pH 3.5–8.5. As shown in Figure 1C, the binding of VN to collagen at pH 7.5 was sensitive to ionic strength, indicating that the interaction at pH 7.5 depends largely on electrostatic interactions. At pH 4.5, which is close to the isoelectric point of human VN, pI = 4, the binding was maximum around the physiological ionic strength, suggesting that other binding forces, such as hydrophobic interaction, in addition to electrostatic interaction were involved in the binding at this pH.

Collagen monomers aggregate under neutral pH at more than 20 °C, but alkaline treatment of collagen changes the charge of the peptide to form a stable monomer at neutral pH at room temperature (Nakashima et al. 1992). The effect of collagen aggregation on the binding of VN is shown in Figure 1D. The binding of VN to alkaline-treated collagen was diminished at neutral pH, indicating that the aggregation of collagen is essential for interaction with VN.

Differential s-value of VN under various temperatures and pH

Activation of VN has been shown to be accompanied by transition to a multimer from the inactive monomer (Gebb et al. 1986; Izumi et al. 1989). Therefore, we measured the relationship between the multimer size of VN and collagen binding under various conditions by analytical ultracentrifugation. As shown in Figure 2A, the sedimentation velocity indicated that the *s*-values of the peak position in c(*s*), distribution funciton of sedimentation coefficients, becomes larger as the temperature increases, $s_{20,w} = 17$ S (4 °C), $s_{20,w} = 18$ S (20 °C), and $s_{20,w} = 21$ S (37 °C). Monomer human VN was reported to have a $s_{20,w}$ of 4.1 S (20 °C) (Zhuang, Blackburn, et al. 1996). VN at 37 °C, a better temperature for collagen binding than 4 °C (Figure 1A and B), exhibits a larger *s*-value for the multimer than at 4 °C.

In our preceding studies, the collagen-binding activity of VN was enhanced by treatment with neuraminidase or peptide *N*-glycosidase (PNGase F) in vitro (Yoneda et al. 1998). As shown in Figure 2B–D, a new component peak of larger *s* values appeared in the c(*s*) profiles of neuraminidase or PNGase F treated VNs when compared with those of control VN. At pH 4.5, neuraminidase-treated VN contained molecular species with a high *s*-value of 22 S (Figure 2B), suggesting a clear effect of glycan structure on the multimerization of VN. As shown in Figure 2B and C, neuraminidase-treated VN formed multimers of larger *s*-value, 52 S, at pH 4.5 than at pH 7.5. Together with the results shown in Figure 1, these results indicate that the collagen-binding activity correlates with the degree of multimerization of VN, i.e., the interaction between VN and collagen must be affected by the multimerization of VN.

Effects of glycosidase-trimming on multimerization and collagen-binding of VN

Various glycans on VN were trimmed stepwise with exoglycosidase to elucidate the effect of alterations of glycans on collagen binding, as indicated in Figure 3A. The involvement of disulfide linkage in the multimerization of VN was estimated from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) under reducing and nonreducing conditions using 5–20% gradient gel. From the intensities of bands (Figure 3B), the relative amounts of monomer (65 and 75 kDa) and multimer VNs (top of the gel) were calculated by using Scion Image. As shown in Figure 3C, the intensities

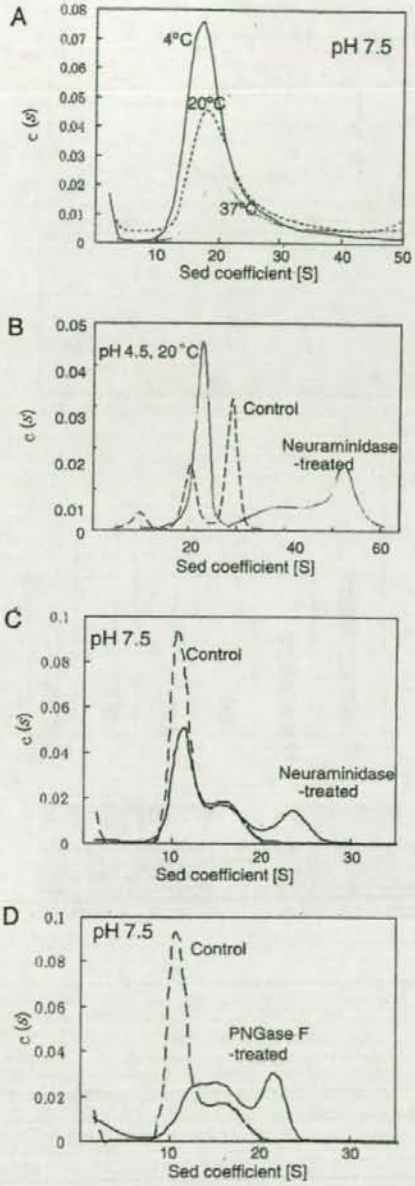

Fig. 2. Distribution of *s*-values of VN changes by temperature, pH, and deglycosylation. (A) Solutions of human VN in PBS containing 5 mM EDTA (A280 = 0.2) were centrifuged for sedimentation velocity analysis at 4 °C (solid line), 20 °C (dashed line), or 37 °C (dotted line) at pH 7.5 as described in the text. (B–D) The effects of enzymatic deglycosylation of VN on *s*-value. Human VN treated with neuraminidase in citrate-phosphate buffer (B and C, solid line), PNGase F in PBS–EDTA (D, solid line), or controls incubated without enzyme (dashed line) were centrifuged at 20 °C at pH 4.5 (B), and pH 7.5 (C, D). The time-dependent moving boundaries recorded at 280 nm were fitted to the numerical solution of the Lamm equation by using the program Sedfit in order to obtain the distribution function of the apparent sedimentation coefficient *s*, c(*s*).

