DNA-binding and splicing regions. The *N*- and *O*-glycosylation of human FN [20-22] and the effects of glycosylation on the interaction with integrin receptors have been reported [23,24], suggesting that the glycosylation of FN is probably involved in its physiological function.

There have been several reports that oligosaccharides play an important role in the interaction between cells and the ECM. For example, the *N*-type glycan is necessary to form an assembly complex between the α and β subunits of integrins [25]. In particular, the oligosaccharides of integrin α5β1 are involved in the adhesion of several cells to fibronectin [24], suggesting that a specific glycan structure is required for effective adhesion. Our study clarified that the fucosylation of *N*-glycans in rat plasma FN was increased after partial hepatectomy (Table II) in contrast to plasma VN, in which the relative molar ratios of fucose hardly changed among NO, SH, and PH rats, though both of these glycoproteins are synthesized in the liver. The fucosylation of glycans is known to take part in many biological processes such as embryonic growth, differentiation, cell recognition, cancer formation, and inflammation [26]. Although the glycans of FN synthesized during liver regeneration may regulate tissue remodeling by increasing fucosylation through a change in binding to other ligands, we found that the alterations of glycosylation and the biological activities of FN after PH are distinct from those of

VN, indicating a difference in biological function of the glycoproteins in the promotion of tissue remodeling processes.

On the one hand, because of its minus charge and structural diversity, including *O*-acetylation, sialic acid has important biological functions, such as regulation of the metabolism of erythrocytes and providing proteins with resistance to protease [27-29]. It was reported that sialic acids from total membranes of rat liver have surprisingly high levels (approximately 20%) of *O*-acetylation at the 7- or 9-position [30] and that these modifications have very specific functions modulating many biological interactions. For instance, the binding of Siglec-1 (sialoadhesin) and Siglec-2 (CD22), which recognize α2,3-linked and α2,6-linked sialic acids, respectively, on glycoconjugates is hindered by 9-*O*-acetylation [31,32]. In this study, we showed that the *O*-acetylation of neuraminic acids was decreased after partial hepatectomy, suggesting that the immune system in the early stage of liver regeneration is regulated by this modification due to the interaction of FN glycan with Siglecs.

An important area for future investigation is delineation of the signal transduction pathway that regulates assembly of the FN matrix. It is necessary to consider matrix formation to understand the physiological functions of glycosylation of FN. This research showed the glycan structure of rat FN in the early stage of liver

regeneration and the insight of the individual regulation of glycosylation in each ECM glycoproteins. It will provide a basis for a new functional study of FN and other glycoproteins concerned to the tissue remodeling.

4. Experimental

4.1. General methods

4.1.1. Animals

Male Wistar rats aged 5 weeks (weighing about 110 g; Nihon Clea, Tokyo, Japan) were maintained in a room at a constant temperature (23.5 °C) with 12 hours each light (6:00-18:00) and darkness. Two-thirds partial hepatectomy was performed under diethyl ether anesthesia as described previously [33]. Sham-operated rats were anesthetized, and their livers were completely exposed outside the peritoneum and manipulated, but no tissue was removed. Plasma was collected from partially hepatectomized and sham-operated rats at the indicated times after the operation. Plasma samples were stored at -80 °C until use.

4.1.2. Preparation of gelatin-Sepharose

Rat FN was purified from plasma of partially hepatectomized (PH), sham-operated (SH), or non-operated (NO) rats by gelatin-affinity chromatography. Gelatin-Sepharose was prepared as described below. Epoxy-activated Sepharose (40 g) was prepared as described previously [34], 200 ml of 0.1 M NaOH including 40 mg of $NaBH_4$ was added to the gel, and it was incubated at 40 °C overnight. Then 0.1M $NaIO_4$ was added, and it was incubated for 1 hour at 4 °C in the dark. Formyl Sepharose was obtained, and 1 g gelatin in 20 ml of PBS and 240 mg of $NaCNBH_3$ were added and incubated at 4 °C overnight. Glutaraldehyde (1%, w/w, 80 ml) and 1.92 g of $NaCNBH_3$ were added and incubated at 4 °C overnight. Then 80 ml of 1 M Tris-HCl, pH 7.4, and 248 mg of $NaCNBH_3$ were added and reacted for 1 hour at room temperature to block residual formyl residues.

4.1.3. Purification of rat FN from plasma

FN was eluted under a denaturing condition as described previously [35]. The protease inhibitors PMSF/EtOH (1 mM, final conc.), EDTA· Na (pH 7.0, final conc. 5 mM), and ε-ACA (50 mM, final conc.) were added to the plasma and mixed, and centrifuged at 3,000 rpm for 10 minutes at 4 °C. The supernatant was used for the following procedures.

