in parentheses in Fig. 3. Fractions after sequential digestion with β -galactosidase and β -N-acetylhexosaminidase were analyzed again and plotted in the Fig. 3. The structures of the standard PA-asialooligosaccharides in

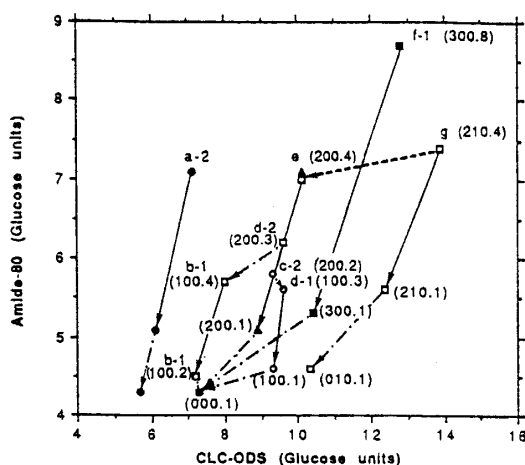


Fig. 3.

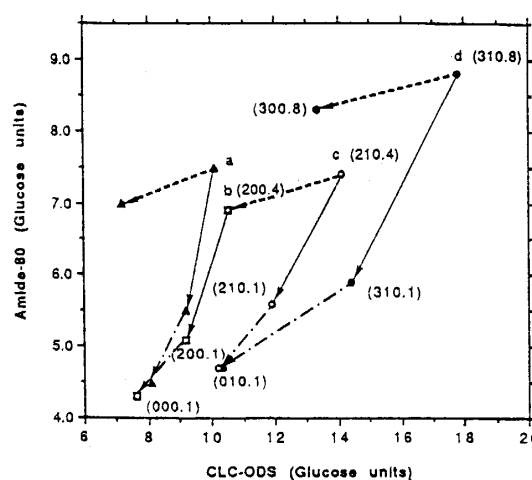
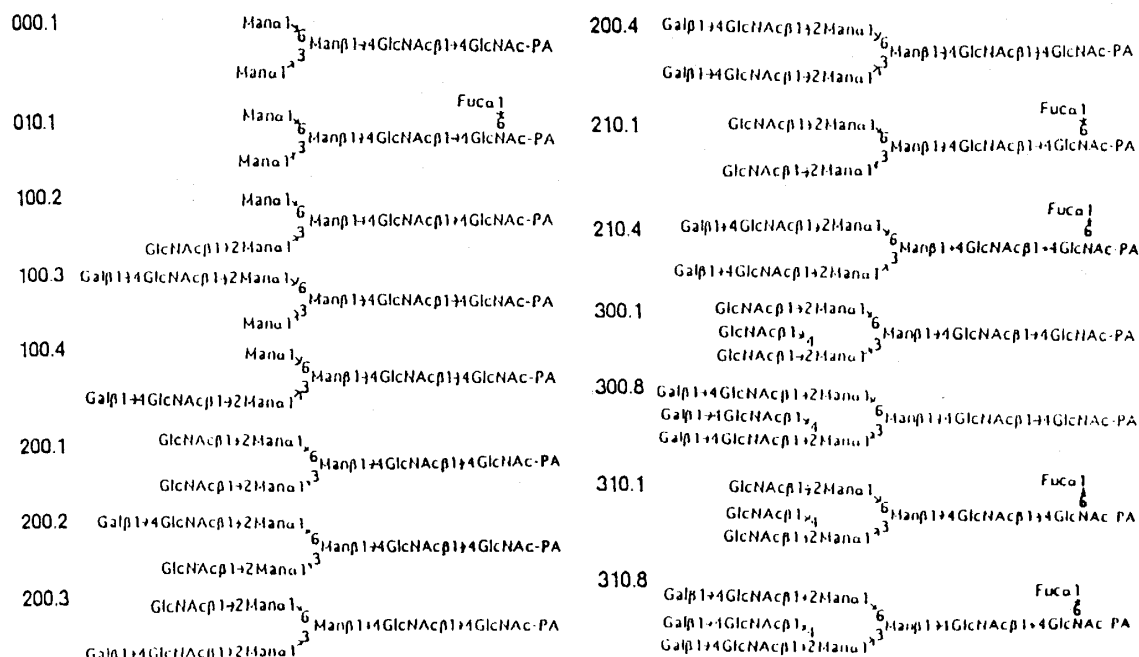


Fig. 3.

Fig. 3. The 2-D sugar map of intact and exoglycosidase-digested PA-asialooligosaccharides of hVN. The scales for the 2-D sugar map are numbers of glucose units obtained on CLC-ODS and Amide-80 columns for X and Y axes, respectively. Arrows show the direction of change in the position after digestion with the glycosidases: β -galactosidase (—), β -N-acetylhexosaminidase (---), and α -L-fucosidase (-----). The number of standard PA-oligosaccharides corresponding to the elution position of each fraction from hVN are shown in parentheses.

Fig. 4. The 2-D sugar map of intact and exoglycosidase-digested PA-asialooligosaccharides of pVN. The scales are the same as in the legend to Fig. 3. Arrows show the direction of change in the position after digestion with the glycosidases as shown in the legend to Fig. 3. The number of standard PA-oligosaccharides corresponding to the elution position of each fraction from pVN are shown in parentheses.



Scheme 1. Structures of PA-asialooligosaccharides in 2-D sugar map (21).

Fig. 3 and Fig. 4 are listed in Scheme 1.