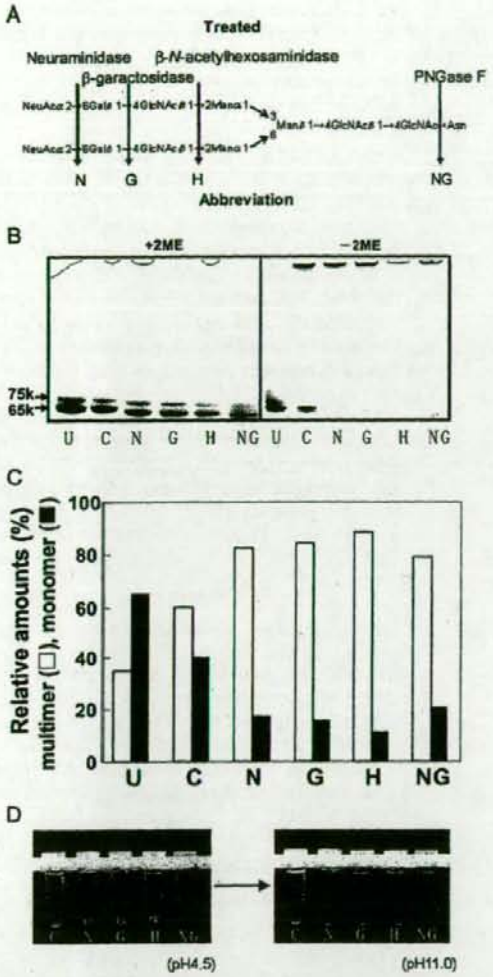

Fig. 3. Multimerization and sedimentation of VN changes by glycosidase-trimming. (**A**), Cleavage sites of enzymatic deglycosylation in the structure of the major *N*-glycan of human VN. (**B**) Glycosidase-trimmed human VNs (5 μg/lane) were loaded on each lane of 5–20% polyacrylamide gel and run for SDS–PAGE in the presence or absence of 2-mercaptoethanol (2ME). The gel was stained with Coomassie brilliant blue (CBB). (**C**) Relative amount of multimer (white bar) to monomer (black bar) under nonreducing conditions was calculated from the band intensities using the software program Scion Image, and expressed by taking the intensities of the monomer band of each VN under reducing condition as 100%. (**D**) Sedimentation of glycosidase-trimmed VNs at pH 4.5. Right, pH 11. U, Untreated VN; C, control VN incubated without enzyme; N, VN treated with neuraminidase; G, VN treated with neuraminidase and β-galactosidase; H, VN treated with neuraminidase, β-galactosidase and β-N-acetylhexosaminidase; NG, VN treated with PNGase F.

of multimer bands were remarkably increased by neuraminidase treatment. The results indicate that formation of multimer VN by intermolecular disulfide bonds was gradually increased with stepwise glycan trimming. Supportingly, as shown in Figure 3D, the amount of sediment from glycosidase-trimmed VNs was gradually increased with the glycan trimming at pH 4.5, but the sediment was solubilized by adding 0.1 M NaOH to pH 11, irrespective of the glycan trimming of VN in the five tubes, showing that the sedimentation is caused by secondary aggregation by ionic interaction due to the charges of the protein surface. These results indicate that VN aggregation was enhanced as the stepwise glycan trimming proceeded. It suggests that the glycans of intact VNs contribute to the suppression of multimer formation and the deglycosylation of VN leads to promotion of multimerization.

As shown in Figure 4, the increase in multimerization of VN by glycan trimming accompanied the enhancement of average molecular weights as measured by sedimentation equilibrium. The multimer size of human VN had increased by 1.5-fold on total deglycosylation of intact VN (Figure 4B). These results may indicate that the enzymatic deglycosylation of VN leads to the promotion of multimer formation in both size and amount.

As shown in Figure 5, the binding of VN to collagen was found to gradually increase with stepwise glycan trimming. The increase due to treatments with neuraminidase and PNGase F was especially remarkable. The observation indicates that the collagen binding activity correlated with the extent of multimerization of VN and that the changes in glycosylation altered the collagen-binding activity of VN through a multivalent effect.

Multimerization of NO-, SH-, and PH-VNs

VNs were purified from rat plasma before and after partial hepatectomy, and the multimerization degree of the purified VN was analyzed. As shown in Figure 6A, SDS–PAGE under reducing and nonreducing conditions showed that multimer VNs were completely dissociated by 2-mercaptoethanol, indicating that multimers are cross-linked by disulfide linkage. The intensities of each band under the nonreducing condition were quantified by using Scion image (Figure 6B). The amount of multimers increased more in PH-VN (91%) than in NO (79%) and SH-VNs (82%).

Whether the relationship between the glycosylation change and multimerization that was observed for human VN holds for rat VN or not, the effects of enzymatic deglycosylation of NO-VN on the multimerization were examined. As shown in Figure 6C and D, both neuraminidase- and PNGase F-treated VNs showed an increase in the ratio of multimers to monomers compared with the control VN on SDS–PAGE, similar to that observed for human VN (Figure 3). Therefore, the conclusion that the glycosylation change causes the increases of multimer VN and collagen binding, which were obtained by using human VN, was shown to be a common attribute of rat and human VNs.

The weight average molecular weights of multimer sizes of NO-, SH-, and PH-VNs were measured by sedimentation equilibrium. As shown in Figure 7, the weight average molecular weights of PH-, and SH-VNs were 420 and 380 kDa, respectively, which were increases of 45 and 31%, respectively, compared with that of NO-VN, 290 kDa. The degrees of polymerization of PH-, SH-, and NO-VNs were calculated to be 7.0, 6.1, and 4.2-mer, respectively, on the basis of the molecular weights of each VN monomer, 68.8, 61.9, and 59.6 kDa, respectively, that were calculated from the cDNA sequence and sugar composition (Yoneda et al. 1998). The multimerization states of VNs indicate that the size and number of multimers are increased in PH-VN, which would

