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Matrix Restructuring During Liver Regeneration is Regulated by Glycosylation of the Matrix Glycoprotein Vitronectin

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1. Introduction

There are three major approaches for regenerative medicine. The most innovative approach among them is: induction of target cells from various stem cells such as induced pluripotent stem cells (iPS cells) or embryonic stem cells (ES cells) and implantation of them to regenerate the organ. The second approach is: in vitro tissue regeneration that involves preparation of artificial tissue by combining human cells with scaffolding biomaterials and growth factors. The third is: promotion of self-regeneration through controlling the repair activity of each tissue, which most organisms do naturally, is a more fundamental approach, but it will also be important in cell therapy to regulate tissue organization after induction of differentiation.

Because tissue homeostasis depends on spatially and temporally controlled expression of multifunctional adhesive glycoproteins and receptors, many studies have examined the changes of expression of extracellular matrix (ECM) molecules during tissue remodeling, inflammation and invasion by cancer cells (DeClerck, Y.A., et al. 2004; Seiffert, D. 1997; Kato, S., et al. 1992; Hughes, R.C. 1997) on the one hand. On the other hand, there is increasing evidence that glycosylations post-translationally modulate various biological phenomena by altering the activity and specificity or the stability of glycoproteins through the biosignaling functions of oligosaccharides (Varki, A. 1993; Varki, A., et al., 2009). During tissue remodeling, the glycosylated ECM molecules are different from those of normal tissue owing to the changes in the expression of many proteins that are responsible for glycan synthesis (Dalziel, M., et al. 1999). However, the glycan modulation of most glycoproteins that are involved in tissue remodeling has remained unknown.

When the three big lobes of a liver are excised, the remaining liver recovers its former mass and function within about two weeks in humans or 7 to 10 days in rats (Diehl, A.M. and Rai, R.M. 1996). ECM degradation occurs in the early stage of this process, followed by biosynthesis of the matrix, cell proliferation, and cell differentiation. During this process, many glycosyl transferases (Bauer, C.H., et al. 1976; Serafini-Cessi, F. 1977; Okamoto, Y., et

al. 1978; Ip, C. 1979; Oda-Tamai, S., et al. 1985; Miyoshi, E., et al. 1995) and total glycoconjugates in the liver have been reported to change (Okamoto, Y. and Akamatsu, N. 1977; Kato, S. and Akamatsu, N. 1984; Kato, S. and Akamatsu, N. 1985; Ishii, I., et al. 1985). However, the nature of the links between such glycans changes, and the process of tissue remodeling has remained unclear. We consider it important to identify which molecules play important roles in the tissue remodeling during liver regeneration, and we will discuss the glycan modulation of one extracellular molecule, vitronectin, in this chapter. Vitronectin is a multifunctional adhesive glycoprotein that plays a central role in tissue remodeling by connecting pericellular tissue lysis with cell adhesion and motility.

We found that the glycans of vitronectin drastically change during liver regeneration after partial hepatectomy. In our studies to determine the glycan structures during the initial stage of the liver regeneration after partial hepatectomy of rats, we found that alterations in glycosylation, especially decreased sialylation of vitronectin, modulate the biological activities of vitronectin during tissue-remodeling processes by multiple steps (Uchibori-Iwaki, H., et al. 2000; Sano, K., et al. 2010). Liver regeneration is a normal repair process, while fibrosis and cirrhosis are considered to be excessive and abnormal repair processes that often give rise to cancer. In this context, elucidating how alterations of glycans occur and understanding how glycans modulate the glycans on vitronectin is useful in order to develop a strategy to regulate matrix remodeling in regeneration and deposition in liver cirrhosis. Therefore, we aimed to elucidate glycan modulation during liver regeneration after partial hepatectomy. We focused on the changes in vitronectin during liver regeneration, especially the changes of the glycan moiety, which plays a crucial role in controlling survival of hepatic stellate cells.

1.1 Structure and function of vitronectin

Vitronectin is a multifunctional adhesive glycoprotein that originates mainly in hepatocytes and circulates in the blood at high concentrations (0.2 mg/ml in human and no more than 0.1 mg/ml in rats). Vitronectin was first isolated from human serum by Holmes in 1967 as an ' α -1 protein' (Holmes, R. 1967), and has been referred to as 'serum spreading factor', 'epibolin', or 'S-protein'. It induces cell growth *in vitro* and is known as a major cell-adhesive component in cell culture mediums (Hayman, E.G., et al. 1983). Vitronectin is present as an ECM component in the liver, as well as various other organs, including skeletal muscle, kidney, and brain (Seiffert, D. 1997; Seiffert, D., et al. 1991). Most vitronectin in normal plasma is present as an inactive monomer form that does not bind to various ligands in the plasma (Gebb, C., et al. 1986; Izumi, M., et al. 1989). *In vivo*, vitronectin is activated in the presence of certain ligands such as heparin, type-1 plasminogen activator inhibitor (PAI-1), thrombin-AT-III, membrane attack complex of complements, and through a partial conformational change and multimerization process (Preissner, K.T. and Muller-Berghaus, G. 1987). Tissue vitronectin is considered to be present as an active multimeric form. Conformation-dependent binding of vitronectin was also observed for sulfatide (Gal(3-SO₄) β 1-1ceramide), cholesterol 3-sulfate, and various phospholipids including phosphatidylserine, but not gangliosides, while vitronectin bound to cholesterol 3-sulfate regardless of its conformational state (Yoneda, A., et al. 1998). The binding of vitronectin to the membrane lipids and β -endorphin-binding activities were found to be attributable to hemopexin domain 2 and hemopexin domain 1, as well as type I collagen and heparin (Yoneda, A., et al. 1998).

The ligand-binding sites on the domain structure of vitronectins are shown in Fig. 1. Vitronectin regulates the proteolytic degradation of matrix and fibrinolysis through binding with urokinase-type plasminogen activator, PAI-1, and urokinase receptor (Seiffert, D. 1997; Schwartz, I., et al. 1999; Preissner, K.T. 1991; Pretzlaff, R.K., et al. 2000). Besides this function, vitronectin plays a key role in cell adhesion and cellular motility during tissue remodeling through binding to major ECM receptors, integrins such as $\alpha\text{v}\beta 1$, $\alpha\text{v}\beta 3$, $\alpha\text{v}\beta 5$, $\alpha\text{v}\beta 6$, $\alpha\text{v}\beta 8$, and other ECM components like collagen and proteoglycans (Schwartz, I., et al. 1999). In plasma, vitronectin has been shown to regulate coagulation and thrombolytic and complement systems (Preissner, K.T. 1991). By providing a link between plasmin-regulated matrix proteolysis and integrin-mediated cell migration, activated vitronectin is considered to play a central role in matrix remodeling.

Fig. 1 also shows the glycosylation structures of vitronectins and ligand-binding sites. *N*-glycosylation sites of vitronectins are well conserved among mammals, and vitronectin contains almost fully sialylated complex-type *N*-linked glycans at these sites. Most vitronectins, except normal human, additionally contain various amounts of *O*-linked glycans, which differ by species: bovine, rabbit, and especially chicken vitronectins possess more *O*-glycans than *N*-glycans. Human vitronectin does not contain *O*-glycans in the normal state, while rat vitronectin contain several *O*-glycans in the connecting region (Sano, K., et al. 2010).