The sample was applied to a gelatin-Sepharose 4B column (10 mL) equipped with a pre-column of Sepharose 4B (20 mL) that had been equilibrated by buffer solution A (10 mM ε-ACA, 0.02% NaN_3, 50 mM Tris-HCl, pH 7.4) beforehand. After the sample had been passed through, the column was washed with buffer A, followed by buffer B (1 M NaCl, 10 mM ε-ACA, 20 mM sodium citrate, 50 mM Tris-HCl. pH 7.4), and eluted by buffer C (0.1 M NaCl and 100 mM citric acid-NaOH, pH 5.2). Finally, the FN remaining in the column was eluted completely by buffer F (2 M NaCl, 6 M urea, 50 mM Tris-HCl, pH 8.5). Purified FN was obtained from the fraction eluted with buffer F, and it was for 2-3 days dialyzed against PBS at 4 °C.

4.2. Determination of FN concentration in plasma (sandwich ELISA)

Wells of microtiter plates (Immulon 1, Dynatech Laboratories Inc., Chantilly, VA) were coated with 50 μl aliquots of a solution of rabbit anti-human FN IgG antibody (1/5400 of 0.5% BSA/PBS) and stored at 4 °C overnight. The wells were washed, and various concentrations of rat plasma (50 μl) were added to each well, followed by incubation for 2 h at room temperature. After they were washed three times, the wells were blocked by 0.5% BSA/PBS for 1 h at room temperature. The amounts of FNs bound to immobilized antigen were detected with horse radish peroxydase (HRP)-conjugated rabbit

anti-human FN IgG. 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 and stopped by adding 4 M H_2SO_4. Absorbance was measured at 490 nm using a microplate reader (model 680, Bio-Rad, Hercules, CA). The amount of FN in the plasma was determined from a standard curve using purified rat FN instead of plasma (a second-order approximation).

4.3. Assays of binding of FN to immobilized ligands

The ligand-binding activities of FN were studied by ELISA essentially according to the method reported previously [36,37]. Briefly, the wells of microtiter plates (Immulon 1, Dynatech Laboratories Inc., Chantilly, VA) were coated with 50 µl aliquots of solutions of gelatin, heparin-BSA, or collagen types I-V (10 µg/ml in 0.1 M carbonate buffer, pH 9.0) and kept overnight at 4 °C. After the wells were washed and blocked, various concentrations of human or rat FN were added to each well, followed by incubation for 2 h. After washing the plates with PBS three times, the amounts of FN bound to immobilized ligands were detected with unconjugated rabbit anti-human FN IgG and HRP-conjugated goat anti-rabbit IgG at room temperature for 1 hour. 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 the addition of 4 M H_2SO_4, and measured at 490 nm using the microplate reader.

4.4. Interaction of FNs with HRP-lectins

FNs were dot-blotted onto a PVDF membrane and reacted with HRP-lectins as previously described [38]. The bound HRP-lectins were developed with diaminobenzidine/H_2O_2. The staining intensities were measured by the software program Scion Image.

4.5. Carbohydrate analysis

FNs (4.5 μg) were placed in glass tubes that had been washed with boiling 50% nitric acid and water and dried *in vacuo*. Hydrolysis was carried out *in vacuo* with a vapor of 2 M HCl and 2 M trifluoroacetic acid for 4 hours at 100 °C. After hydrolysis, the mixture was *N*-acetylated, then reacted with a fluorescent probe, 2-aminopyridine, and the carbohydrate concentration was analyzed as previously reported [39] by using a column for anion exchange chromatography, TSK gel sugar AXI (Tosoh, Tokyo, Japan).

4.6. Reduction to alditols of *N*-glycans of FNs

FN (30 μg/about 200 μl) was dialyzed against 10 mM phosphate buffer (pH 7.5) containing 0.13 M NaCl and 5 mM EDTA. After the addition of 2-mercaptoethanol to a final concentration of 2%, the mixture was boiled for 1 min. Peptide-*N*4-(*N*-acetyl-β-$_D$-glucosaminyl) asparagine amidase from *Fravobacterium meningosepticum* (PNGase F) (4.8 U) was added and incubated at 37 ℃ for 7 days. Cold ethanol (66%, v/v) was added, and the mixture was centrifuged at 6400 rpm for 5 min. After the supernatant was dried, the samples were dissolved in 60 μl of H_2O, 60 μl of 0.5 M $NaBH_4$ was added, and they were incubated for 2 hours at the room temperature. The reaction was stopped by the addition of 15 μl of 30% acetic acid. ENVI-Carb C (100 mg, Supelco Co., Ltd., Bellefonte, PA, USA) was used to remove the reagents, and the elutes were lyophilized.