Major fraction, Fr. e (72%) was eluted at the position corresponding to that of standard (200.4): $\text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \rightarrow 6 (\text{Gal}\beta \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \rightarrow 3) \text{Man}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 4 \text{GlcNAc-PA}$. After β -galactosidase and β -N-acetylhexosaminidase digestion, the decrease in the glucose units of Fr. e assessed on amide-silica HPLC were 2 units and 1 unit and 1 unit, respectively. It has been shown that each mannose, galactose, and xylose residue behaves like a 1.0 glucose unit, and each fucose and N-acetylglucosamine residue behaves like a 0.5 glucose unit on an Amide-80 column [21]. Therefore, the result is interpreted as that two β -galactoside and two β -N-acetylhexosaminide residues were released to yield a core structure, suggesting that Fr. e is a biantennary complex-type oligosaccharides. The elution positions of Fr. e in the 2-D map after β -galactosidase and β -N-acetylhexosaminidase digestions corresponded to those of standard (200.2) and (000.1), respectively. These chromatographic behavior of Fr. e after exoglycosidase digestions agreed with the structure of (200.4) proposed initially. In a similar way, the structures of other fractions were analyzed. Frs. a-1, b-1, c-1, c-2, d-1, d-2, and f-1 yielded a common core structure of (000.1): $\text{Man}_3 \text{GlcNAc}_2 \text{PA}$ after β -galactosidase and β -N-acetylhexosaminidase digestion. Only Fr. g yielded a fucosylated core structure, (010.1): $\text{Man}_3 \text{GlcNAc}_2 \text{FucPA}$. The elution position of intact Fr. g was changed to that of Fr. e after α -L-fucosidase digestion. These chromatographic behavior of each fraction after exoglycosidase digestion agreed with the structure initially proposed by comparing the elution position

with those of standards.

The elution position of Fr. a-2 (5%) did not correspond to any of standard oligosaccharides hitherto reported. The changes of elution position of Fr. a-2 by exoglycosidase digestions suggested that a-2 is a biantennary non-fucosylated oligosaccharide. PA-sugar analysis of a-2 indicated, however, the presence of PA-ManNAc but not PA-GlcNAc, suggesting that a-2 is an artifact generated by epimerization of the reducing end of Fr. e oligosaccharide during the pyridylation [26]. All the proposed structures for PA-asialooligosaccharides of hVN are summarized in Table I.

Structure of PA-asialooligosaccharides of pVN

The values of elution positions before and after exoglycosidase digestions were plotted on the 2-D sugar map as shown in Fig. 4. The elution positions of Frs. a, c and d were drastically changed by α -L-fucosidase digestion and that of Fr. c shifted to the same position of Fr. b. For Frs. a, b and c, the decrease in the size by β -galactosidase and β -N-acetylhexosaminidase digestion assessed on amide-silica HPLC were 2 units and 1 unit, respectively, suggesting that these fractions have biantennary structures. While those for Fr. d were 3 units and 1.5 units, respectively, which suggests that Fr. d has triantennary structure. Frs. c and d yielded a core structure with fucose (010.1), whereas only Fr. b yielded non-fucosylated core (000.1). These chromatographic behavior of each fraction except Fr. a agreed with the structure initially proposed by the elution position. On the other hand, the elution position of Fr. a did not correspond to any standard oligosaccharides and yielded different core structure. PA-sugar analysis of Fr. a indicated the presence of PA-ManNAc but not PA-GlcNAc, suggesting that Fr. a is an artifact generated by epimerization of Fr. c during the pyridylation. All the proposed structures for asialo PA-oligosaccharides of pVN are summarized in Table I.

¹H NMR analysis of major PA-asialooligosaccharides of hVN and pVN

The structures of major PA-asialooligosaccharides of hVN and pVN were further confirmed by 400 MHz ¹H NMR spectroscopy as listed in Table II, in comparison with the chemical data for oligosaccharides of human normal IgG, Fr. D: Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 6(Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 3)Man β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4GlcNAc-PA, Fr. H: Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 6(Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 3)Man β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4(Fuc α 1 \rightarrow 6)GlcNAc-PA [27]. From these results, the structures of hVN Fr. e and pVN Fr. c are determined to be as shown in Table I. Other fractions could not be analyzed due to the limited amounts of the samples.

Table I. Proposed structures of PA-asialooligosaccharides of VNs.