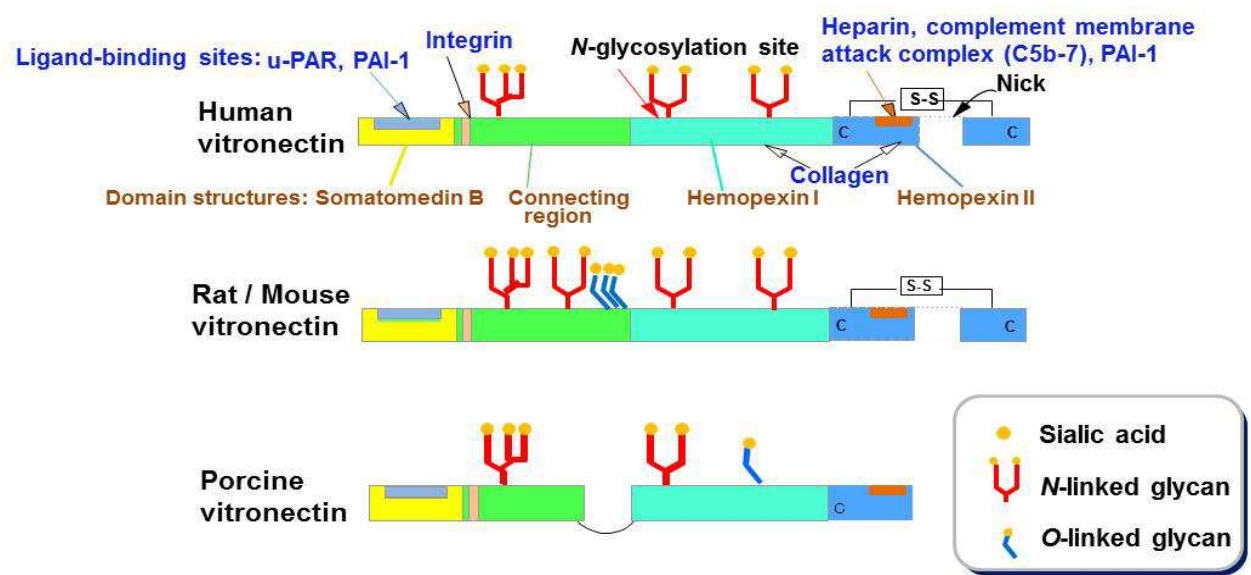


Fig. 1. Glycosylation and domain structure models of vitronectins. Human, rat/mouse, and porcine vitronectin contain three, four, and two *N*-glycosylation sites, respectively (Ogawa, H., Yoneda, A., et al. 1995) (Yoneda, A., Ogawa, H., et al. 1993) (Sano, K., Miyamoto, Y., et al. 2010). The numbers of *O*-glycosylation sites differ by animal species. The major ligand-binding sites that have been identified are indicated at the top of the figure (Yoneda, A., Ogawa, H., et al. 1998) (Preissner, K.T. 1991) (Preissner, K.T. and Seiffert, D. 1998).

2. Modulation of vitronectin activities by glycosylation

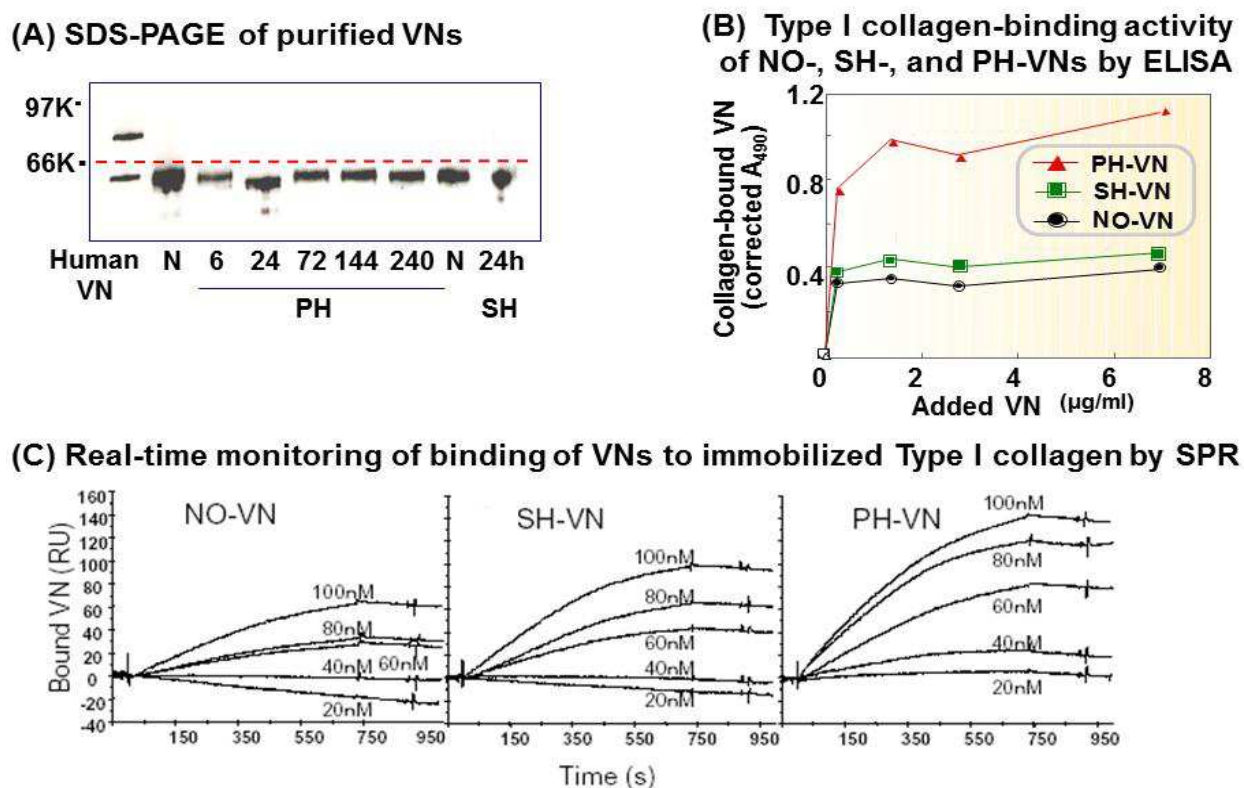
De-*N*-glycosylation of plasma vitronectin by enzyme treatment significantly attenuated the cholesterol sulfate-binding activity while it increased the collagen-binding activity. De-*O*-glycosylation or desialylation of vitronectin contributed to the stability for proteolysis (Uchibori, H., Ogawa, H., et al. 1992). These findings suggest that glycosylations modulate the ligand binding activities and the half-life of vitronectin *in vivo*. We prepared various recombinant domains of human vitronectin and mutants with certain domains deleted and expressed them separately in *E. coli* as fusion proteins. Using these recombinants, sulfatide-, phosphatidylserine-, cholesterol 3-sulfate-, type I collagen-, heparin-, and beta-endorphin-binding activities were found to be attributable to hemopexin domains 2 and 1. The possibility was suggested that the presence of a somatomedin B domain and/or connecting region flanking hemopexin domain 1 inactivated its heparin binding. Further, it was indicated that some of the ligand binding activities were modulated by glycosylation of plasma vitronectin, which enables modulation of its biological activities by a change in glycosylation accompanying the physiological or pathological state of the liver.

2.1 Changes in vitronectin during liver regeneration

2.1.1 Changes in collagen-binding activity of plasma vitronectin during early stage of liver regeneration

In this study, we used the liver regeneration of rats induced by two-thirds partial hepatectomy as a model system to study whether and how vitronectin plays a role in tissue remodeling after hepatectomy and how glycosylations are involved in the physiological processes. Vitronectin was purified from plasma at different times during the liver regeneration process and analyzed by SDS-PAGE (Uchibori-Iwaki, H., et al. 2000). As shown in Fig. 2A, each vitronectin showed one band on SDS-PAGE, and 24 h after partial hepatectomy (PH-VN) plasma vitronectin had shifted to a low migration position compared to vitronectins purified from plasma of non-operated (NO-VN) or partially hepatectomized rats and sham-operated rats (SH-VN), suggesting that the molecular mass of vitronectin had shrunk to 65 kDa at 24 h after partial hepatectomy from the 68-69 kDa of other vitronectins. At 24 h after operation, the yield of PH-VN had decreased to 1/3 that of sham-operated rats, and it was restored by 240 h, when liver regeneration was completed. At this time point, the amino acid composition did not change significantly, and the composition divergence (Black, J.A. and Harkins, R.N. 1977) of PH-VN was 0.040 when taking that of SH-VN as 0.0. All three vitronectins had the same N-terminal sequence, indicating that the three vitronectins had high homology among the primary sequence (Uchibori-Iwaki, H., et al. 2000).