4.7. Analysis of *N*-glycan structures by mass spectrometry (LC/MS^n)

N-glycans of FNs were analyzed by LC/MS^n. LC/MS was performed using a Fourier transformation ion cyclotron resonance/ ion trap type mass spectrometer LTQ-FT (Thermo Fisher Scientific, Waltham, MA, USA) connected to a Magic 2002 HPLC system (Michrom BioResource, Inc., Auburn, CA). The eluents were 5 mM

CH_3COONH_4 (pH 9.6)-2% CH_3CN (pump A), and 5 mM CH_3COONH_4 (pH 9.6)-80% CH_3CN (pump B). The borohydride-reduced *N*-linked oligosaccharides were separated on a Hypercarb column (Thermo Fisher Scientific, 0.2×150 mm) with a 5-30% linear gradient of eluent B in 60 min and 5-20% of B in 15 min, followed by 20-70% of B in 20 min at a flow rate of 2 μl/min. Mass spectra were recorded with the sequential scans: a full MS scan (m/z 700-2000) by FT-ICR MS in positive ion mode followed by data-dependent MS/MS and MS/MS/MS for the most abundant ion, and a full MS scan (m/z 700-2000) by FT-ICR MS in negative ion mode followed by data-dependent MS/MS for the most abundant ion. The ESI voltage was set at 2.0 kV, and the capillary temperature was 200 °C.

Abbreviations

AAL, *Aleuria aurantia* lectin; BSA, bovine serum albumin; Con A, Concanavalin A; ε-ACA, ε-aminocaproic acid; ECM, extracellular matrix; EDTA, ethylenediamine tetraacetic acid; E-PHA, Phaseolus vulgaris erythroagglutinin; FN, fibronectin; HRP, hoseradish peroxidase; HPLC, high-performance liquid chromatography; IgG, immunoglobulin G; LC, liquid chromatography; LCA, *Lens culinaris* agglutinin; MAM,

Maackia amurensis mitogen; MS; mass spectrometry; NO-, non-operated; PBS, 10 mM phosphate buffer (pH 7.5) containing 0.13 M NaCl; PMSF, phenylmethylsulfonyl fluoride; PH-, partially hepatectomized; PNA, peanut agglutinin; PNGase F, Peptide-*N*4-(*N*-acetyl-β-$_D$-glucosaminyl) asparagine amidase from *Fravobacterium meningosepticum*; PVL, *Psathyrella velutina* lectin; SH-, sham-operated; SPR, surface plasmon resonance; RCA, *Ricinus communis* agglutinin; SBA, soybean agglutinin; SSA, *Sambucus sieboldiana* agglutinin; TBS, 10 mM Tris-HCl (pH 7.5) containing 0.14 M NaCl; VN, vitronectin. WGA, wheat germ agglutinin

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Tables

Table I. Reactivity of various lectins with FNs.

	NO-FN	SH-FN	PH-FN	specificity
Con A	++	++	++	*N*-linked biantennary
SNA	++	++	++	sialyl α2-6 Gal
MAM	+	+	++	sialyl α2-3 Gal
L-PHA	+++	+++	+++	*N*-linked tri- or tetraantennary
E-PHA	++	++	+++	*N*-linked di- or triantennary
	(+++)	(++)	(+)	
AAL	++	+	+++	core or outer fucosylated
LCA	++	++	++	*N*-linked, core-fucosylated
PNA	-	-	-	O-linked, unsialylated Gal
	[++]	[+++]	[+++]	
PVL	-	-	-	tri- or tetraantennary sialyl
	(-)	(-)	(-)	α2-3 Gal or nonreducing
	[++]	[++]	[+++]	terminal GlcNAc
RCA	+++	+++	+++	non reducing terminal Gal
WGA	++	++	++	GalNAc

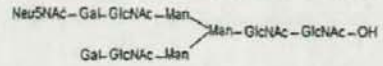
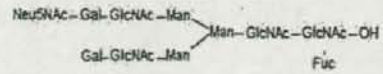
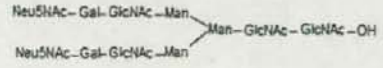
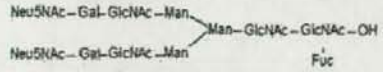
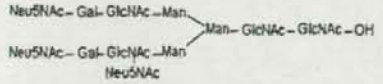
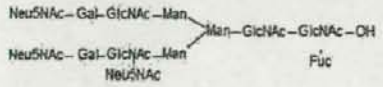
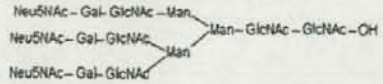
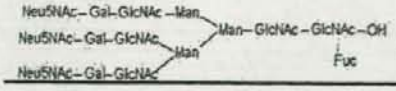
Reactivity of FNs with biotinyl lectins was measured by dot blotting on a PVDF membrane and is expressed as staining intensity. +, indicates positive staining; -, negative staining; w, positive but weak staining. Reactivity after digestion with hexosaminidase is indicated in parentheses, and reactivity after mild acid desialylation is indicated in brackets.