hVN	
Fr. ^a	Structure
a-1 (0.2%)	$\begin{array}{c} \text{Man}\alpha 1 \searrow 6 \\ \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \nearrow 3 \text{Man}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 4 \text{GlcNAcPA} \end{array}$
b-1 (3.4%)	$\begin{array}{c} \text{Man}\alpha 1 \searrow 6 \\ \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \nearrow 3 \text{Man}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 4 \text{GlcNAcPA} \end{array}$
c-2 (1.1%)	$\begin{array}{c} \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \searrow 6 \\ \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \nearrow 3 \text{Man}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 4 \text{GlcNAcPA} \end{array}$
d-1 (0.6%)	$\begin{array}{c} \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \searrow 6 \\ \text{Man}\alpha 1 \nearrow 3 \text{Man}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 4 \text{GlcNAcPA} \end{array}$
d-2 (1.2%)	$\begin{array}{c} \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \searrow 6 \\ \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \nearrow 3 \text{Man}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 4 \text{GlcNAcPA} \end{array}$
e ^b (76%)	$\begin{array}{c} \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \searrow 6 \\ \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \nearrow 3 \text{Man}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 4 \text{GlcNAcPA} \end{array}$
f-1 (2.9%)	$\begin{array}{c} \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \searrow 6 \\ \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \searrow 4 \nearrow 3 \text{Man}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 4 \text{GlcNAcPA} \\ \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \nearrow 3 \end{array}$
g (2.8%)	$\begin{array}{c} \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \searrow 6 \\ \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \nearrow 3 \text{Man}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 4 \text{GlcNAcPA} \\ \text{Fuc}\alpha 1 \downarrow 6 \end{array}$
pVN	
Fr. ^a	Structure
b (9.1%)	$\begin{array}{c} \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \searrow 6 \\ \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \nearrow 3 \text{Man}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 4 \text{GlcNAcPA} \\ \text{Fuc}\alpha 1 \downarrow 6 \end{array}$
c ^c (72%)	$\begin{array}{c} \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \searrow 6 \\ \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \nearrow 3 \text{Man}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 4 \text{GlcNAcPA} \\ \text{Fuc}\alpha 1 \downarrow 6 \end{array}$
d (5.0%)	$\begin{array}{c} \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \searrow 6 \\ \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \searrow 4 \nearrow 3 \text{Man}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 4 \text{GlcNAcPA} \\ \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \nearrow 3 \\ \text{Fuc}\alpha 1 \downarrow 6 \end{array}$

^aThe amount of each PA-asialooligosaccharide fraction was estimated from the fluorescence intensity in Fig. 1 and Fig. 2, and is shown in parentheses as mol % of total PA-asialooligosaccharides.

^bThe content of Fr. a-2 (4.2%), which is considered to be an epimerization product of Fr. e has been included in the content.

^cThe content of Fr. a (7.4%), which is considered to be an epimerization product of Fr. c has been included in the content.

Table II. Chemical Shifts (ppm) of Structural Reporter Signals of Major Asialooligosaccharides of pVN and hVN.

$ \begin{array}{cccccc} \text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 & & & & & \text{Fuc}\alpha 1 \\ 6' & 5' & 4' & \searrow 6 & & \downarrow 6 \\ & & & \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc}-\text{PA} \\ & & & \nearrow 3 & 3 & 2 & 1 \\ \text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 & & & & & & \\ 6 & 5 & 4 & & & & \end{array} $					
Reporter Group	Residue	hVN Fr. e ^a	pVN Fr. c ^a	human IgG	
				compound D ^b	compound H ^b
H-1 of	2	(4.658)	(4.716)	4.621	4.675
	3	(4.752)	(4.753)	[4.746]	[4.749]
	4	5.115 (5.132)	5.115 (5.130)	5.110	5.110
	4'	4.918 (4.913)	4.914 (4.912)	[4.912]	4.916 [4.909]
	5	4.577	4.577	4.572	4.573
	5'	4.577	4.577	4.572	4.573
	6	4.465	4.465	4.461	4.461
	6'	4.465	4.465	4.461	4.461
	Fuc	—	4.849	—	[4.848]
H-2 of	3	4.226 (4.217)	4.226 (4.216)		
	4	4.181 (4.181)	4.175 (4.178)		
	4'	4.102 (4.089)	4.093 (4.095)		
NAc of	1	1.964 (1.959)	1.957 (1.972)		
	2	2.067 (2.065)	2.075 (2.072)	2.065	2.075
	5	2.048	2.048	2.047	2.047
	5'	2.040 (2.043)	2.040 (2.043)	2.037	2.037
CH ₃ of Fuc		—	1.162 (1.163)	—	1.178

^aChemical shifts are expressed in ppm from internal 3-(trimethylsilyl) propionic-2, 2, 3, 3-d-acid, sodium salt but were actually measured with internal acetone ($\delta=2.22$ ppm in D₂O at 30°C). Values in parentheses were measured at 65°C by using internal acetone ($\delta=2.22$ ppm in D₂O).

^bData from Ref. 27. Values were measured with internal acetone ($\delta=2.216$ ppm in D₂O). Values in brackets were measured at 60°C by using internal acetone ($\delta=2.213$ ppm in D₂O).

Isolation of PA-oligosaccharides of hVN

PA-oligosaccharides purified by gel-filtration on a Sephadex G-15 were separated by anion-exchange HPLC (DEAE-5PW column) into three fractions (Fig. 5(A)). The elution positions of fractions hM, hD and hT corresponded to those of PA-mono-, di- and tri-sialooligosaccharides, respectively. Although Fr. N was eluted at the position of neutral PA-oligosaccharides, no detectable peak corresponding to PA-oligosaccharides was observed on following reversed-phase HPLC (CLC-ODS column). These results suggested that N-linked oligosaccharides of hVN were nearly completely