As shown in Fig. 2B, the purified vitronectins bound to type I collagen by ELISA in a concentration-dependent manner, and PH-VN was found to exhibit much greater binding to collagen, about 3 times higher than that of SH-VN and NO-VN (Uchibori-Iwaki, H., et al. 2000). The enhanced binding of PH-VN to immobilized collagen shown by ELISA was supported by surface plasmon resonance (SPR), as shown in Fig. 2C. The relative affinity per monomer of PH-VN is remarkably high compared with those of NO- and SH-VN, especially at the lower concentrations (Sano, K., et al. 2007).



remarkably decreased reactivity of PH-VN with *Psathyrella velutina* lectin (PVL), together with the decrease in the reactivities with concanavalin A (Con A) and *Lens culinaris* lectin (LCA), indicate that the sialylated *N*-glycans markedly decreased after partial hepatectomy, which agrees well with the decreased amounts of carbohydrates including sialyl residues of the PH-VN. The changes in branching glycans would be attributable to the activity of several glycosyltransferases, which have been reported to increase (Miyoshi, E., et al. 1995; Okamoto, Y., et al. 1978), while the decreased *N*-glycosylation of vitronectin at 24 h after partial hepatectomy could be attributed to the attenuation of the oligosaccharide transferase activity in microsomes (Oda-Tamai, S., et al. 1985).

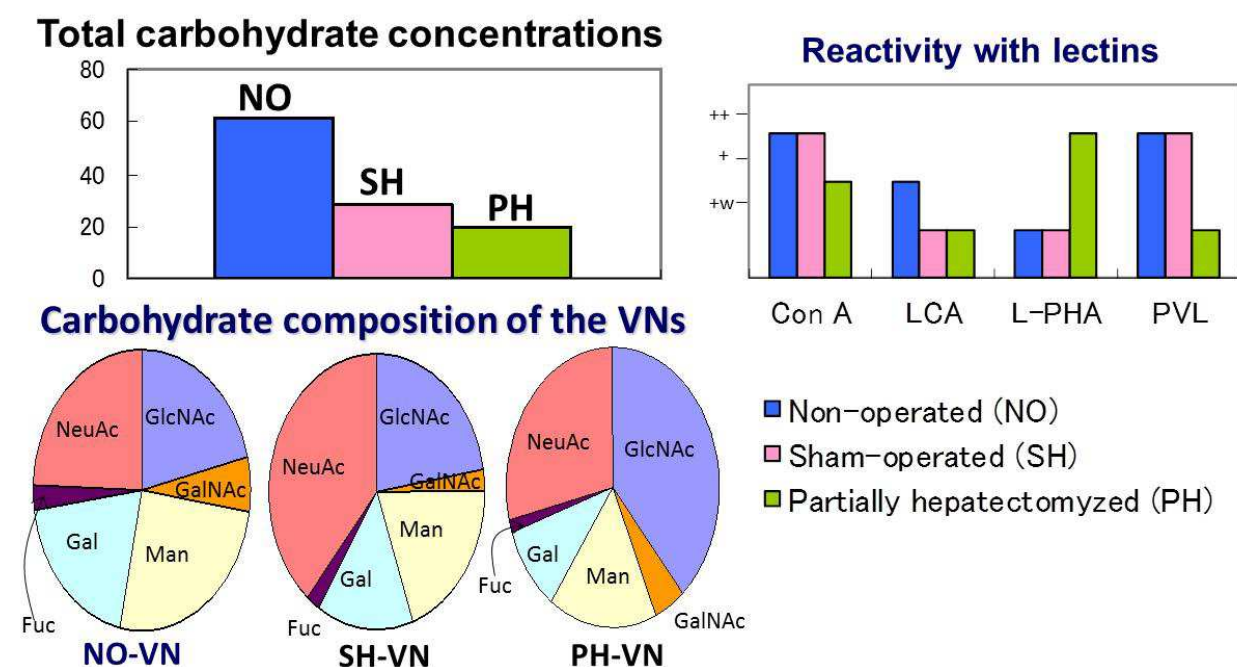


Fig. 3. The carbohydrate concentration, composition, and reactivity with lectins of vitronectins (VN) from non-operated (NO), sham-operated (SH), and partially hepatectomized (PH) rats.

2.1.3 Mechanism of enhanced collagen binding by change of vitronectin glycosylation

To study the enhancement of the mechanism for collagen binding, NO-VN was deglycosylated by sequential exoglycosidase treatments and collagen binding activity was analyzed by ELISA. As shown in Fig. 4A, collagen-binding of vitronectin gradually increased with step-wise trimming of glycans. Deglycosylated vitronectin (NG) showed collagen-binding activity three times higher than that of control vitronectin, suggesting that the enhancement of collagen binding of PH-VN is due to the changes in glycosylation (Sano, K., et al. 2007).

The deglycosylated NO-VNs were analyzed for multimer formation by ultracentrifugation, and the multimer sizes were calculated from the weight average molecular weight of vitronectin (Fig. 4B). The multimer sizes were gradually increased by step-wise deglycosylation, accompanied with an increase of the amounts of multimer vitronectins,

which were cross-linked by disulfide-bonds, as measured by the intensity ratio of bands in SDS-PAGE before and after reduction, as shown in Fig. 4C. The enhanced collagen-binding activity of PH-VN was attributable to a multivalent effect that was due to the increase in the sizes and amounts of multimer vitronectins. The increase in multimer vitronectins in active form in various ligand-binding activities will accelerate the matrix incorporation of PH-VNs.