Table II. Carbohydrate composition of rat FNs.

Carbohydrate	NO-FN	SH-FN	PH-FN
GalNAc	92 (9.3)	61(5.3)	33(4.1)
GlcNAc	33(3.4)	37(3.2)	21(2.6)
Man	30(3.0)	35(3.0)	24(3.0)
Fuc	1.9(0.19)	2.5(0.22)	4.5(0.56)
Gal	37(3.7)	57(5.0)	44(5.5)
Total	134	193	127

Values are expressed as mol/mol FN. Relative molar ratios of each carbohydrate are expressed in parentheses by taking the value of Man as 3.0.

Table III. Oligosaccharide structures of rat FN.

Glycan structures	Abbreviation
	BiNA(1)
	BiNA(1)F
	BiNA(2)
	BiNA(2)F
	BiNA(3)
	BiNA(3)F
	TiNA(3)
	TriNA(3)F

The *N*-glycan structure of rat plasma FN was analyzed by LC/MS. Abbreviations used for glycan backbone structures are: BiNA(1), biantennary glycan with one *N*-acetylneuraminic acid; BiNA(1)F, biantennary glycan with one *N*-acetylneuraminic acid and a fucose linked to a penultimate GlcNAc; BiNA(2), biantennary glycan with two *N*-acetylneuraminic acids; BiNA(2)F, biantennary glycan with two

N-acetylneuraminic acids and a fucose linked to a penultimate GlcNAc; BiNA(3), biantennary glycan with three *N*-acetylneuraminic acids; BiNA(3)F, biantennary glycan with three *N*-acetylneuraminic acids and a fucose linked to a penultimate GlcNAc; TiNA(3), triantennary glycan with three *N*-acetylneuraminic acids; and TiNA(3)F, triantennary glycan with three *N*-acetylneuraminic acids and a fucose linked to a penultimate GlcNAc.

Figure legends

Fig. 1. Changes in FN concentration in plasma. Ratios of the concentrations of SH- and PH-FN in plasma to that of NO-FN. The plasma FN concentration was measured by sandwich ELISA, as described in the text, and expressed as a ratio taking that of NO-FN as 1.0. *Open bar*, NO plasma; *dotted bar*, SH plasma; *solid bar*, PH plasma.

Fig. 2. Purification of FN from NO , SH , and PH plasma at 24 h after operations. Plasma (25 ml) was applied to a gelatin-Sepharose column (10 mL, 15x57 mm). FNs were eluted with 0.1 M NaCl in 100 mM citric acid-NaOH (pH 5.2), and FNs still bound to the column were eluted with 2 M NaCl-6 M urea in 50 mM Tris-HCl (pH 8.5). *(A),* Elution curves of FN purification. Symbols used are: *circle,* NO plasma; *square,* SH plasma; *triangle,* PH plasma. *(B),* FN concentration in plasma and *(C)* ratios of purified FN to total FN in plasma taking that of NO-FN as 1.0. *Open bar*, NO; *dotted bar*, SH; *solid bar*, PH.

Fig. 3 Binding activity of FNs in NO, SH, and PH plasma (A) and purified FNs (B) to gelatin, collagen types I-V, and heparin-BSA by ELISA. *(A),* Each plasma was

incubated in microtiter plates coated with gelatin, collagen types I-V, and heparin-BSA. Bound FNs in plasma were detected with rabbit anti-rat FN IgG and HRP-labeled secondary antibodies as described in the text. *(B),* Purified FNs were incubated in microtiter plates coated with each ligand. Bound FNs were detected by the same procedure as described in (A). Symbols used are: *circle,* NO FN; *square,* SH FN; *triangle,* PH FN.

Fig. 4. Effect of de-N-glycosylation on binding activity of FN to collagen types I-V and heparin-BSA. Ligand-binding activities were assayed by ELISA before and after PNGase F treatment. Purified human FNs treated with PNGase F or untreated were incubated in microtiter plates coated with collagen types I-V and heparin-BSA. ELISA was carried out as described in Fig. 3. Bound FNs were detected with rabbit anti-rat FN IgG and HRP-labeled secondary antibodies as described in the text. Symbols used are: *circle,* human FN; *square,* de-*N*-glycosylated human FN.

Fig. 5. Ratios of each FN glycans. The *N*-glycan structure of rat plasma FN was analyzed by LC/MS, and the ratio of each glycan was calculated from the relative peak