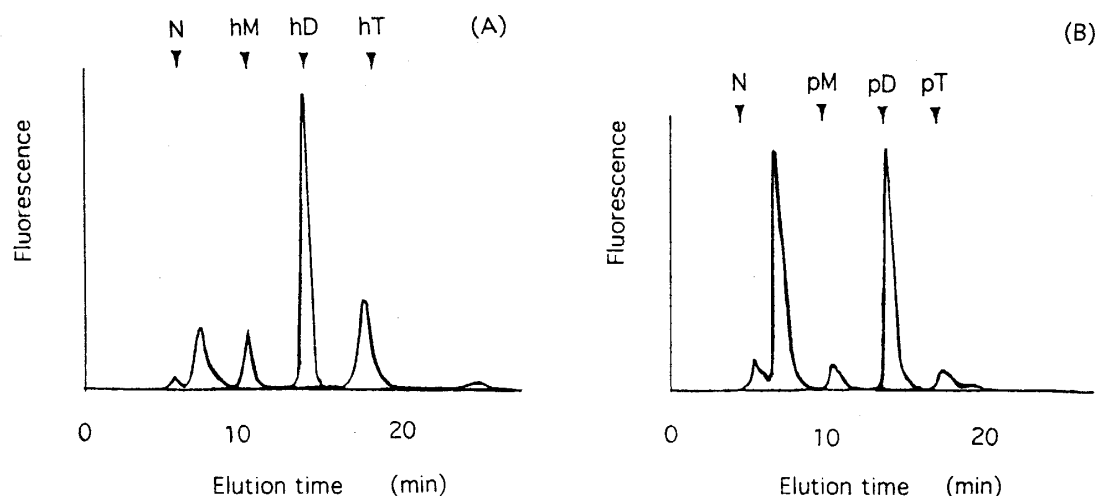


Fig. 5. Anion-exchange HPLC of PA-oligosaccharides of hVN (A) and pVN (B). PA-oligosaccharides of hVN and pVN were separated by anion exchange HPLC (DEAE-5PW column) according to the number of sialic acids. The elution was performed as described by Yamamoto et al. (23). The elution positions of peak hM, hD and hT in (A) pM, pD and pT in (B) were those of mono-, di- and tri-PA-sialooligosaccharide, respectively. Though peaks N of both hVN and pVN were eluted at the neutral PA-oligosaccharide, no detectable peak corresponding to PA-oligosaccharide was observed on reversed-phase HPLC (CLC-ODS column).

sialylated. The molar ratio of hM:hD:hT was 8:70:22.

Frs. hM, hD and hT were analyzed by reversed-phase HPLC (Cosmosil 5C18-P column) as shown in Fig. 6(A), 6(B) and 6(C), respectively. Fr. hM was separated into three major fractions (in parentheses indicate % molar ratio): hM-1(3.4), hM-2(2.7) and hM-3(1.0); hD into four: hD-1(9.6), hD-2(45.8), hD-3(3.1) and hD-4(7.9); and hT into three: hT-1(7.1), hT-2(9.0), and hT-3(3.8).

Structures of PA-oligosaccharides of hVN

Each fraction desialylated by mild acid hydrolysis was analyzed by the 2-D mapping method described above and compared with the asialo PA-oligosaccharides identified as Frs. a~g. In the case of hD-2, acid-desialylated product, asialo hD-2, was eluted at the identical position of PA-asialooligosaccharide Fr. e on ODS-silica and amide-silica HPLC, respectively. The identity of asialo hD-2 and Fr. e was supported by the following observations: Co-injection of asialo hD-2 with Fr. e onto these HPLC showed a single peak on each HPLC, and the elution position of the digest with β -galactosidase indicated the decrease of two galactose residues on size fractionation HPLC. In the similar ways, other desialylated fractions were tested for matching with respective PA-asialooligosaccharides. Results are shown in Table III.

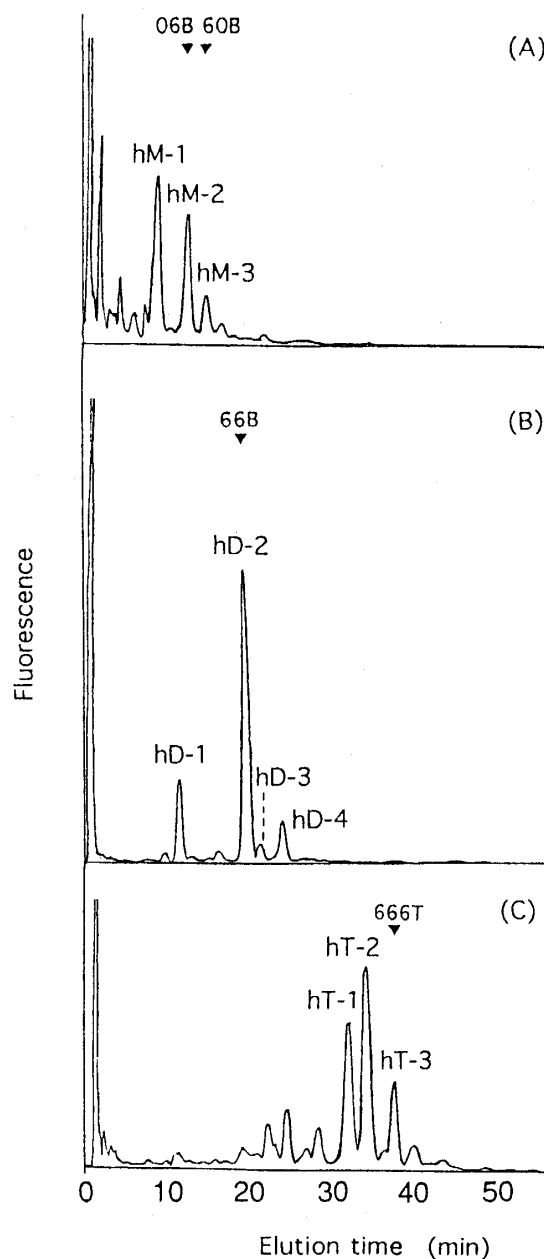


Fig. 6. Reversed-phase HPLC of PA-sialooligosaccharide fraction, hM (A), hD (B), and hT (C) on Cosmosil 5C18-P column. The elution was done with 0.1 M acetic acid containing a gradient of 1-butanol for 55 min at 23°C. Arrows, 06B, 60B, 66B and 666T, are the elution positions of standard PA-oligosaccharides. The structures of standard PA-sialooligosaccharides are listed in Scheme 2.

