Identification of disialic acid-containing glycoproteins in mouse serum: a novel modification of immunoglobulin light chains, vitronectin, and plasminogen

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Received on January 28, 2006; revised on March 31, 2006; accepted on April 4, 2006

Serum glycoproteins are involved in various biologic activities, such as the removal of exogenous antigens, fibrinolysis, and metal transport. Some of them are also useful markers of inflammation and disease. Although the amount of sialic acid increases following inflammation, little attention has been paid to the presence of linkage-specific epitopes in serum, especially the α2,8-linkage. In a previous study, we demonstrated that four components in mouse serum contain α2,8-linked disialic acid (diSia), based on immunoreactivity with monoclonal antibody 2-4B, which is specific to N-glycolylneuraminic acid (Neu5Gc)α2→(8Neu5Gcα2→)n−1, n ≥ 2 [Yasukawa et al., (2005) Glycobiology, 15, 827–837]. In this study, we purified three components, 30-, 70-, and 120-kDa gp, and identified them as an immunoglobulin (Ig) light chain, vitronectin, and plasminogen, respectively, using matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy analyses. Modifications of these proteins with α2,8-linked diSia were chemically confirmed by fluorometric Cα/Cβ analyses and mild acid hydrolysates-fluorometric anion-exchange chromatography analyses. We also demonstrated that the IgG, IgM, and IgE light chains are commonly modified with α2,8-linked diSia. In addition, both mouse and rat vitronectin contained diSia, and the amount of disialylation in vitronectin dramatically decreased after hepatectomy. These results indicate that a novel diSia modification of serum glycoproteins is biologically important for immunologic events and fibrinolysis.

Key words: disialic acid/immunoglobulin light chain/plasminogen/serum glycoprotein/vitronectin

Introduction

The sialic acids are a family of 9-carbon carboxylated sugars, containing nearly 50 members that are derivatives of N-acetyleneuraminic acid (Neu5Ac), N-α-glycolylneuraminic acid (Neu5Gc), and deaminoneuraminic acid (2-keto-3-deoxy-D-glycero-D-galacto-nononic acid) (Angata and Varki, 2002). Sialic acid is an important non-reducing terminal residue in glycoconjugates and is involved in a wide variety of biologic activities in animals (Schauer, 2004). Sometimes, sialic acid links to another sialic acid to form disialic acid (diSia). DiSia is often present in glycolipids and is involved in cell adhesion, cell signaling, and tumor-antigen expression (Nagai and Iwamori, 1995; Sharon and Lis, 1997). Recently, we demonstrated that the α2,8-linked diSia structure is a common carbohydrate antigen not only in glycolipids but also in glycoproteins, using newly developed methods to detect di/oligo/polysialic acid structures in glycoproteins (Sato, Kitajima et al., 1998, Sato et al., 1999, 2000), and identified some molecules as diSia-containing glycoproteins (Sato, 2004).

In serum, sialic acids have an important role in clearance of serum glycoproteins (Drickamer, 1991). Sialic acid residues often cap the terminal galactose residues of serum glycoproteins to prohibit binding to the asialoglycoprotein receptor on hepatocytes (Ashwell and Morell, 1974). Serum glycoproteins are also involved in various biologic activities: immunoglobulins (Igs) and/or complements involved in exclusion of foreign invaders, plasminogen in fibrinolysis, and transferrin and ceruloplasmin in metal transport. They are useful markers of inflammation and disease (Browning et al., 2004; Petersen et al., 2004). The amount of sialic acid in serum is sometimes used as a marker of inflammation because acute-phase proteins such as α1-acid glycoprotein and α1-antitrypsin are capped with sialic acid and increase dramatically after induction of inflammation (Sillanaukee et al., 1999). Previously, we focussed on changes in the expression of α2,3-, α2,6-, and α2,8-linked sialic acid glycotopes in serum glycoproteins, especially under inflammatory conditions, using Maackia amurensis (specific for Siaα2,3Galβ1,4GlcNAc) and Sambucus sieldoldiana (specific for Siaα2,6Gal/GalNAc) lectins, and monoclonal antibody 2-4B (mAb.2-4B), which specifically recognizes Neu5Gcα2→(8Neu5Gcα2→)n−1, n ≥ 2 (Sato et al., 1998). Based on mAb.2-4B immunoreactivity, the presence of diSia was strongly suggested in four components of mouse serum (Yasukawa et al., 2005). To
RESULTS

Purification of the mAb.2-4B-immunoreactive molecules, 30-, 70-, and 120-kDa gp from mouse serum and MALDI-TOF MS analyses