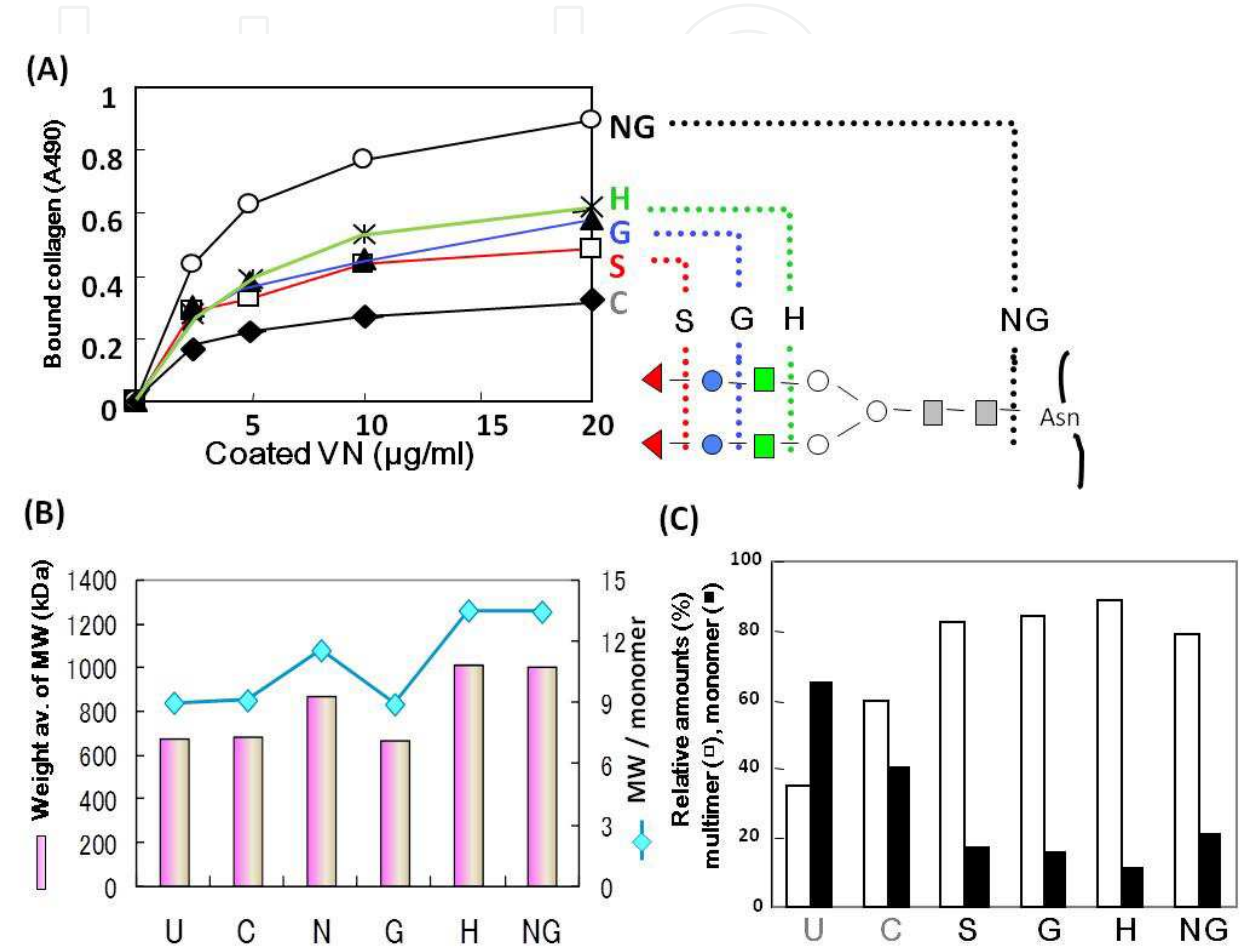


Fig. 4. The collagen binding activities (A), molecular weight and multimerization (B), and relative amounts of multimer (white bar) to monomer (black bar) of glycan-trimmed human vitronectin. The typical complex-type glycan structures of mammalian vitronectin was sequentially trimmed by sialidase (S), β-galactosidase (G), β-hexosaminidase (H), and N-glycosidase F (NG). U: untreated vitronectin; C: control vitronectin incubated without enzyme.

2.1.4 Effects of glycosylation of vitronectin on hepatic stellate cell spreading

Hepatic stellate cells are fibrotic cells that are induced during hepatic inflammation and are the major source of the newly synthesized ECM during hepatic fibrosis, whereas the survival or apoptosis of hepatic stellate cells is critical for the development or resolution, respectively, of liver fibrosis in chronic liver diseases (Benyon, R.C. and Arthur, M.J. 2001). In the normal liver, hepatic stellate cells have a low proliferation rate and produce trace amounts of ECM. As liver fibrosis progresses, hepatic stellate cells proliferate, but during

the resolution of fibrosis there is extensive apoptosis that coincides with degradation of the liver scar (Benyon, R.C. and Arthur, M.J. 2001). It was reported that the activation of hepatic stellate cells increased the expression of integrin $\alpha\beta3$, which is the major receptor of vitronectin on the cell surface, and promotes their proliferation and survival (Kato, S. and Akamatsu, N. 1985). We determined the structure and changes of rat vitronectin glycans during liver regeneration, and observed the relationship between the survival signaling of hepatic stellate cells and glycosylation of vitronectin.

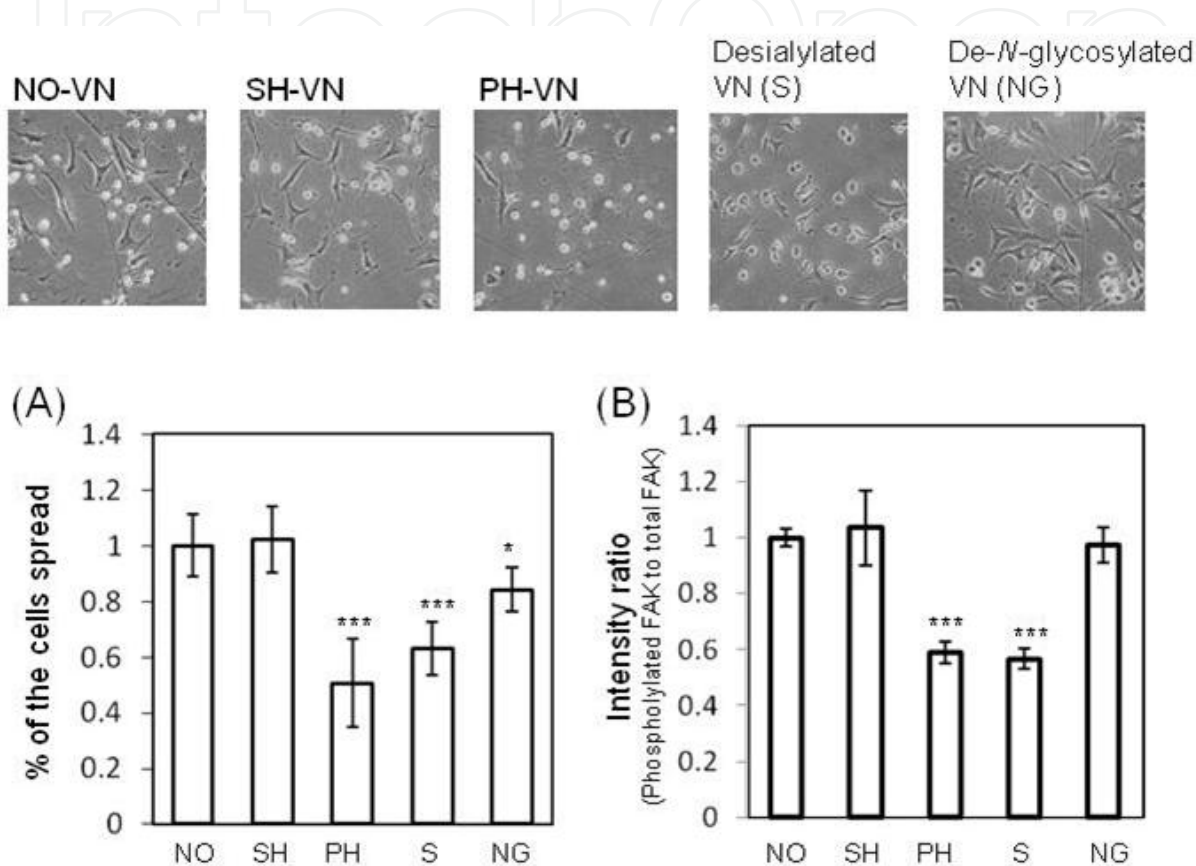


Fig. 5. Spreading and FAK-phosphorylation of HSCs on vitronectins. HSCs were plated on substrates coated with 10 $\mu\text{g}/\text{mL}$ of vitronectins purified from NO, SH, or PH rats or desialylated (S) or de-N-glycosylated (NG) vitronectin. After 90-min incubation at 37°C in 5% CO_2 , the % of the cells spread and FAK-phosphorylated were assessed by taking those on NO-VN as 1. Photomicrographs at $\times 40$ magnification are shown on the upper panel. The data were analyzed by Student's *t*-test. The data represent the means S.D. ($n=6$); ***, $p<0.001$; *, $p<0.05$ compared with that on NO-VN.

In this study, vitronectins were purified from rat plasma at 24 hours after partial hepatectomy, sham-operation, or non-operated and designated as PH-, SH-, and NO-VN, respectively. The effect of PH-VN on HSC spreading was decreased to 1/2 of that of NO- or SH-VN (Fig. 5A). HSC spreading was also decreased on neuraminidase-treated vitronectin compared with untreated vitronectin, whereas it was decreased less on de-N-glycosylated NO-VN. These results indicate the importance of glycosylation, particularly sialylation, of vitronectin in HSC spreading. The effect of de-N-glycosylation was small compared with that of desialylation, because many sialic acid residues still remained on the O-glycans after

N-glycosidase F (PNGase F) treatment. In addition, PNGase F converts asparagine to aspartate, which may reduce the effect of the decrease of the negative charge of sialic acids. Because a clear difference between de-*N*-glycosylated and non-treated samples in cell spreading was still observed, suggesting the contribution of fibronectin-glycans to some extent, it cannot be concluded from this result that *O*-glycans contribute more to the decreased HSC spreading activity of PH-VN than *N*-glycans do.