To analyze the linkage of sialic acid, the elution position of each fraction was compared with those of standard PA-sialooligosaccharides on the Cosmosil 5C18-P column. The structures of the standard PA-sialooligosaccharides are listed in Scheme 2. When hD-2 was co-injected with the

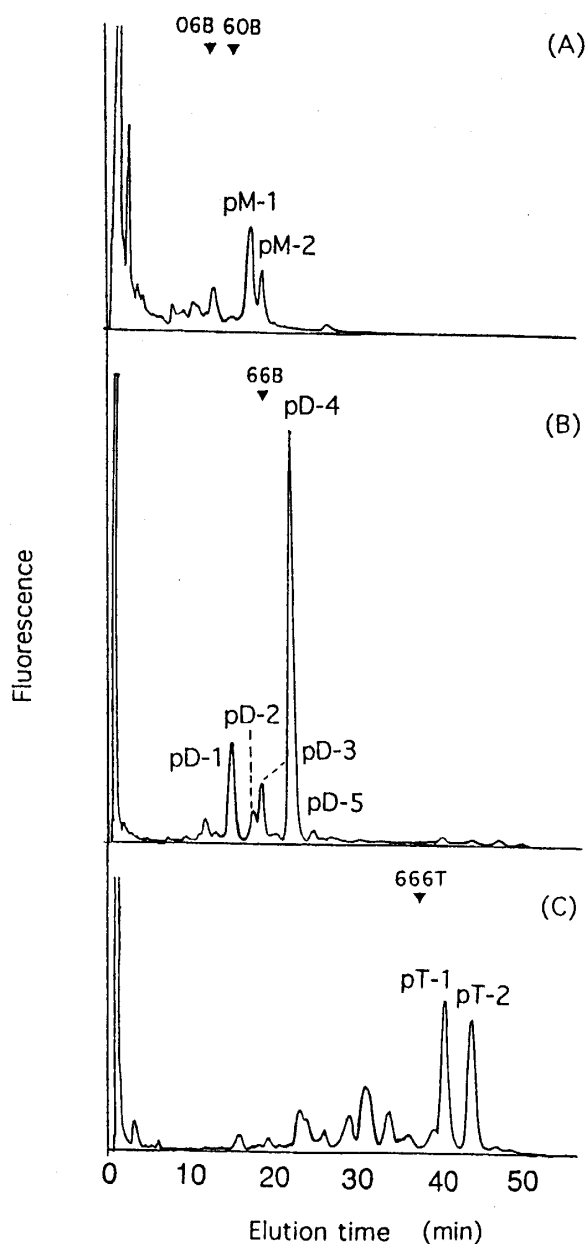


Fig. 7. Reversed-phase HPLC of PA-sialooligosaccharide fraction, pM (A), pD (B), and pT (C) on Cosmosil 5C18-P column. The elution condition and arrows, see the legend to Fig. 6.

standard 66B, a single peak was observed. The asialo hD-2 was Fr. e as mentioned above, indicating that hD-2 is 66B. In similar ways, hM-2, hM-3 and hT-3 were assigned to be 06B, 60B and 666T, respectively. The results are summarized in Table III and the proposed structures for PA-sialooligosaccharides are listed in Table IV. When the reaction product of hD-4 by α -L-fucosidase digestion was co-injected with standard 66B, a single peak was obtained. As the asialo hD-4 was Fr. g, hD-4 is 66B with fucose linked to the innermost *N*-acetylglucosamine by α 1 \rightarrow 6 linkage.

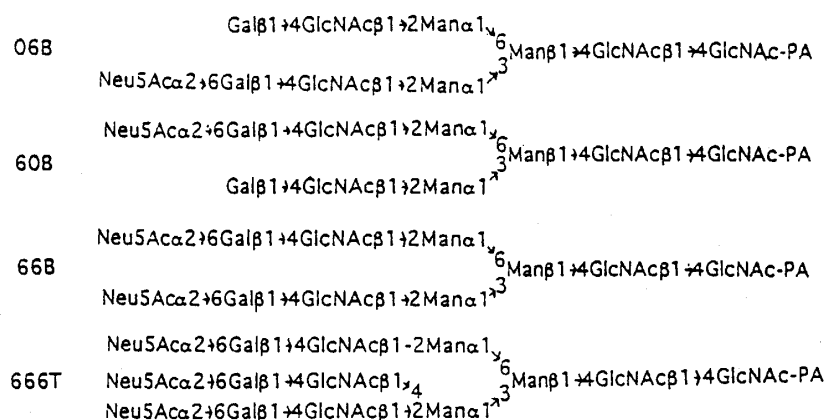
Table III. Characteristics of PA-sialooligosaccharides.