In a previous study (Yasukawa et al., 2005), we showed that four components in mouse serum were immunoreactive with mAb.2-4B (Sato, Kitajima et al., 1998) (Figure 1). Specifically, 32-kDa gp was an acute-phase protein during inflammation (Yasukawa et al., 2005) and was identified as carbonic anhydrase II. We developed this antibody using phosphatidylethanolamine-conjugated Neu5Gc structure as immunogen. Using Neu5Gc–n–PE (n is defined), glycoprotein containing Neu5Gc and the periodate-treated antigens, mAb.2-4B was shown to react specifically with (→8Neu5Gcα2→)n, (n ≥ 2) (Sato, Kitajima et al., 1998; Sato et al., 2000). Based on mAb.2-4B immunoreactivity, other components, 30-, 70-, and 120-kDa gp, are considered to have di/oligoNeu5Gc residues. To identify these glycoproteins, we purified them from mouse serum as described below.

Purification and identification of the 30- and 120-kDa gp

The 30- and 120-kDa gp were salted out from mouse serum with 50% (NH₄)₂SO₄. The proteins in the 50% (NH₄)₂SO₄ precipitate were separated by Sephacryl S-100 gel filtration chromatography. The elution was monitored by absorbance at 280 nm, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Coomassie brilliant blue (CBB) staining, and immunoblotting with mAb.2-4B (Figure 2a). The 30-kDa gp was detected in fractions 41–45, and the 120-kDa gp was detected in fractions 45–49. Fractions 39–45 were pooled and further separated by DEAE-Toyopearl 650 M anion-exchange chromatography with a linear NaCl gradient. The elution was monitored as described in Figure 2a. The 30-kDa gp was detected in fractions 63 and 81, whereas the 120-kDa gp was detected in fraction 63. The CBB-stained band of the 30-kDa gp in fraction 81 and the 120-kDa gp in fraction 63 was excised from the polyacrylamide gel and destained. The gels were digested with trypsin, and the obtained peptides were analyzed by MALDI-TOF MS analyses (Figure 2c for the 30-kDa gp and Figure 2e for the 120-kDa gp). The observed peaks at m/z 940.43, 990.54, 1361.64, and 2176.11 from the 30-kDa gp were considered to come from the Ig light-chain κ-constant region by peptide mass fingerprinting followed by database searches (Figure 2d and Table I). All the observed peptide sequences were confirmed by tandem mass spectrometry (MS/MS) analyses (Table I, boldface-type sequences, data not shown). The observed sequences covered 32% of the Ig light-chain κ-constant region sequence (CAC20700). From the tryptic digests of the 120-kDa gp, 10 discrete peaks were obtained by MALDI-TOF MS analyses (Figure 2e), and these peak components were determined to be parts of plasminogen using peptide mass fingerprinting followed by database searches (Figure 2f and Table I). Of 10 sequences, six were also confirmed by MS/MS analyses (Table I, boldface-type sequences, data not shown). The observed sequences covered 13% of the plasminogen sequence (AAA50168).