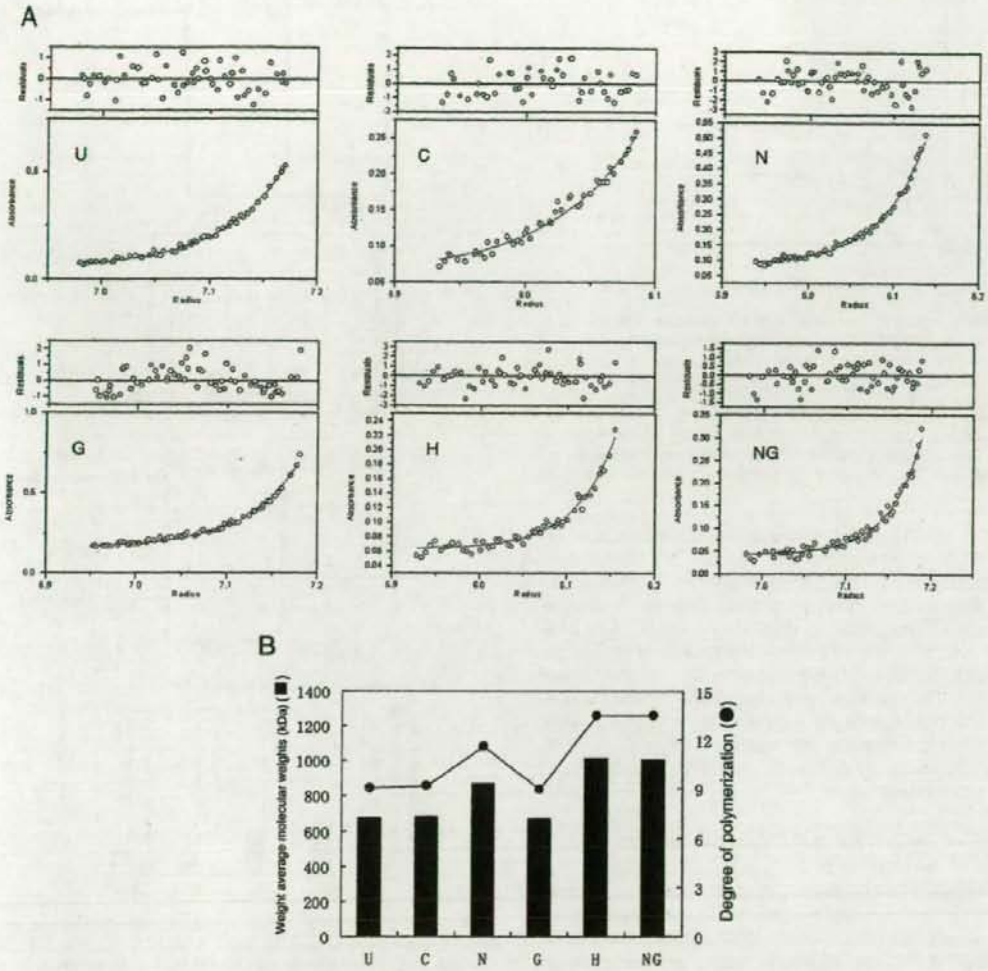

Fig. 4. Molecular weights of glycosidase-trimmed VNs by sedimentation equilibrium. Sedimentation equilibrium analysis of deglycosylated human VNs was performed at a concentration of 0.3 mg/mL in PBS containing 5 mM EDTA at 20 °C. Data were collected after equilibrium had been established at rotor speeds of 3000 and 5000 rpm by absorbance at 280 nm, and the data were analyzed using program, "nonlin" to calculate the averages molecular weights. (**A**) Radial distribution of absorbance at equilibrium at 5000 rpm for VNs. Individual absorbance readings are shown by open circles, with solid lines through the data representing the fit to a single species model. The residuals for the fit are shown in the upper panel. (**B**) The calculated weight average molecular weights of VNs. U, Untreated VN; C, control VN incubated without enzyme; N, VN treated with neuraminidase; G, VN treated with neuraminidase and β-galactosidase; H, VN treated with neuraminidase, β-galactosidase and β-N-acetylhexosaminidase; and NG, VN treated with PNGase F.

be attributable to the changes in glycosylation according to the results of glycosidase-trimming of VN (Figures 3 and 4).

SPR measurements of collagen-binding activity of NO-, SH-, and PH-VNs

Previously, we reported that ELISA showed that the collagen-binding activity of VN increased three-fold with the glycan change after partial hepatectomy (Yoneda et al. 1998). In this study, the interaction of NO-, SH-, and PH-VN with type I collagen was quantitatively analyzed by SPR. As shown in Figure 8A, the resonances were monitored when NO-, SH-, or PH-VN was placed in contact with immobilized collagen. The amount of type I collagen immobilized on one flow cell of a sensor chip was 4312 RU. As shown in Figure 8A–C, the binding of collagen to each VN was concentration-dependent, and the dissociation was very slow. Among the three VNs, PH-VN bound best to type I collagen. Under these conditions, VN is in a multimer form (Figure 7), and multivalent–multivalent interaction with type I collagen cannot be analyzed to obtain kinetic parameters. However, when injected into a single flow cell at the same concentration, the amount of each bound VN on the sensor chip correlates

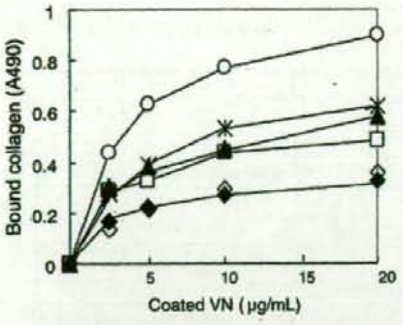

Fig. 5. The collagen-binding activities of glycosidase-trimmed VN. Microtiter wells were coated with type I collagen and incubated with glycosidase-trimmed human VNs. The amounts of bound VNs were measured with ELISA at pH 7.5 at 4 °C, as described in the text. The data are averages of four experiments. U, Untreated VN (◇); C, control VN incubated without enzyme (◆); N, VN treated with neuraminidase (□); G, VN treated with neuraminidase and β-galactosidase (▲); *H*, VN treated with neuraminidase, β-galactosidase and β-*N*-acetylhexosaminidase (*); and NG, VN treated with PNGase F (○). The data of untreated VN and control VN completely overlapped each other.

with the total affinity of VN to the collagen immobilized on the sensor chip. To calculate the bound VN (femtomole/square millimeters), the maximum responses monitored at the end of the injection phase were divided by the molecular weight of each VN monomer, as shown in Figure 8D. The relative affinity per monomer of PH-VN is remarkably high compared with NO- and SH-VNs, especially at the lower concentrations. The results support the relative binding activities of VNs to type I collagen suggested by ELISA (Yoneda et al. 1998) and indicate that the binding activity of PH-VN, which is much enhanced compared with NO- and SH-VNs, is due to a multivalent effect.

Elution profile of NO-, SH-, and PH-VNs in sera on gel-filtration chromatography

Most VN in plasma has been reported to be an inactive monomer form, and a small portion of plasma VN is in an active multimer form (Izumi et al. 1989). During the purification in this study, the originally active molecules were removed by the first heparin affinity chromatography, and then the originally inactive VN in serum was activated with urea during the purification procedure to be purified by the second heparin affinity chromatography. Therefore, the sizes and amounts of VNs in native NO-, SH-, and PH- sera were analyzed by gel-filtration high-performance liquid chromatography (HPLC) in combination with ELISA detection to elucidate the state of the VN multimers in each serum. As shown in Figure 9A, immunological detection of the eluted fractions using anti-VN immunoglobulins G (IgGs) indicated that most VN was eluted in the major protein peak that corresponds to a monomer VN. The major protein peak of each serum was detected at A280 at the elution time of 11.3 min in HPLC chromatograms, which corresponds to a molecular weight of about 65 kDa. The difference in the three sera was found in the elution positions of multimer VN at the elution time of 6–11 min, corresponding to more than 150 kDa. The amount of multimer VN was increased in the PH serum compared with those in NO and SH sera. A peak of significantly larger

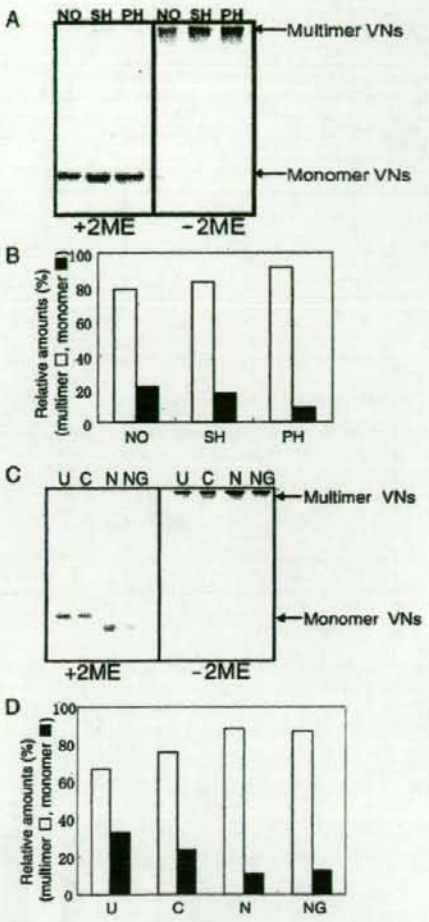

Fig. 6. Amount of rat VN multimer changes after partial hepatectomy (**A** and **B**) and due to enzymatic deglycosylation (**C** and **D**). (A) NO-, SH-, and PH-VN (each 5 μg) were loaded on each lane of 7.5% polyacrylamide gel and run for SDS–PAGE in the presence or absence of 2-mercaptoethanol (2ME). The gels were stained with CBB. (C) Neuraminidase-, PNGase F-treated or untreated VNs (each 4 μg) were subjected to SDS–PAGE under the same condition as in *(A)*. (B) and (D) The relative amount of multimer (white bar) to monomer (black bar) was calculated from the intensities of each band in (A) and (C), respectively, under nonreducing conditions as described in Fig. 3, and expressed by taking the intensities of monomer band of each VN under reducing condition as 100%. Abbreviation used in (C) and (D): N, VN treated with neuraminidase; and NG, VN treated with PNGase F; C, control VN incubated without enzyme; and U, Untreated VN.

molecular weight was eluted at 6.5–7 min in only PH serum (Figure 9A). There was little difference between the elution patterns of NO and SH sera, except that the VN eluted at 10–11 min was a little lower in SH serum.