Fraction	Molar ratio (%)	Fraction of PA-asialooligosaccharide after desialylation ¹	Number of $\alpha 2 \rightarrow 3$ linked sialic acid ²	Number of standard PA-sialooligosaccharide ³
hVN				
hM-1	3.4	b-1	0	de(Gal-GlcNAc)-06B
hM-2	2.7	e	0	06B
hM-3	1.0	e	0	60B
hD-1	9.6	a-2	0	
hD-2	45.8	e	0	66B
hD-3	3.1	e	1	
hD-4	7.9	g	0	66B (after defucosylation)
hT-1	7.1	f-1, f-2	1	
hT-2	9.0	f-1	1	
hT-3	3.8	f-1	0	666T
pVN				
pM-1	3.0	c	0	06B (after defucosylation)
pM-2	1.7	c	0	60B (after defucosylation)
pD-1	15.6	a	0	
pD-2	5.0	c	0	
pD-3	7.6	b	0	66B
pD-4	51.8	c	0	66B (after defucosylation)
pD-5	2.2	c	1	
pT-1	2.3	d	1	
pT-2	2.1	d	0	666T (after defucosylation)

1 Desialylated fraction was co-injected with corresponding PA-asialooligosaccharide.

2 The *Salmonella* sialidase digest of each PA-oligosaccharide was applied on anion-exchange HPLC (DEAE-5PW column).

3 Intact or defucosylated fraction was co-injected with corresponding standard PA-sialooligosaccharide onto reversed-phase HPLC (Cosmosil 5C18-P column). Standard PA-sialooligosaccharides were listed in Scheme 2.



The naming is according to Yamamoto et al.(38).

Scheme 2. Structures of standard PA-sialooligosaccharides.

Table IV. Proposed structures of PA-sialooligosaccharides in hVN and pVN.

hVN	
hM-1	$\begin{array}{c} \text{Man}\alpha 1 \\ \swarrow \\ \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \swarrow \\ \text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \end{array} \begin{array}{c} \nearrow \\ \nearrow \\ \nearrow \end{array} \begin{array}{c} 6 \\ 3 \end{array} \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc-PA}$
hM-2	$\begin{array}{c} \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \swarrow \\ \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \end{array} \begin{array}{c} \nearrow \\ \nearrow \end{array} \begin{array}{c} 6 \\ 3 \end{array} \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc-PA}$
hM-3	$\begin{array}{c} \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \swarrow \\ \text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \end{array} \begin{array}{c} \nearrow \\ \nearrow \end{array} \begin{array}{c} 6 \\ 3 \end{array} \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc-PA}$
hD-2	$\begin{array}{c} \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \swarrow \\ \text{Neu5Ac}\alpha 2 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \end{array} \begin{array}{c} \nearrow \\ \nearrow \end{array} \begin{array}{c} 6 \\ 3 \end{array} \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc-PA}$
hD-3	$\begin{array}{c} \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \swarrow \\ \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \end{array} \begin{array}{c} \nearrow \\ \nearrow \end{array} \begin{array}{c} 6 \\ 3 \end{array} \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc-PA}$
hD-4	$\begin{array}{c} \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \swarrow \\ \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \swarrow \\ \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \end{array} \begin{array}{c} \nearrow \\ \nearrow \\ \nearrow \end{array} \begin{array}{c} 6 \\ 3 \end{array} \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc-PA}$
hT-2	$\begin{array}{c} \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \swarrow \\ \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \swarrow \\ \text{Neu5Ac}\alpha 2 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \end{array} \begin{array}{c} \nearrow \\ \nearrow \\ \nearrow \end{array} \begin{array}{c} 6 \\ 3 \end{array} \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc-PA}$
hT-3	$\begin{array}{c} \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \swarrow \\ \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \swarrow \\ \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \end{array} \begin{array}{c} \nearrow \\ \nearrow \\ \nearrow \end{array} \begin{array}{c} 6 \\ 3 \end{array} \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc-PA}$

When hM-1 was co-injected with the digestion product of standard 06B treated with β -galactosidase and then β -N-acetylhexosaminidase on reversed-phase HPLC, a single peak was observed. As the asialo hM-1 was Fr. b-1, the structure of hM-1 is $\text{Man}\alpha 1 \rightarrow 6(\text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow 3)\text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc-PA}$.

To study the presence of $\alpha 2 \rightarrow 3$ linked sialic acid, each fraction was digested with *Salmonella* sialidase, and then the digest was analyzed on anion-exchange HPLC. The digestion product of hD-3 was eluted at the position of PA-monosialooligosaccharide, those of hT-1 and hT-2 were at the position of disialoderivatives. The results suggested that hD-3, hT-1,

Table IV. (Continued)

pVN ^a	
pM-1	$\begin{array}{c} \text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \searrow \begin{array}{c} 6 \\ 3 \end{array} \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc-PA} \\ \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \end{array}$
pM-2	$\begin{array}{c} \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \searrow \begin{array}{c} 6 \\ 3 \end{array} \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc-PA} \\ \text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \end{array}$
pD-3	$\begin{array}{c} \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \searrow \begin{array}{c} 6 \\ 3 \end{array} \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc-PA} \\ \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \end{array}$
pD-4	$\begin{array}{c} \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \searrow \begin{array}{c} 6 \\ 3 \end{array} \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc-PA} \\ \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \end{array}$
pD-5	$\begin{array}{c} \text{Neu5Ac}\alpha 2 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \searrow \begin{array}{c} 6 \\ 3 \end{array} \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc-PA} \\ \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \end{array}$
pT-1	$\begin{array}{c} \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \searrow \begin{array}{c} 6 \\ 3 \end{array} \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc-PA} \\ \text{Neu5Ac}\alpha 2 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc-PA} \\ \searrow 4 \end{array}$
pT-2	$\begin{array}{c} \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \searrow \begin{array}{c} 6 \\ 3 \end{array} \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc-PA} \\ \text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \searrow 4 \end{array}$

^a As a sialic acid, pVN contains Neu5Ac and Neu5Gc at a molar ratio of 10:1. Neu5Gc was found on all major oligosaccharides. The location of Neu5Gc on each oligosaccharide is not known and Neu5Ac represents sialic acid in this table.

and hT-2 contained one sialyl $\alpha 2 \rightarrow 3$ linkage, respectively (Table III).