Purification of the 70-kDa gp

The 70-kDa gp was salted out with 70% (NH₄)₂SO₄ using the supernatant derived from the 50% (NH₄)₂SO₄ solution of the inflamed mouse sera, as described in Materials and Methods. The precipitate was separated by Sephacryl S-100 gel filtration chromatography. The elution was monitored by absorbance at 280 nm (Figure 3a) and by SDS–PAGE followed by CBB staining and western blotting (data not shown). Fractions 44–61, which contained the mAb.2-4B-immunoreactive 70-kDa gp, were pooled and further separated by DEAE-Toyopearl 650 M anion-exchange chromatography and eluted by stepwise elution. The elution was monitored by absorbance at 280 nm (Figure 3b, left panel) and by SDS–PAGE followed by CBB staining and western blotting (data not shown). The 70-kDa gp was purified to homogeneity as a single band (Figure 3b, CBB) in the fractions eluted by 1.0 M NaCl, and the 70-kDa band was immunostained with mAb.2-4B (Figure 3b, 2-4B). The CBB-stained band of the 70-kDa gp was excised from the gel, destained, and digested with trypsin. The peptides were analyzed by MALDI-TOF MS (Figure 3c). The peptide mass fingerprints obtained by MALDI-TOF MS followed by database searches identified the 70-kDa gp as vitronectin (Figure 3d and Table I). Of nine sequences, four were also confirmed by MS/MS analyses (Table I, boldface-type sequences, data not shown). The observed sequences covered 23% of the vitronectin sequence (AAA40558).
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Fig. 2. Purification of the 30- and 120-kDa gp from normal mouse serum and MALDI-TOF MS analyses of their tryptic digest. (a) Sephacryl S-100 chromatography of 50% (NH₄)₂SO₄ precipitate of normal mouse serum (upper panel). Mouse serum was subjected to ammonium sulfate precipitation as described in Materials and Methods. The 50% (NH₄)₂SO₄ precipitate was applied to the column (1.3 × 75 cm) and eluted with 0.01 M NaCl in 10 mM Tris–HCl (pH 8.0). Elution was monitored by absorbance at 280 nm (V₀, void volume; Vₜ, total volume). Fractions were also monitored SDS–PAGE CBB staining (denoted as CBB, middle panel) and western blotting with mAb.2-4B (IB: 2-4B, lower panel). Aliquots (1 μL) of fractions 33–81 were loaded on a 10% polyacrylamide gel. Positions of the molecular mass markers are indicated on the left. The mAb.2-4B-immunoreactive proteins at 30- and 120-kDa gp-containing fractions (fractions 39–45) indicated by the bars were pooled. (b) DEAE-Toyopearl 650 M chromatography of the major 30- and 120-kDa gp-containing fractions in (a). The pooled fraction was applied to a DEAE-Toyopearl 650 M (Cl– form; column size 1.2 × 17 cm) and eluted with a linear gradient of NaCl (0.01–0.07 M) in 0.01 M Tris–HCl (pH 8.0) (upper panel) as described in Materials and Methods. The NaCl concentration is shown by the dotted line. Elution profile was monitored by the absorbance at 280 nm. SDS–PAGE/CBB staining (denoted as CBB, middle panel) and western blotting with mAb.2-4B (IB: 2-4B) (lower panel) of fractions 41, 63, and 81 eluted from DEAE-Toyopearl 650 M chromatography were loaded on 10% polyacrylamide gel. Positions of the molecular mass markers are indicated on the left. The 30-kDa gp-containing fractions (fractions 56–79 at 0.050–0.065 M NaCl) were pooled as indicated by the bars. (c) MS spectrum (reflectron mode) of a tryptic digest of the 30-kDa gp. The 30-kDa gp band seen in (b) at fraction 81 was excised, digested in gel by trypsin, and analyzed by MALDI-TOF MS as described in Materials and Methods. (d) Sequence of the immunoglobulin light-chain κ-constant region (CAC20700). The putative sequences of observed peptides are summarized in Table I and are given in boldface type. (e) MS spectrum (reflectron mode) of a tryptic digest of the 30-kDa gp. The 30-kDa gp band seen in (b) at fraction 81 was excised, digested in gel by trypsin, and analyzed by MALDI-TOF MS as described in Materials and Methods. (f) Plasminogen sequence (AAA50168). The putative sequences of observed peptides are summarized in Table I and are underlined in boldface type.
Fig. 2. continued
Identification of disialic acid-containing glycoproteins in mouse serum

MALDI-TOF MS analyses indicated that 30-, 70-, and 120-kDa gp mAb.2-4B-immunoreactive components were an Ig light chain, plasminogen, and vitronectin, respectively. To confirm these results, we purified these components from mouse serum by conventional methods and analyzed them for the presence of di/oligosialic acid using chemical analyses.

Affinity purification of the mouse Igs and demonstration of the presence of diSia

The partially purified 30-kDa gp obtained after DEAE-Toyopearl 650 M chromatography (Figure 2b) was applied to a Protein G-Sepharose column. After washing, Igs bound to the Protein G-Sepharose column were eluted with 50 mM diethylamine–HCl (pH 11.5). The purified IgGs were analyzed by western blotting using mAb.2-4B. The mAb.2-4B-positive 30-kDa gp(s) were bound to the Protein G-Sepharose column, which specifically traps the Fc region of IgG, and recovered in the diethylamine-eluted fraction (Figure 4a, elute). In SDS–PAGE/CBB analysis, the 30-kDa band moved up to the 170-kDa band under non-reducing conditions (data not shown). These findings together with the results obtained from MALDI-TOF MS (Figure 2c and d; Table I) confirmed that the 30-kDa gp is the IgG light chain.