After serum proteins were separated into heparin-bound and unbound fractions by heparin affinity chromatography, each fraction was applied to gel-filtration HPLC. As shown in Figure 9B, active VNs in the heparin-bound fraction of NO and SH sera were eluted in multimer form at around

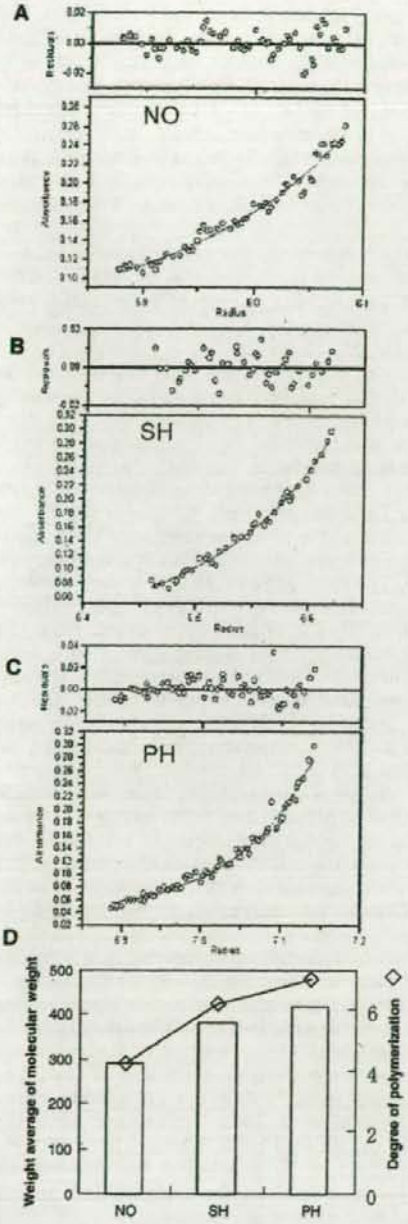

Fig. 7. Molecular weights of NO-, SH-, and PH-VN by sedimentation equilibrium. Sedimentation equilibrium analysis of NO-, SH-, and PH-VNs was performed at 20°C. Data were collected and analyzed as described in Fig. 4. (A–C) Show the radial distribution of absorbance at equilibrium at 5,000 rpm for NO- (A), SH- (B), and PH-VN (C). Individual absorbance readings are shown by open circles, with solid lines through the data representing the fit to a single species model. The residuals for the fit are shown in the upper panels. (D) shows the calculated weight average molecular weight (bars) and degree of polymerization of VNs (diamonds). NO, NO-VN; SH, SH-VN; and PH, PH-VN.

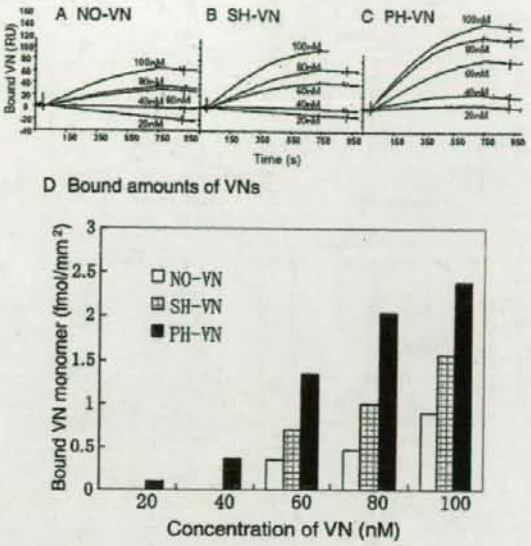

Fig. 8. Interaction between NO, SH-, or PH-VNs and type I collagen by SPR. Type I collagen was immobilized on a CM5 sensor chip as described in the text. NO- (A), SH- (B), and PH-VN (C) in PBS were injected onto the sensorchip at concentrations of 20, 40, 60, 80, or 100 nM at a flow rate of 20 μL/min at 20°C. The response is expressed as the change of resonance units induced by the binding of each VN to the collagen-immobilized flow cell, which was corrected for bulk effect by subtracting the change on the BSA-immobilized reference cell. Sensorgrams of NO- (A), SH- (B), and PH-VN (C). (D) Amounts per each VN monomer (femtomole/square millimeters) bound at various concentrations.

9.2 min, which corresponds to around 670 kDa, about 10-mer of VN. The active VN in PH serum was eluted faster by 0.3 min than those of NO and SH-VNs, suggesting that the multimer size of VN is larger in PH serum than in NO and SH sera, whereas the inactive VNs of the heparin-unbound fraction were mainly monomers in all three sera (Figure 9B). The ratio of active VN to total VN in each serum was estimated to be 25, 21, and 21% for PH, SH, and NO sera, respectively, from the integrated peak areas in Figure 9A. These results indicate that active multimer VNs are a larger size and higher numbers in PH plasma than in SH and NO sera.

Discussion

This study clarified the molecular mechanism by which ECM glycoprotein is activated by changes in glycans by analyzing VNs purified from PH-, SH-, and NO-rats in comparison with the deglycosylated VNs. PH-VN exhibited increased amounts and sizes of multimers compared with NO- and SH-VNs (Figures 6, 7, and 9) and markedly enhanced collagen binding (Figure 8). These changes in the multimerization and activity of PH-VN were reproduced by deglycosylation of VN, especially desialylation (Figures 3–6), and the relationship between glycosylation and the multimerization state was elucidated. Together with our previous observation that the carbohydrate concentration of PH-VN decreased to one-third of that of NO-VN and that the oligosaccharide structure