To address the effects of glycosylation of vitronectin on integrin-mediated signaling, the focal adhesion kinase (FAK) of HSCs was compared among NO-, SH-, and PH-VN. As shown in Fig. 5B, the amount of phosphorylated FAK on PH-VN was decreased in proportion to cell spreading. These results suggest that the change in vitronectin glycosylation due to partial hepatectomy is able to regulate activation of the integrin-mediated signaling pathway. In addition, the effect of phosphorylated FAK on neuraminidase-treated NO-VN was decreased to an extent similar to that on PH-VN.

2.1.5 Site-specific glycosylations of rat vitronectin

In the early stage of liver regeneration, the synthesis of total DNA increased while the synthesis of total glycoproteins decreased within 48 h after partial hepatectomy (Okamoto, Y. and Akamatsu, N. 1977). The contradictory decrease of total glycoprotein synthesis in regenerating rat liver is due to the attenuation of oligosaccharide transferase activity in microsomes (Oda-Tamai, S., et al. 1985). Alterations in the glycan structure of total hepatic glycoproteins have been also suggested during liver regeneration (Kato, S. and Akamatsu, N. 1985; Ishii, I., et al. 1985). However, the changes in the glycans of a particular glycoprotein have not been well characterized. For these reasons, we investigated the site-specific glycosylations and changes after partial hepatectomy in rat plasma vitronectin.

Liquid chromatography/mass spectrometry analysis (LC/MSⁿ) of Glu-C glycopeptides determined the site-specific glycosylation of each vitronectin. Four potential sites, Asn⁸⁶, Asn⁹⁶, Asn¹⁶⁷, and Asn²⁴⁰ were revealed to be *N*-glycosylated, while the peptides of residues Thr¹¹⁰-Thr¹²⁴ were *O*-glycosylated. The most frequent *N*-glycan structures site-specifically found for each site are shown in Fig. 6. At Asn⁸⁶, Asn⁹⁶, and Asn²⁴⁰, biantennary complex-type trisialoglycans with or without core fucosylation and with different amounts of *O*-acetylated NeuNAc were deduced (Fig. 6), whereas biantennary hybrid-type *N*-glycans were found to be the major structures at Asn¹⁶⁷. In the Thr¹¹⁰-Thr¹²⁴ region, the highly sialylated glycans were detected in the negative ion mode spectrum, and analysis in positive ion mode revealed that a Hex-HexNAc unit was located in the inner region of the glycans. From the results of lectin reactivity (18), it was inferred that one to three sialylated core-1 type molecules were attached.

These *O*-glycans contained disialic acid, which was chemically confirmed by fluorometric C7/C9 analyses and mild acid hydrolysate-fluorometric anion-exchange chromatography (Yasukawa, Z., Sato, C., et al. 2005). PH-VN had less disialyl *O*-glycans and complex-type *N*-glycans, but more core-fucosylated *N*-glycans than NO-VN (Sano, K., et al. 2010).

At the same time, alterations in the glycosylation of fibronectin (FN) after PH were different from those of vitronectin. The carbohydrate concentration of PH-FN decreased to 66% of that of NO- and SH-FNs. LC/MSⁿ revealed that eight kinds of complex-type *N*-glycan structures were present in NO-, SH-, and PH-FNs, and that bi- and trisialobiantennary

glycans were the major structures (Sano, K., et al. 2008). Hybrid-type *N*-glycans and disialyl *O*-glycans were not detected. These results indicate that the alterations in the glycosylations of fibronectin and vitronectin were significantly different in the early stage of liver regeneration and demonstrate that these glycoproteins play different biological roles in the promotion of tissue remodeling processes.

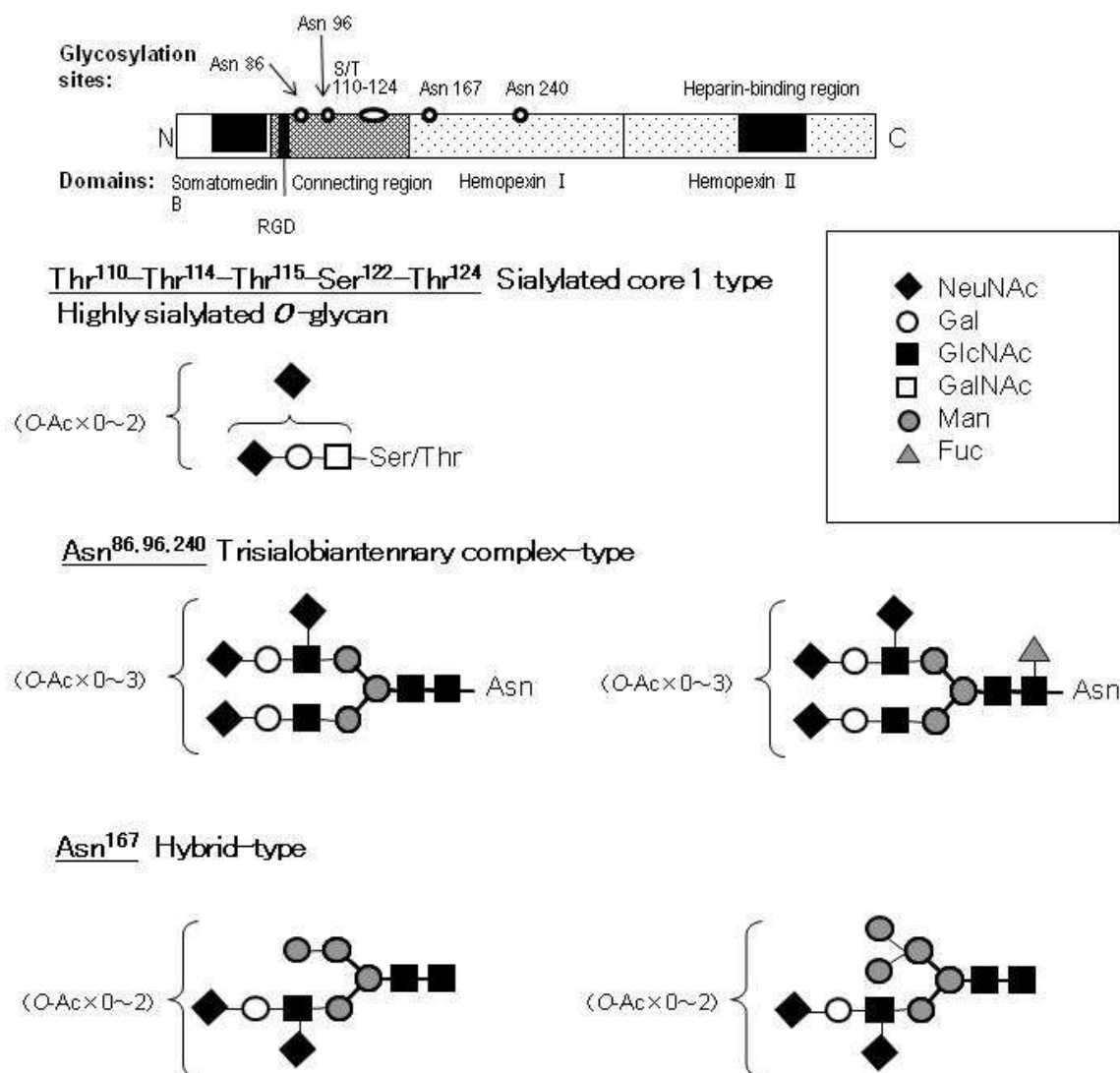


Fig. 6. Site-specific glycosylation of rat plasma vitronectin. The four glycosylation sites on rat plasma vitronectin and the glycan structures at each glycosylation site were determined by glycopeptide analyses using LC/MSⁿ. The most frequent glycan structures that were present at each site are presented.