The structures of hD-3 and hT-2 were further analyzed by comparing their exoglycosidase-treated products with standards. One sialic acid of hD-3 was released by *Salmonella* sialidase digestion and the digestion product (monosialoderivative) was eluted at the identical position of standard 06B on reversed-phase HPLC (Cosmosil 5C18-P column). As asialo hD-3 was Fr. e, these results indicated that the structure of hD-3 is Neu5Ac $\alpha 2 \rightarrow 3$ Gal $\beta 1 \rightarrow 4$ GlcNAc $\beta 1 \rightarrow 2$ Man $\alpha 1 \rightarrow 6$ (Neu5Ac $\alpha 2 \rightarrow 6$ Gal $\beta 1 \rightarrow 4$ GlcNAc $\beta 1 \rightarrow 2$ Man $\alpha 1 \rightarrow 3$)Man $\beta 1 \rightarrow 4$ GlcNAc $\beta 1 \rightarrow 4$ GlcNAc-PA. The digest of hT-2 with *Salmonella*

sialidase, which was a disialoderivative, was sequentially digested with β -galactosidase and then β -N-acetylhexosaminidase. When the final product was co-injected with standard 66B on reversed-phase HPLC (Cosmosil 5C 18-P column) only a single peak was observed. As asialo hT-2 was Fr. f-1, it should be Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4 branch on Man α 1 \rightarrow 3 arm that was trimmed by sequential exoglycosidase digestion. These results indicated that the structure of hT-2 is Neu5Ac α 2 \rightarrow 6Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 6(Neu5Ac α 2 \rightarrow 3Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4)(Neu5Ac α 2 \rightarrow 6Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2)Man α 1 \rightarrow 3Man β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4GlcNAc-PA.

The asialo hD-1 was Fr. a-2, indicating that hD-1 is an artifact generated by epimerization. hT-1 contained one sialyl α 2 \rightarrow 3 linkage, but asialo hT-1 was Frs. f-1 and f-2 in almost equal amount. The precise structure could not be presumed for hT-1.

Isolation of PA-oligosaccharides of pVN

In the same manner as hVN, PA-oligosaccharides of pVN were prepared and separated by anion-exchange HPLC into three fractions (Fig. 5(B)). The elution positions of pM, pD and pT were those of PA-mono-, di- and trisialooligosaccharides, respectively. As well as the case of hVN, Fr. N gave no detectable peak corresponding to PA-oligosaccharides on a CLC-ODS column. These results suggested that N-linked oligosaccharides of pVN were almost completely sialylated. The molar ratio of pM:pD:pT was 7:89:4.

On reversed-phase HPLC (Cosmosil 5C18-P column), major fractions of pM were separated into two fractions (in parentheses indicate % molar ratio): pM-1(3.0) and pM-2(1.7); those of pD into five: pD-1 (15.6), pD-2 (5.0), pD-3(7.6), pD-4(51.8), and pD-5(2.2); and those of pT into two: pT-1 (2.3) and pT-2(2.1), as shown in Fig. 7(A), 7(B), and 7(C), respectively.

Structures of PA-oligosaccharides of pVN

Each fraction desialylated by mild acid hydrolysis was analyzed for matching with respective PA-asialooligosaccharide by the 2-D mapping method as described in the case of hVN oligosaccharides. The results are summarized in Table III.

The digestion product of pD-4 with α -L-fucosidase was co-eluted with standard 66B on reversed-phase HPLC (Cosmosil 5C18-P column). As asialo form of pD-4 was Fr.c, these results indicated that the structure of pD-4 is Neu5Ac α 2 \rightarrow 6Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 6(Neu5Ac α 2 \rightarrow 6Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 3)Man β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4(Fuc α 1 \rightarrow 6)GlcNAc-PA. In similar ways, pM-1, pM-2 and pT-2 were assigned to be; pM-1: Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 6(Neu5Ac α 2 \rightarrow 6Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 3)Man β 1 \rightarrow 4Glc

Nac β 1 \rightarrow 4(Fuca1 \rightarrow 6)GlcNAc-PA, pM-2: Neu5Ac α 2 \rightarrow 6Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 6(Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 3)Man β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4(Fuca1 \rightarrow 6)GlcNAc-PA, pT-2: Neu5Ac α 2 \rightarrow 6Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 6(Neu5Ac α 2 \rightarrow 6Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4)(Neu5Ac α 2 \rightarrow 6Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2)Man α 1 \rightarrow 3Man β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4(Fuca1 \rightarrow 6)GlcNAc-PA, respectively. pD-3 was co-eluted with standard 66B on reversed-phase HPLC. As asialo pD-3 was Fr. b, these results indicated that pD-3 is 66B.