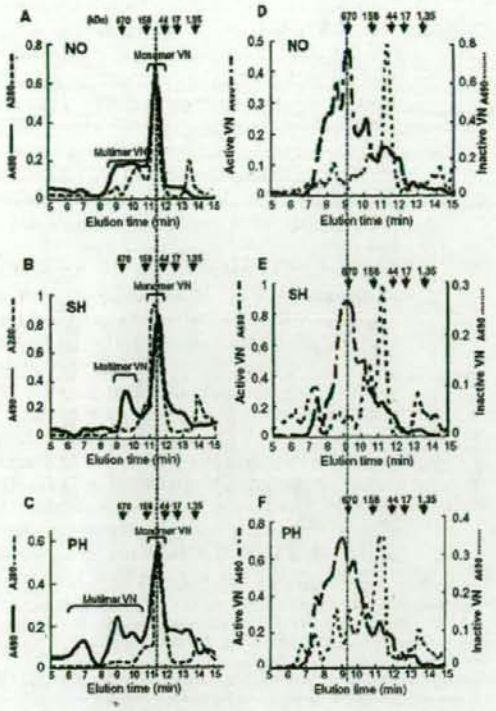

Fig. 9. Gel-filtration HPLC of serum VN from NO, SH, and PH rats. (**A**–**C**), rat sera were diluted to one-fourth, and a 50 μL aliquot was directly applied to a Shodex Protein KW-804 column (8 mm × 300 mm) and eluted with PBS containing 5 mM EDTA. The eluted fractions were analyzed by ELISA to detect VNs. The elution profile was monitored by absorbance at 280 nm for protein, as indicated by dashed lines and the amount of rat VNs were detected by ELISA, as indicated by solid lines. Samples are: NO (A), SH (B), and PH (C) rat sera. (**D**–**F**), Each serum was applied to a heparin-Sepharose column to separate it into the heparin-bound (bound and eluted with 0.5 M NaCl) and unbound (pass-through) fractions. Aliquots (50 μL) of the heparin-bound fraction, which contains active VNs, and heparin-unbound fraction, which contains inactive VNs, were applied separately to the HPLC column. Active VNs (dashed-and-dotted lines) and inactive VNs (dotted lines) in the heparin-unbound fractions were detected by ELISA. Samples are fractions of NO- (*D*), SH- (E), and PH- (F) rat sera. *Arrows* indicate the elution position of molecular weight standards: 670 kDa (thyroglobulin), 158 kDa (bovine gamma globulin), 44 kDa (chicken ovalbumin), 17 kDa (equine myoglobin), and 1.35 kDa (vitamin B-12).

changed from those of SH- and NO-VNs (Uchibori-Iwaki et al. 2000), the enhanced collagen binding of PH-VN is, therefore, attributable to a mechanism very similar to that caused by enzymatic deglycosylation.

VN multimers cross-linked via intermolecular disulfide bonds were shown to be increased in PH-VN compared with NO-VN (Figures 8 and 9) or on enzymatic deglycosylation of VN (Figure 3B). The formation of cross-linked multimers of VN was attributable to free sulfhydryl residues, Cys411 and Cys453, in the hemopexin I and II domains (Gibson et al. 1999), respectively. According to previous studies on activation of VN, native VN acquires binding activities to various biological ligands through a conformational transition and subsequent multimerization in the presence of heparin, thrombin-AT-III, membrane attack complex, or due to chemical treatments (McKeown-Longo and Panetti 1996; Preissner 1991). It was demonstrated that the decrease in multimer VN was correlated with the increase in ionic strength by gel filtration (Zhuang, Li, et al. 1996), suggesting that multimerization proceeds with the help of ionic interaction. The changes in glycosylation of VN after partial hepatectomy may contribute electrostatically and sterically to the multimerization process to enhance VN activity because it included both changes of sialylation and other intrinsic structures (Nakashima et al. 1992).

The interaction between VN and collagen is known to be sensitive to ionic strength (Gebb et al. 1986; Izumi et al. 1988), and VN had been considered to be inactive with collagen under physiological saline concentrations (Izumi et al. 1988). This study indicated that VN in a changed glycosylation state binds well to collagen under the physiological concentration, and that binding was enhanced by the multivalent effect caused by increased multimerization (Figures 3–6). Besides the multimerization of VN, electrostatic interaction is changed by glycosylation, especially sialylation. As shown in Figure 1C, the collagen binding of human VN was less affected by ionic strength at pH 4.5 than at pH 7.5, because pH 4.5 is close to the pI of mammalian VN. The pI of PH-VN changed to 6 from the 4 of NO-VN and slightly above 4 of SH-VN. The elevated pI of PH-VN is attributable mainly to the reduced sialylation (Yoneda et al. 1998), which would reduce the contribution of ionic interaction to the collagen at neutral pH. In this way, the change in glycosylation, especially sialylation alters the binding force between VN and collagen.

On the other hand, the effect of collagen aggregation on the interaction is notable, too. Collagen is known to aggregate at neutral pH and room temperature, but alkali-treated collagen aggregates at acid pH (Hattori et al. 1999). The binding of multimer VN to alkali-treated collagen was significantly reduced at neutral pH compared with binding to untreated collagen, but it recovered at pH 4 (Figure 1D). Thus, VN-collagen interaction is significantly affected by aggregation of collagen as well as the multimerization of VN, indicating that multivalencies of both VN and collagen are required for the interaction.

There have been several cases reported in which the oligosaccharide plays an important role in the interaction of the protein molecule. For example, the *N*-type glycan is necessary to form an assembly complex between the α and β subunits of integrins (Zheng et al. 1994). Particularly, the oligosaccharides of integrin α5β1 are involved in adhesion of several cells to fibronectin, suggesting that a specific glycan structure is required (Pretzlaff et al. 2000; Nadanaka et al. 2001; Isaji et al. 2004). On the other hand, examples in which the multimerization relates to the function of the molecule are also reported. For example, integrin, described earlier, is known to be activated following clustering (Li et al. 2003). However, very few examples have proven that the glycosylation is related to functional multimerization of the molecule in ECM. As far as we know, VN is the first case of it.

Human and rat VN have 73% sequence identity with respect to the common domain organization and ligand binding activities. All three glycosylation sites of human VN (Asn 86, 169, and 242) are conserved in rat VN, which has one additional glylcosylation site (Asn 96). Therefore, the effects of

glycosylation change on the activity of human VN are expected to be similar to those of rat VN, besides the possibility that an additional effect might be exhibited by glycosylation at Asn 96. This study showed that the change in glycosylation frequently increased the ratio and size of multimers in human and rat VNs and demonstrated the mechanism of how VN shows increased collagen binding due to glycan changes. We also observed that collagen binding increased by neuraminidase or PNGase F treatments for porcine VN (A Yoneda et al., unpublished results), which suggests that this property is common to mammalian VNs, in spite of the fact that the number of potential *N*-glycosylation sites is different among these VNs.

VN is known to be related to wound healing and to the progression of hepatic fibrosis and cirrhosis (Inuzuka et al. 1992; Kobayashi et al. 1994). The collagen binding of VN must be significantly charged for these biological and pathological processes to occur because the collagen binding of VN provides a scaffold for the progression of these events. Increased depositions of VN have been observed in areas of hepatic injury and necrosis, whereas little VN immunoreactivity is detectable in most normal livers. Although the targeted deletion of VN in mice resulted in normal development and normal coagulation parameters (Zheng et al. 1995), the VN-null mice showed increased fibrinolysis and decreased angiogenesis on tissue injury, causing delayed wound healing (Jang et al. 2000). These observations indicate the importance of VN in tissue remodeling by binding with matrix molecules such as collagen, PAI-1, urokinase-type plasminogen activator, urokinase receptor, and integrin that are involved in the remodeling process. It well coincides with the observations that VN binds with fibrinolytic factors such as fibrinogen, thrombin, and PAI-1 (Preissner and Seiffert 1998), and that expression of VN increases in acute phases (Seiffert 1997). We propose that alteration of glycosylation is very significant in modulation of the biological activity of VN during these tissue-remodeling processes. Modulation of glycans on VN could contribute to development of a strategy to regulate matrix deposition and fibrinolysis after fibrosis.