2.1.6 Change in isoelectric points and oligosialylation of vitronectin

Highly sialylated *O*-glycans, which have a diNeuAc structure and were markedly decreased in PH-VN, affect the isoelectric points of vitronectins. Immunostaining of vitronectins after two-dimensional PAGE showed that each vitronectin has two components, pI 4.0 and 5.7 in NO-VN, that both shifted to higher pI, pI 4.3 and 6.0 in SH-VN, and further to pI 4.6 and 6.0 in PH-VN (Fig. 7). The pI of NO-VN was converted to one basic point, pI 6, after

neuraminidase treatment, and only the more acidic component of pI 4.1 reacted with mAb S2-566, which specifically recognizes the Neu5Ac α 2,8Neu5Ac α 2,3Gal structure (Yasukawa, Z., et al. 2006).

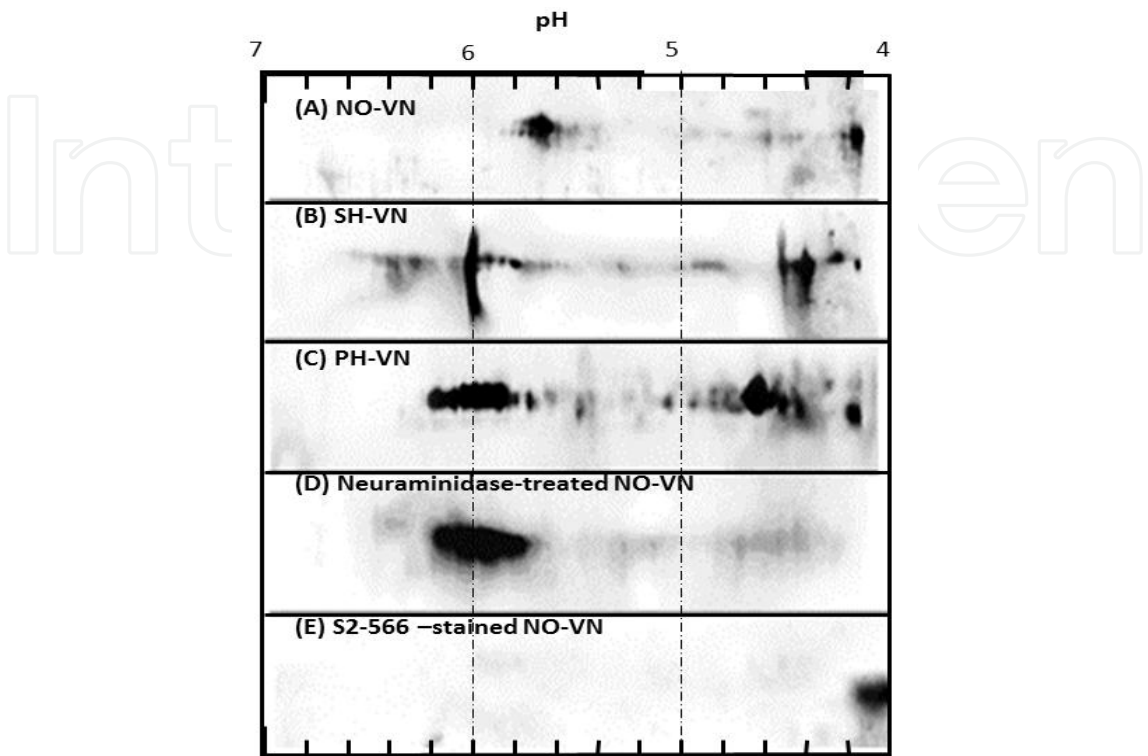


Fig. 7. Two-dimensional PAGE and western blotting of vitronectins. The first electrophoresis was isoelectric focusing. The second electrophoresis was SDS-PAGE under reducing conditions on 7.5% polyacrylamide gel, followed by blotting onto a polyvinylidene difluoride membrane and immunostaining using sheep anti-vitronectin IgGs (A-D) or the anti-oligosialic acid monoclonal antibody S2-566 (E), and HRP-conjugated secondary antibodies. Membranes were developed with ECL-Plus.

The results were also supported by the immunodetection of each vitronectin after 2D-PAGE where both hyper- and hyposialylated molecules were present and the hypersialylation in PH-VN was markedly attenuated (Fig. 3). These analyses showed that the oligosialic acid on the *O*-glycan significantly affected the pI of the acidic partially hepatectomized vitronectin fraction. In addition to the decrease of oligosialyl epitopes in PH-VN, undersialylation of both *N*- and *O*-glycans was found in the basic PH-VN fraction.

The presence of disialic acid structures in some glycoproteins was previously described (Finne, J., et al. 1977), and neural cell adhesion molecules have been very thoroughly studied (Finne, J., Krusius, T., et al. 1977). Changes in expression of the oligosialic epitope on serum glycoproteins under inflammatory conditions were also reported (Yasukawa, Z., et al. 2005) (Yasukawa, Z., et al. 2007). The fact that the amounts of disialic acid structures in PH-VN were decreased compared with those in NO-VN indicates that the inflammation caused by partial hepatectomy reduces disialylation on vitronectin. The results indicate that the decreased sialylation plays a key role in regulating the function of vitronectin in liver regeneration.

In contrast to PH-VN, the glycosylation and biological activities of vitronectin in cirrhotic plasma were differentially changed. Yamada's group reported that vitronectin, which is active in collagen-binding in plasma, increased and correlated with certain fibrous markers (Yamada, S., et al. 1997, Yamada, S., et al. 1996), that the concentration of vitronectin in liver tissue was significantly increased in chronic liver disease compared with that in normal controls, and that vitronectin was colocalized at fibrous sites (Kobayashi, J., et al. 1994). Several reports supported the observation; therefore, the immunoreactivity of vitronectin in liver can be considered a marker of chronic/mature fibrosis. The vitronectins in untreated plasma exist mainly in native inactive form and exhibit low collagen binding. Urea treatment of cirrhotic and normal plasma revealed that the ratio of active to inactive vitronectin in cirrhotic plasma increased to more than twice that in normal plasma, promoting the incorporation of vitronectin from plasma into the matrix proceeds in spite of the fact that the vitronectin concentration in cirrhotic plasma was only 70% of that in normal plasma (Suzuki, R., et al. 2001). It is important to elucidate the changes in the glycosylation and biological activities of vitronectin in cirrhotic plasma and compare them with those of liver regeneration.

2.2 Summary

The present study attempted to determine how alterations of glycans modulate the biological activities of vitronectin during the initial stage of liver regeneration. Plasma vitronectin was purified from partially hepatectomized and sham-operated rats at 24 hours after operation and from non-operated rats. We found that the glycosylation of vitronectin changed and decreased markedly after surgery. The multimer sizes of PH- and SH-VNs significantly increased compared with NO-VN, and the change was accompanied by an increase in collagen binding. It was indicated that these changes were due to the changes in glycosylation of vitronectin, especially decreased sialylation, which increased the size and amount of the multimers to enhance the collagen-binding activity by a multivalent effect. Adhesion and spreading of rat hepatic stellate cells on PH-VN was decreased to 1/2 of that on NO- or SH-VN. Similarly, desialylated NO-VN decreased the spreading of rat hepatic stellate cells to 1/2 of that of control vitronectin, indicating the importance of sialylation of vitronectin for activation of rat hepatic stellate cells. LC/MSⁿ of vitronectin glycopeptides determined the site-specific glycosylation and the presence of highly sialylated O-glycans, which dramatically decreased after partial hepatectomy. Understanding the functional modulation of glycans on vitronectin may contribute to development of a strategy to regulate liver regeneration and matrix fibrosis in liver cirrhosis.

3. New method to detect changes in sialylation

As described in the previous sections, in the initial stage of the liver regeneration, alterations in sialylation of vitronectin modulate the important biological activities of vitronectin during tissue-remodeling processes by multiple steps. Like vitronectin, the sialylation of other glycoproteins changes under pathological conditions as well as during developmental stages, and altered sialylation often has significant implications in the physiological role of glycoproteins (Varki, A., Cummings, R.D., Esko, J. D., Freeze, H. H., Stanley, P., H., Bertozzi, C.R., Hart, G.W., Etzler, M.E. 2009)(Rutishauser, U. 1998). The aim of this section is to describe a fundamental method using chromatofocusing that enables detection of the

changes in sialylation and separation of the matrix glycoproteins according to their pI in small amounts. The strategy is technically feasible and is applicable to various glycoproteins for a number of biological systems.