DEAE-HPLC of each fraction before and after digestion with *Salmonella* sialidase showed that pD-5 and pT-1 have one α 2 \rightarrow 3 sialyl linkage (Table III). pD-5 was digested with *Salmonella* sialidase followed by α -L-fucosidase digestion. When the product was co-injected with standard 06B on reversed-phase HPLC (Cosmosil 5C18-P column), a single peak was obtained. As asialo pD-5 was Fr. c, these results indicated that the structure of pD-5 is Neu5Ac α 2 \rightarrow 3Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 6(Neu5Ac α 2 \rightarrow 6Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 3)Man β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4(Fuca1 \rightarrow 6)GlcNAc-PA. In the case of pT-1, the reaction product of pT-1 by α -L-fucosidase digestion was co-eluted with hT-2 from a Cosmosil 5C18-P column. As asialo pT-1 was Fr. d, these results indicated that the structure of pT-1 is Neu5Ac α 2 \rightarrow 6Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 6(Neu5Ac α 2 \rightarrow 3Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4)(Neu5Ac α 2 \rightarrow 6Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2)Man α 1 \rightarrow 3Man β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4(Fuca1 \rightarrow 6)GlcNAc-PA. All the proposed structures for PA-sialooligosaccharides of hVN and pVN are listed in Table IV. The asialo pD-1 was Fr. a, indicating that pD-1 is an artifact generated by epimerization. Although asialo pD-2 was Fr. c, linkage and location of sialic acids is not yet clarified.

Distribution of Neu5Gc in oligosaccharides of pVN

As a sialic acid, pVN contained Neu5Gc besides Neu5Ac at a molar ratio of 1:10 [15]. To know the distribution of Neu5Gc in sialooligosaccharides of pVN, major PA-oligosaccharides were analyzed for sialic acid composition. As a result, Neu5Gc was found in each PA-oligosaccharide fraction together with Neu5Ac at a similar molar ratio. Whereas only Neu5Ac was found in hVN oligosaccharide analyzed in the same way as a control.

Discussion

This paper describes the complete structures of the N-linked carbohydrate chains attached to hVN and pVN. The chemical structures of 8 and 7 oligosaccharides of hVN and pVN, respectively, have been determined by comparing the elution positions of their PA-derivatives with those of stan-

dard oligosaccharides before and after exoglycosidase digestions (Table III). The structures of the major oligosaccharides of both vitronectins agreed well with the carbohydrate compositions [15]. The amino acid sequence of hVN predicted from cDNA contains 3 potential asparagine glycosylation sites [1,3], while the number of glycosylation sites of pVN is unknown. The size of the carbohydrate moieties of hVN and pVN calculated from carbohydrate contents [15] are 6 kDa and 5 kDa, respectively, and by deglycosylation analysis [16], 7 kDa and 4 kDa, respectively. Since the molecular weights of major oligosaccharides of hVN and pVN calculated are 2.2kDa and 2.4kDa, respectively, these data suggest that hVN has 3 carbohydrate chains while pVN has 2 on a molecule.

The most abundant oligosaccharide of hVN was of biantennary lactosamine type with 2 or 1 NeuAc. A smaller amounts of tri- and mono-antennary type with 1~3 NeuAc were also present. In contrast, the most abundant oligosaccharide of pVN was of biantennary type with a fucose linked by $\alpha 1 \rightarrow 6$ to the innermost *N*-acetylglucosamine and 2 or 1 sialic acid. A smaller amounts of trisialotriantennary type with a fucose and disialobiantennary type without fucose were also present. The linkage of sialic acid was mainly $\alpha 2 \rightarrow 6$ on both vitronectins. Sialic acids linked to terminal Gal by $\alpha 2 \rightarrow 3$ linkage besides $\alpha 2 \rightarrow 6$ linked sialic acid were also found on 19.2% of hVN oligosaccharides and on 4.5% of pVN. For both vitronectins, $\alpha 2 \rightarrow 3$ linked sialic acid was found on the $\beta 1 \rightarrow 4$ branching lactosamine of $\alpha 1 \rightarrow 3$ linked mannose on trisialooligosaccharides, and on the branch of $\alpha 1 \rightarrow 6$ arm on disialooligosaccharides. These locations of $\alpha 2 \rightarrow 3$ and $\alpha 2 \rightarrow 6$ linked sialic acids agree with the relative preference of $\alpha 2 \rightarrow 6$ sialyltransferase for addition of Neu5Ac to peripheral branches [28].

Since this is the first report elucidating the carbohydrate structure of vitronectins, it is interesting to compare our results with those obtained for other plasma glycoproteins. The oligosaccharide structure of fibronectin, another major cell-adhesive glycoprotein, has been reported for human [29], hamster [30], and bovine [31]. Plasma fibronectins from three animal species commonly contain biantennary lactosamine-type oligosaccharide but no triantennary type, and the diversity was found mainly in sialylation including linkage, location and degree of sialylation. Vitronectin oligosaccharides showed higher heterogeneities than those of fibronectin while sialylation patterns were very similar between hVN and pVN. The most remarkable difference between hVN and pVN are absence or presence of fucose at the innermost *N*-acetylglucosamine. Only porcine fibronectin has been reported to contain fucose [32,33] as well as porcine transferrin [34], suggesting that core fucosylation may commonly take place during *N*-glycosylation process of porcine plasma glycoproteins.

Important finding in this report is the distribution of Neu5Gc on porcine VN. pVN contain Neu5Gc at a molar ratio of one to tenth of Neu5Ac [15]. The sialic acid composition of purified PA-oligosaccharides were analyzed to know whether Neu5Gc is localized on particular oligosaccharide or not. As a result, Neu5Gc was found equally on all major oligosaccharides. Neu5Gc is biosynthetically generated from Neu5Ac by hydroxylase before carbohydrate chain synthesis [35,36]. Higa and Paulson showed that purified rat liver sialyltransferase can utilize CMP-Neu5Gc and CMP-Neu5Ac as equally good donor substrates *in vitro* [37]. Our result strongly suggest that sialyltransferases working in the glycosylation of pVN actually transfer Neu5Gc as equally as Neu5Ac to each oligosaccharide present on vitronectin *in vivo*.

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