Materials and methods

Materials

Rat and human VNs were purified from each plasma by two-step heparin affinity chromatography before and after urea treatment as described previously (Yatohgo et al. 1988). Neuraminidase (*Vibrio cholerae*) and recombinant peptide-*N*4-(*N*-acetyl-β-D-glucosaminyl) asparagine amidase from *Fravobacterium meningosepticum*, (PNGase F) were purchased from Roche Diagnostics (Mannheim, Germany). β-galactosidase (jack bean) and β-*N*-acetylhexosaminidase (jack bean) were purchased from Seikagaku Corporation (Tokyo, Japan). Unconjugated rabbit anti-human VN IgGs and horseradish peroxidase (HRP)-conjugated sheep anti-rabbit IgGs were purchased from The Binding Site (Birmingham, UK). Type I collagen from calfskin and other reagents of special grade were from Wako (Osaka, Japan). Alkali-treated type I collagen, a monomeric form that does not aggregate even at neutral pH and 37°C, was prepared as described previously (Hattori et al. 1999). *Psathyrella velutina* lectin was prepared in our laboratory as previously described (Ueda et al. 1999). HRP-labeled Con A was purchased from Seikagaku Corporation.

Animals

Male Wistar rats aged 5 weeks (weighing about 110 g; Nihon Clea, Tokyo, Japan) were maintained at a constant temperature (23.5 °C) with 12 h each light (6:00–18:00) and darkness. Two-thirds partial hepatectomy was performed under diethyl ether anesthesia as described previously (Higgins and Anderson 1931). Sham-operated rats were anesthetized, and their livers were completely exposed outside the peritoneum and manipulated but not resected.

Glycosidase digestion of VN

Enzymatic deglycosylation of VN was performed as described previously (Uchibori-Iwaki et al. 2000). For desialylation, VN (32 μg/200 μL) was dialyzed against 50 mM acetate buffer containing 4 mM $CaCl_2$ (pH 5.5) and treated with neuraminidase (0.001 U) at 37 °C overnight. For stepwise glycan trimming, VN was treated with neuraminidase alone, neuraminidase and β-galactosidase, or neuraminidase, β-galactosidase, and β-*N*-acetylhexosaminidase in 50 mM acetate buffer containing 4 mM $CaCl_2$ (pH 5.5). For de-*N*-glycosylation, VN (32 μg/200 μL) was dialyzed against 10 mM phosphate buffer (pH 7.5) containing 0.13 M NaCl and 5 mM ethylenediaminetetraacetic acid (EDTA) [phosphate-buffered saline (PBS)–EDTA], and digested with PNGase F (0.2 U) at 37 °C for 48 h. The treatments of VN were performed without the enzymes for controls. The treated VNs were dialyzed against 10 mM Tris-buffer (pH 7.5) containing 0.14 M NaCl [tris-buffered saline (TBS)]. Desialylation or de-*N*-glycosylation was ascertained by both the behavior on SDS–PAGE and the loss of reactivity with biotinylated *P. velutina* lectin for desialylation and ahexosaminylation (Ueda et al. 2002), or HRP-Con A on western blotting.

Analytical ultracentrifugation

Sedimentation velocity and equilibrium measurements were performed with a Beckman Optima XL-A analytical ultracentrifuge (Beckman Instruments, Palo Alto, CA) in a 8-hole An50Ti rotor with standard double-sector centerpieces and quartz windows at 4, 20, or 37°C. Concentration profiles of samples were monitored by absorbance at 280 nm. Sedimentation velocity experiments were performed using VN solutions of 0.2 mg/mL in PBS–EDTA (pH 7.5) or citrate-phosphate buffer (pH 4.5, 7.5) at a rotor speed of 40 000 or 45 000 rpm. Data acquisition was performed without time intervals with about 50 scans collected per run. The data were analyzed using a software program, Sedfit (Schuck 1998), to obtain the distribution function of the apparent sedimentation coefficient, s; $c(s)$. Sedimentation equilibrium experiments were performed using 0.3 mg/mL VN in PBS–EDTA at 20 °C at rotor speeds of 3000 and 5000 rpm. Scans were recorded every 2 h, and the equilibrium of the system was judged by the superimposition of the last three scans. The two datasets were globally fitted to a single species model to determine the weight average molecular weight using the "nonlin" program preinstalled by the manufacturer. The partial specific volumes, 0.6768 cm^3/g, for VN

were based on the amino acid and sugar compositions of the glycoprotein.

Assays of binding of VN to immobilized type I collagen

Collagen-binding activities were studied by ELISA essentially according to the method reported previously (Gebb et al. 1986; Yoneda et al. 1998) at 4 °C or 37 °C. Briefly, the wells of microtiter plates (Immulon 1, Dynatech Laboratories Inc., Chantilly, VA) were coated with 100 μL aliquots of a solution of type I collagen (10 μg/mL in 0.1 M carbonate buffer, pH 9.0) for 3 h. The wells were washed and blocked with 0.5% skim milk in TBS (pH 7.5) for 2 h. Various concentrations of human or rat VN in TBS (pH 7.5 or 8.5) or 10 mM citrate-phosphate buffer (pH 3.5, 4.5, 5.5, or 6.5) containing 0.14 M NaCl (50 μL) were added to each well, followed by incubation for 2 h. For measurement of the binding dependency on ionic strength, VN was dissolved in 10 mM citrate-phosphate buffer (pH 4.5 or 7.5), each containing 0–2.24 M NaCl and used for the binding assay at 4 °C. To assess the effect of aggregation of collagen, alkali-treated monomeric collagen was immobilized on a microtiter plates and measured for VN binding at 37 °C.

After washing the plates with the same buffer three times, the amounts of VN bound to immobilized collagen were detected with unconjugated rabbit anti-human VN IgG and HRP-conjugated sheep anti-rabbit IgG at room temperature (15 °C). The reaction was developed using *O*-phenylenediamine (2.3 mg/ml) in 0.1 M phosphate-citrate buffer (pH 5.0) containing 0.007% H_2O_2, stopped by addition of 4 N H_2SO_4, and measured at 490 nm using a microplate reader, model 680 (Bio-Rad, Hercules, CA).

Quantification of interactions between VN and immobilized collagen by surface plasmon resonance

The interaction between VNs and type I collagen was analyzed by surface plasmon resonance (SPR) using a BIAcore2000 SPR apparatus (BIAcore AB, Uppsala, Sweden). After equilibration with *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES)-buffered saline, type I collagen (15 μg/mL) in sodium acetate buffer (pH 4:3) was injected onto a CM5 sensor chip (BIAcore) preactivated with the amine coupling kit, and then the remaining *N*-hydroxysuccinimide esters were blocked by the addition of 1.0 M ethanolamine hydrochloride (pH 8.0). Each step was performed for 14 min at a flow rate of 10 μL/min. The reference flow cell was prepared with bovine serum albumin (BSA) as a ligand.