3.1.1 Principle of chromatofocusing

Chromatofocusing was originally a method of purification by separating proteins according to their pI. The pI of a protein is the pH at which the protein has zero surface charge. If a buffer, initially adjusted to the first pH, is run through an ion exchange column (see Fig. 8A) and followed by another buffer of a second pH, a pH gradient is formed in the column (Fig. 8B) (Sluyterman, L.A.A. E., O 1978; Sluyterman, L.A. and Wijdenes, J. 1978). If this pH gradient is used to elute proteins bound to the ion exchanger, the proteins in the column are eluted in the order of their pI. If the pH of the mobile phase around a protein is higher than the protein's pI, the protein has a positive charge. Therefore, it is dissociated from the anion exchange column and eluted from the column (Fig. 8A-C). If the pH of the mobile phase is lower, the protein has a negative charge and remains in the column (Shan, L. and Anderson, D.J. 2001). In this study, we applied chromatofocusing to detect the changes in sialylation of glycoproteins during liver degeneration.

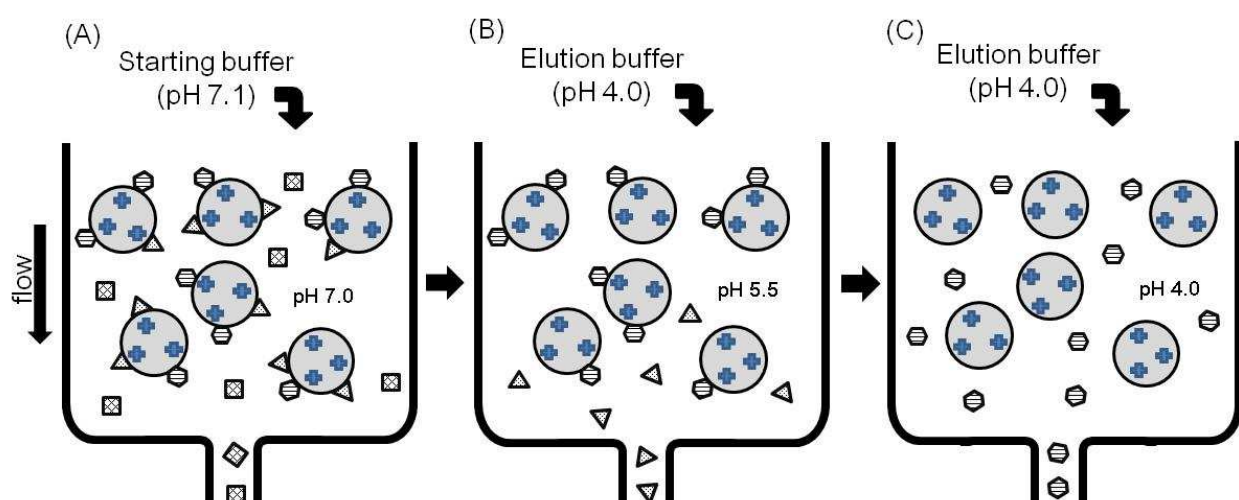


Fig. 8. Proteins having different pI are separated from each other as they pass through the anion exchanger column. A sample containing proteins that are \square (pI = 7.0), \triangle (pI = 5.5), and \hexagon (pI = 4.0) was applied to the column. (A) When the first buffer (pH 7.0) was poured into the column, the pH in the column is 7.0; therefore, rectangle proteins (pI > 7.0) are eluted. Triangle and hexagon proteins (pI < 7.0) are still bound to the column because they are negatively charged at pH 7.0. (B), When the second buffer (pH 4.0) was added, the pH in the column becomes lower, and this is halfway through the elution. (C), When the second buffer (pH 4.0) was continuously loaded, the pH in the column became pH 4.0 throughout the column, and then the hexagon proteins (pI = 4.0) were eluted at the end of the elution.

3.1.2 Method

Rat plasma samples were collected at 24 h, 48 h, 72 h, 5 d, or 7 d after two-thirds hepatectomy or sham operation and stored at -80°C until use. Each plasma sample (200 $\mu\text{g}/100 \mu\text{l}$) was applied to chromatofocusing using a Mono P5/50 GL column (GE

Healthcare Inc.) and a fast protein liquid chromatograph (FPLC; AKTA Purifier, GE Healthcare, Inc). The starting buffer was 0.025 M bis-Tris, pH 7.1, and the elution buffer was 10% Polybuffer74, pH 4.0. Buffers were filtered through 0.22 μm filters under vacuum and degassed. Samples were adjusted to the pH of the starting buffer, or exchanged by dialysis into the starting buffer. The column was pre-equilibrated with the starting buffer until the eluent was applied to the column, which was the same pH as that of starting buffer. After samples were applied to FPLC, the elution buffer was added. Samples were separated by the pI of each component protein in the column, and sequentially eluted from the column. The effluent was continuously monitored by absorbance at 280 nm and divided by 1 mL/tube. After the pH gradient ended, the column was washed with two column volumes of 2M NaCl to elute the molecules still bound to the column. Finally, the column was re-equilibrated with five column volumes of starting buffer until UV absorbance and pH values reached a plateau. An aliquot (10 μL) of each fraction eluted from the column was subjected to SDS-PAGE on a 7% polyacrylamide gel, and the separated protein bands were electro transferred to a polyvinylidene difluoride membrane and detected with specific antibodies to vitronectin. Membranes were developed with ECL (GE Healthcare Inc.).

3.1.3 Results

An example of the elution pattern of rat plasma is shown in Fig. 9. As the buffer flowed through the chromatofocusing column, the pH decreased and a descending pH gradient was generated. As shown in Fig. 10, when vitronectin in rat plasma was separated by chromatofocusing, NO-VN was eluted at pH 4.7–4.5, while PH-VN (24 h) was eluted at pH 5.2–4.8.

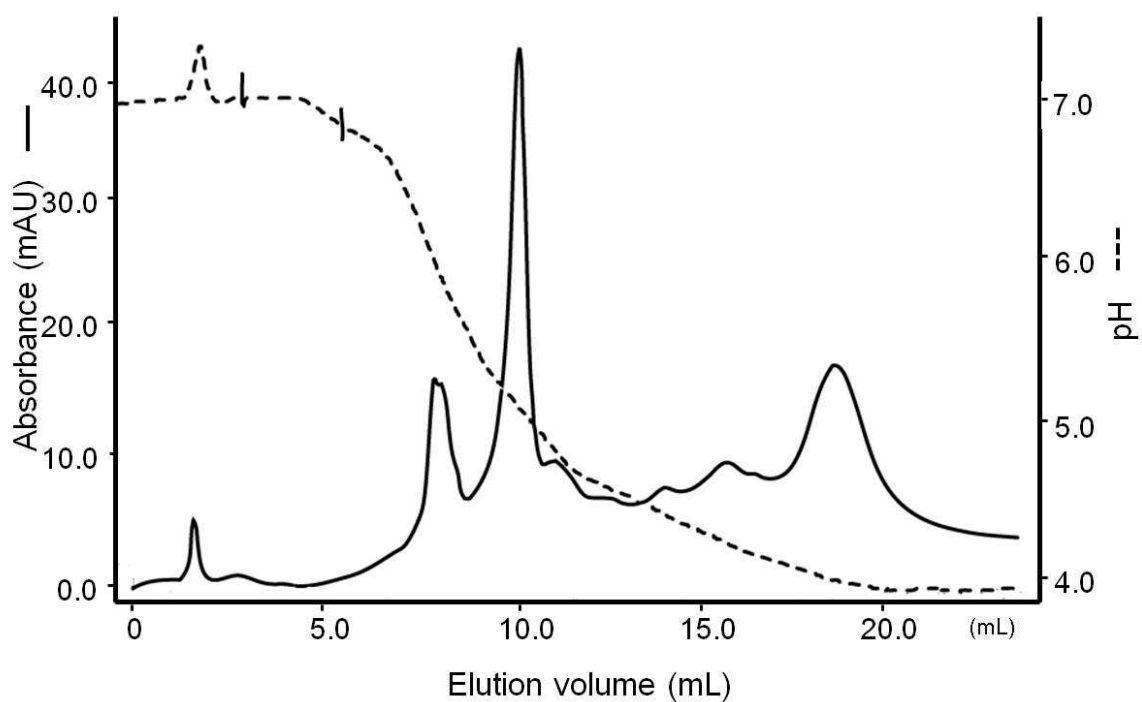


Fig. 9. Chromatogram of non-operated rat plasma. Dotted line, pH; solid line, absorbance at 280 nm. Flow rate: 0.5 mL/min, temperature: 4°C. Other conditions are described in the text.