To measure the binding curves, various concentrations of rat NO-, SH-, or PH-VN in PBS (pH 7.5) were separately injected onto a collagen-immobilized chip with a contact time of 720 s, a dissociation time of 150 s, and a flowrate of 20 μL/min at 25 °C. The sensor chip was regenerated with 0.1 M HCl for 60 s. The bound amounts were expressed in BIAcore resonance units (RU, 1000; RU = 1 ng/mm^2), and the changes in resonance units induced by binding of VN to the collagen-immobilized flow cell were corrected by bulk effect by subtracting the changes on the BSA-immobilized reference cell.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SDS–PAGE was carried out using a 7.5 or 5–20% gradient polyacrylamide gel (ATTO, e-PAGEL, Tokyo, Japan) according to the method of Laemmli (1970) under reducing or nonreducing conditions.

Gel-filtration HPLC and immunodetection of VN by ELISA

Sera of NO, SH, and PH rats or the pass-through and bound fractions from heparin-Sepharose were analyzed by gel-filtration HPLC on a Shodex Protein KW-804 column (8 mm × 300 mm, Showa Denko, Tokyo, Japan). Rat sera were diluted four-fold with the elution buffer (PBS–EDTA), and a 50 μL aliquot of each sample was applied to the HPLC and eluted at a flow rate of 1 mL/min at room temperature. The eluted fraction was monitored by absorbance at 280 nm and collected every 15 or 30 s by a fraction collector. The amounts of VN in each fraction were detected by ELISA at 4 °C. The wells of microtiter plates (Immulon 1, Dynatech Laboratories Inc.) were coated with 100 μL aliquots of the eluted fractions from HPLC at 4 °C for 3 h, and the procedures were performed as described in *Assays of binding of VN to immobilized type I collagen*.

Acknowledgments

This work was supported in part by Hayashi Memorial Foundation for Female Natural Scientists (KS) and Grants-in-Aid for Scientific Research (C) 14580622 and 17570109 (HO) from the Japan Society for the Promotion of Science and Grants-in-aid for Scientific Research on Priority Areas 15040209 and 17046004 (HO) from the Ministry of Education, Culture, Sports, Science, and Technology. We thank our laboratory members for helping the operations and K. Ono for editing the English.

Conflict of interest statement

None declared.

Abbreviations

2ME, 2-mercaptoethanol; BSA, bovine serum albumin; CBB, Coomassie brilliant blue; ECM, extracellular matrix; EDTA, ethylenediamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; NO, nonoperated; PAI-1, plasminogen activator inhibitor-1; PBS, phosphate-buffered saline; PH, partially hepatectomized; PNGase F, peptide *N*-glycosidase F; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SH, sham-operated; SPR, surface plasmon resonance; TBS, tris-buffered saline.

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Characterization of glycosylation and ligand-binding activities of rat plasma fibronectin during liver regeneration after partial hepatectomy

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Abstract

Fibronectin (FN) is a multifunctional glycoprotein present in the extracellular matrix and plasma. We previously reported that the glycosylation and ligand-binding activities of vitronectin (VN) change markedly after partial hepatectomy (PH). To compare the biological functions of FN with VN during liver regeneration, changes in the concentration in plasma, glycosylation, and ligand-binding activities were studied. The concentration of FN in plasma had increased slightly at 24 h after PH and sham-operation (SH), and the yields of purified SH- and PH-FN were about two and one-and-a-half times, respectively, of that of non-operated (NO)-FN. However, binding activities of FNs to gelatin, collagen types I-V, and heparin-BSA increased only slightly after the operations. The carbohydrate concentration of PH-FN decreased to 66% of NO- and SH-FN. Moreover we determined the *N*-glycan structures of each FNs by LC/MS^n. Eight kinds of complex-type *N*-glycan structures were found to be present in all FNs, and bi- and trisialobiantennary glycans were the major structures with a minor amount of trisialotriantennary glycans. Fucosylation was markedly increased, while *O*-acetylation of sialic acid was found to be decreased in PH-FN. The finding that alterations in glycosylation and biological activities of FN after PH are different from those of VN suggests a difference in the biological functions of the glycoproteins in

tissue remodeling.

Key words: fibronectin/ glycosylation/ liver regeneration/ tissue remodeling/ partial hepatectomy

1. Introduction

Numerous biological phenomena are mediated by recognition of specific oligosaccharide signals. This recognition implies quality control in polypeptide folding, cellular interactions, and protein targeting. In contrast, some functions of protein glycosylation seem to be widely applicable to various types of glycosylation, for example, stabilizing active conformations, protecting against proteolysis, and affording solubility to proteins [1]. These functions have been attributed to ambiguous steric effects of glycosylation in the absence of clear structural specificity, but clarification of the molecular mechanism(s) by which glycosylation plays a role in stabilizing or protecting the active conformation of proteins would enable the use of glycosylation in molecular engineering for therapeutic purposes. From this viewpoint, the extracellular matrix glycoproteins are targets for tissue engineering to enable remodeling, regeneration, and repairing of injured tissues. In our preceding study, several important ligand-binding activities of vitronectin (VN) were found to be significantly affected by changes in

N-glycosylation *in vitro* [2]. The marked increase in collagen-binding activity was found to be controlled by the increased multimerization induced by glycosylation alterations during liver regeneration after partial hepatectomy of rats [3].

Fibronectin (FN) is a typical extracellular matrix glycoprotein that plays critical roles in many biological and pathological processes including embryogenesis, wound healing, metastasis, fibrosis, and thrombosis [4]. FN is synthesized by various cells, including hepatocytes, fibroblasts, and vascular endothelial cells, and exists primarily as a soluble dimer in body fluids such as plasma at high concentrations (0.3 mg/ml in humans) [5], while in matrix assembly, it is assembled into insoluble fibrils with the fibrillar polymeric matrix stabilized by a disulfide bond [6].

Alternative splicing is a known regulatory mechanism of the biological activities of FN. FN is composed of a repetition of I-, II-, and III-type modules, and molecular variety is achieved by expression of the III-type modules called EDA (extra domain A) and EDB (extra domain B) and mutation of a segment called V by alternative splicing [7]. Cellular FNs are a mixture of forms, with or without EDA and EDB domains, that regulate the activation of focal adhesion kinase and cell apoptosis [8]. The expression ratio of each type of FN depends on the kind of cell and the pathological state, such as metastasis and fibrosis, of the individual [9]. Moreover, specific cell surface integrins play

important roles in initiation and regulation of the assembly of the FN matrix and cellular signaling [9-12].

The concentration of FN has been reported to triple at the protein and mRNA levels during liver regeneration[13], but a change in glycosylation and its effect on biological activity has not been demonstrated yet. In this study, we focused on the glycan structure and the ligand-binding activity of rat FN during liver regeneration after 70% hepatectomy and found that changes occur differentially in extracellular matrix (ECM) glycoproteins to regulate biological activities.

2. Results

2.1. The time-dependent changes of plasma FN during liver regeneration

Rat plasma was collected from non-operated (NO) rat, or 6, 24, 48, 120 and 168 hours after sham-operation (SH) and partial hepatectomy (PH). The changes in total protein concentration in plasma were similar to our previous report [2]. Briefly, the protein concentration in SH-plasma was increased 6-24 hours after operation, and gradually returned to a normal level. On the other hand, it had decreased markedly in PH-plasma

24 hours after the operation and gradually returned to a normal level (data not shown). The results of measuring the FN concentration in the plasma by sandwich ELISA method are shown in Fig. 1. SH-FN increased 25% compared with NO-FN at 6 hours after sham operation, and returned to a normal level in 7 days. On the other hand, a 10% increase was shown during 24-120 hours after partial hepatectomy.

2.2. Amount of purified FN in early stage of liver regeneration

FN from rat plasma 24 hours after PH, SH, or NO was purified by gelatin-Sepharose affinity chromatography. The elution curves are shown in Fig. 2A. FN was scarcely eluted at pH 5.2 but was eluted with 6 M urea. Because more FN was stably obtained by elution with urea, urea-purified FN was used in the experiments reported below. The purity of the FN was confirmed by SDS-PAGE according to the method of Leammli [14] under reducing conditions. Bands of FN were detected at 230 kDa, and no difference in the molecular weight after the operation was detected (data not shown). Compared with NO-FN, the amounts of purified FN were increased 1.6-2 times and 1.2-1.4 times (Fig. 2B), and the ratio of purified FN to total FN in plasma was also increased about 1.7 times and 1.3 times, respectively, after sham operation and partial hepatectomy (Fig. 2C). Our method of FN purification from plasma utilizes the gelatin-binding activity,

and these results suggest that the gelatin-binding activity of FN was enhanced after surgery.

2.3. Ligand-binding activities of FN in plasma and after purification

The ligand-binding activities of FN in NO, SH, and PH plasma to gelatin, collagen types I-V, and heparin-BSA were analyzed by ELISA. As shown in Fig. 3, FN bound best to collagen types I, II, and III, gelatin, and heparin-BSA, but scarcely bound to collagen types IV and V. The binding to gelatin was slightly increased in SH-FN in accordance with the results shown in Fig. 2C and described above. However, the binding activities to other ligands hardly changed after surgery, though both the concentration in plasma and the purified yield of FN was increased in SH and PH. This is probably because other factors in plasma interfered with FN binding to the ligands.

The binding activities of purified FN to gelatin, collagen types I-V, and heparin were analyzed by ELISA, and the results are shown in Fig. 3B. The binding activities of PH- and SH-FN to collagen types III, IV, and V were higher than those of NO-FN, and the binding of PH-FN to types I and II collagen was slightly increased, suggesting that surgery increased the binding activities. There was no difference among in the binding activities of the three FNs to gelatin and heparin-BSA. However, compared to those

shown Fig. 3A, the binding activities of purified FNs to gelatin and collagen types IV and V were significantly higher than those of FN in plasma before purification. This result suggests that urea denaturation of FN activates the binding activities to some ligands, similar to the urea-activated increases in VN binding activity to heparin [15].

2.4. Effect of PNGase F treatment on ligand-binding activities of FNs

The binding activities of PNGase F-treated human FN and a control incubated without enzymes to collagen types I-V and heparin-BSA were compared by ELISA, as shown in Fig. 4. The ligand-binding activities of de-*N*-glycosylated FN were increased but only slightly. Previously we demonstrated a 2.9-fold increase of binding of VN to type I collagen after de*N*-glycosylation [2,3], which contrasts to FN binding. The difference between the effects of deglycosylation on the ligand-binding activities of FN and VN indicates distinct biological roles of the glycans in each glycoprotein.

2.5. Changes in glycosylation of FN during liver regeneration

2.5.1 Lectin reactivity

The interactions of NO-, SH-, and PH-FN with biotinylated lectins were analyzed by dot blotting. The interaction intensities were quantified by the software

program Scion Image, as shown in Table I. The most conspicuous change after partial hepatectomy was the increase in reactivity to *Aleuria aurantia lectin* (AAL), suggesting an increase in fucosylated glycans in PH-FN. The reactivity to *Peanut lectin* (PNA) after mild acid desialylation of FNs was remarkable, clearly indicating the presence of *O*-type glycans. The reactivity to *Psathyrella velutina* lectin (PVL) was increased in PH-FN and disappeared in all FNs after desialylation, indicating that sialylated glycans having more than trisialyl residues increased after partial hepatectomy.

2.5.2 Carbohydrate composition

As summarized in Table II, the total carbohydrate concentration of PH-FN was markedly decreased compared with those of NO- and SH-FN, with the GalNAc concentration decreased to about one third and one half of that of NO- and SH-FN, respectively, while the Fuc concentration increased to about twice those of NO- and SH-FN. The ratio of the GalNAc concentration to the total carbohydrate concentration was notable, indicating that FN is highly *O*-glycosylated, in accordance with its reactivity to PNA as described above.

2.5.3 Oligosaccharide structure

Rat plasma FN was reported to have seven potential *N*-glycosylation sites. *N*-glycans were released by PNGase F treatment, reduced to sugar alcohol, and analyzed by LC/MS. As shown in Table III and Fig. 5A, the most frequent *N*-glycan structures of rat plasma FN were biantennary complex type disialoglycans (BiNA(2)), and then biantennary trisialoglycans (BiNA(3)), with different amounts of *O*-acetylated Neu5NAc. As summarized in Table III, eight kinds of *N*-glycan backbone structures were found. If variations of the number of *O*-acetylation of sialic acid residues were included, the varieties of structures would amount to more than 15 (Fig. 5). The ratios of fucosylation were increased in PH-FN in accordance with the results of carbohydrate compositions and reactivity with AAL. Rat FN contained BiNA(2) as the most common *N*-glycan, which is very similar to human plasma FN [16], and core-fucosylated (BiNA(2)F), trisialobiantennary (BiNA(3)), and monosialobiantennary (BiNA(1)) glycans were also included as major components. The glycans contained various concentrations of *O*-acetylated Neu5NAc, as shown in Fig. 5B. The ratio of non-*O*-acetylated glycans in each backbone structure was higher in PH-FN than in NO- and SH-FN. This indicates that *O*-acetylation is lower after partial hepatectomy.

3. Discussion

This research is the first to determine the details of glycan structure of particular glycoprotein synthesized in the early stage of liver regeneration after partial hepatectomy. The liver has a strong capacity to regenerate and performs many physiological functions, such as storage, metabolism, biosynthesis, and secretion. Partial (70%) hepatectomy is often used to study liver regeneration mechanisms. The remaining liver recovers its former weight in about two weeks after the operation [17]. During this process, matrix degradation occurs in the early stage, followed by biosynthesis of matrix, cell proliferation, and cell differentiation. However, many aspects of this complex mechanism are unknown, especially the regulation of activity of each ECM glycoprotein by glycosylation.

Previously we reported that the glycosylation and activity of vitronectin, one of the multifunctional extracellular matrix glycoproteins, were significantly changed in the early stage of liver regeneration. FN is another major ECM molecule which has many functional domains that bind to many ligands, such as fibrin, collagen, integrin, and heparin [18,19]. Rat FN has ten potential *N*-glycosylation sites at 430, 528, 524, 876, 1006, 1243, 1290, 1859, 1903, and 2198. Three sites are in the collagen-binding domain, two are in the heparin-binding domain of the C-terminal, and the others exist in