Rat plasma vitronectin at 24 h after partial hepatectomy was shifted to a higher pI than that of NO-VN on two-dimensional PAGE, as shown in Fig. 7. The result of chromatofocusing combined with immunodetection suggests that sialylation of vitronectin remained low with pI over pH 5 for the period from 24 to 72h after partial hepatectomy. The pI of PH-VN after 7d had recovered to pH 4.7–4.5 (data not shown).

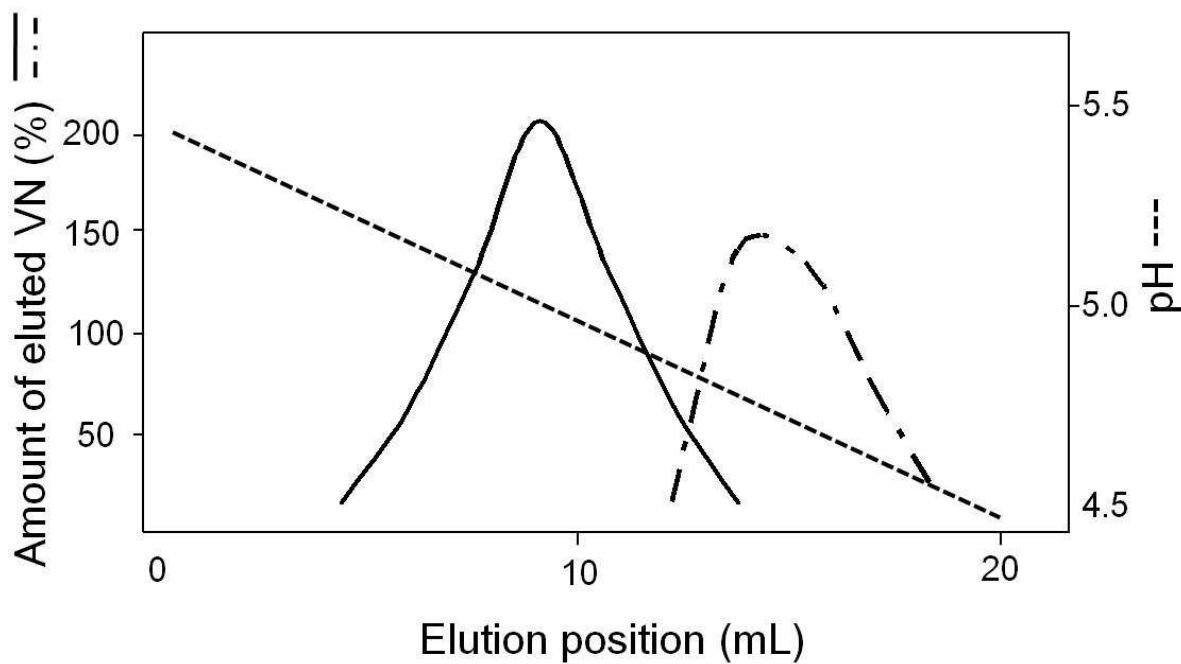


Fig. 10. The elution pattern of vitronectin with descending elution pH. The amount of vitronectin was measured by the band intensity of an immunoblot of the eluted fraction and is expressed as %, when taking that of 1 μ g of purified NO-VN as 100%. Solid line: PH-VN (24 h), chain line: NO-VN, dotted line: pH of the eluted fraction.

3.1.4 Discussion

The elution pattern on chromatofocusing was very reproducible, and the pIs of NO-VN and PH-VN were found to be 4.7–4.5 and 5.2–4.8, respectively (Fig. 10). The immunostaining of PH-VN after two-dimensional PAGE (Fig. 7) indicated the presence of two components (pI 4.6 and 6.0), whereas PH-VN was eluted by chromatofocusing at intermediate pH, although the two methods agreed on the tendency for the pI of PH-VN to be considerably shifted toward alkalinity compared to that of NO-VN. This discrepancy may be due not only to the difference in the plasma sample lots but also because parts of the vitronectin eluted by chromatofocusing are multimerized in the buffer like the vitronectin in physiological plasma, which shows a broad pH range of elution peaks indicating variation in sialylation of the vitronectin molecules contained. Chromatofocusing has the advantage of analyzing the isoelectric property under physiological conditions, especially in detection of changes in sialylation of glycoproteins, and the eluted fractions are utilizable for activity measurements (Kneba, M., et al. 1983).

3.1.5 Summary

Each plasma sample was subjected to chromatofocusing using FPLC, which can separate molecules by their isoelectric point (pI). Fractions eluted from the column were subjected to

SDS-PAGE, electrotransferred to a PVDF membrane, and detected with specific antibodies to vitronectin or fibronectin.

Key results: The chromatofocusing and immunoblotting of plasma before and after sialidase treatment enabled us to demonstrate when and what alteration of sialylation occurs in each glycoprotein at each different stage during liver regeneration. The changes in pI essentially coincided with that of 2D-PAGE; however, we can determine the pI of a sample under physiological condition by using the chromatofocusing technique. Furthermore, this method is facile, quick, and applicable to recovered samples for activity analyses because they are non-denatured and separated by pI.

4. Conclusion

This study proposes that alterations of glycosylation, especially decreased sialylation of vitronectin, modulate tissue remodeling processes in multiple steps, especially HSC spreading and survival. Our findings suggest that the removal of sialic acid from vitronectin suppressed activation of stellate cells, indicating the possibility of a new treatment for or method to prevent liver cirrhosis. This will open new windows to the paradigm of glyco-regenerative medicine, which is based on the modulatory functions of glycans of ECM glycoproteins such as vitronectin.

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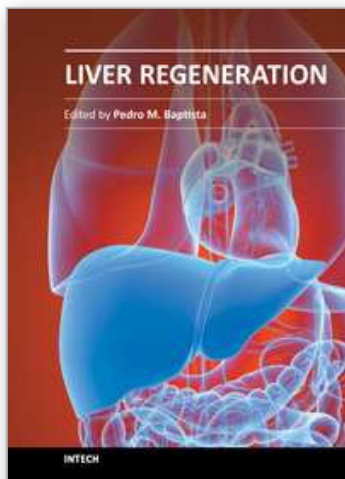
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Liver Regeneration

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Doctors and scientists have been aware of the "phenomenon" of liver regeneration since the time of the ancient Greeks, illustrated by the mythic tale of Prometheus' punishment. Nevertheless, true insight into its intricate mechanisms have only become available in the 20th century. Since then, the pathways and mechanisms involved in restoring the liver to its normal function after injury have been resolutely described and characterized, from the hepatic stem/progenitor cell activation and expansion to the more systemic mechanisms involving other tissues and organs like bone-marrow progenitor cell mobilization. This book describes some of the complex mechanisms involved in liver regeneration and provides examples of the most up-to-date strategies used to induce liver regeneration, both in the clinic and in the laboratory. The information presented will hopefully benefit not only professionals in the liver field, but also people in other areas of science (pharmacology, toxicology, etc) that wish to expand their knowledge of the fundamental biology that orchestrates liver injury and regeneration.

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