To demonstrate the presence of diSia in the 30-kDa gp, we analyzed the purified IgG fraction by mild acid hydrolysis–fluorometric anion-exchange chromatography analysis and the fluorometric C\(_7\)/C\(_9\) analysis. First, we analyzed the mild acid hydrolysate of the 30-kDa gp, which was labeled with a fluorescent reagent using anion-exchange chromatography. A Neu5Gc dimer was clearly observed, and higher oligomers were not observed (Figure 4b, middle panel). Then, we performed the fluorometric C\(_7\)/C\(_9\) analysis. As summarized in Table II, C\(_9\)-Neu5Gc, which indicates the presence of internal sialic acid, was in the IgG fraction with a molar ratio of C\(_9\)-Neu5Gc to C\(_7\)-Neu5Gc of 0.09, suggesting that theoretically, \(\sim \)9% of the sialic acid present on the molecule exists as diSia. These results indicate that the Ig light chain has diNeu5Gc residues. To determine the difference among the Ig classes or IgG subclasses, we analyzed a wide variety of monoclonal Igs. The light chains of IgG1, 2a, and 3, and other Ig classes, IgE and IgM, were immunostained with mAb.2-4B (Figure 4c, upper panel). To exclude the possibility of the non-specific binding of the secondary antibody, we used biotinylated mAb.2-4B and detected it with an avidin–biotin–peroxidase complex system. The same result obtained in Figure 4c was observed (data not shown). These data strongly indicate that the Ig light chains all contain the diNeu5Gc residues.

Table I. Summary of the peptide sequences of peptide fragments obtained by MALDI-TOF MS analysis of the 30, 70, and 120-kDa gp purified from mouse serum

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>MH(^{+}) observed</th>
<th>Mr theoretical</th>
<th>Delta (Da)</th>
<th>Sequence coverage (%)</th>
</tr>
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<tr>
<td>30-kDa gp</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKIDGSE (40–47)</td>
<td>990.54</td>
<td>989.49</td>
<td>0.04</td>
<td>32</td>
</tr>
<tr>
<td>HNSYTECAHK (81–91)</td>
<td>1361.64</td>
<td>1360.58</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>HNSYTECAHKTSISP (81–99)</td>
<td>2175.11</td>
<td>2174.04</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>SFNRNE (100–106)</td>
<td>940.43</td>
<td>939.39</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>70-kDa gp</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CTQGFMAKKK (28–37)</td>
<td>1171.45</td>
<td>1170.55</td>
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<td>23</td>
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<td>GQCYCDAVPRPGYPK (176–193)</td>
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<tr>
<td>LIQDVWIGPDAAFTR (194–211)</td>
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<td>2000.04</td>
<td>0.14</td>
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<td>TYLFF (218–222)</td>
<td>671.96</td>
<td>670.37</td>
<td>0.58</td>
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<tr>
<td>GQYWR (223–228)</td>
<td>796.29</td>
<td>795.36</td>
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<td>FEDGPLYP (229–240)</td>
<td>1364.53</td>
<td>1363.64</td>
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<tr>
<td>SSDGAREPQ (323–335)</td>
<td>1449.58</td>
<td>1448.70</td>
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<tr>
<td>NWHGPV (336–351)</td>
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<td>1664.82</td>
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<tr>
<td>1461.65</td>
<td>1460.76</td>
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<tr>
<td>120-kDa gp</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>QLAAGGSCLAKCETDFV (40–62)</td>
<td>2585.16</td>
<td>2584.17</td>
<td>0.02</td>
<td>13</td>
</tr>
<tr>
<td>WEYCDIPR (254–261)</td>
<td>1152.54</td>
<td>1151.51</td>
<td>0.03</td>
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<tr>
<td>WSEQTPHR (299–306)</td>
<td>1040.51</td>
<td>1039.48</td>
<td>0.02</td>
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<tr>
<td>WSEQTPIRHNR (299–309)</td>
<td>1447.73</td>
<td>1446.69</td>
<td>0.03</td>
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<tr>
<td>CQSWAMFPHIR (398–408)</td>
<td>1404.67</td>
<td>1403.62</td>
<td>0.04</td>
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<tr>
<td>WEYNL (446–453)</td>
<td>1182.60</td>
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<td>0.03</td>
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<td>TAVTAPCQGW (493–512)</td>
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<tr>
<td>HSFTQTPNPR (513–523)</td>
<td>1297.68</td>
<td>1296.66</td>
<td>0.02</td>
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<tr>
<td>VCNVR (730–740)</td>
<td>1450.74</td>
<td>1449.71</td>
<td>0.02</td>
<td></td>
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<tr>
<td>FVDWIER (801–807)</td>
<td>964.50</td>
<td>963.48</td>
<td>0.01</td>
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Boldface-type sequences were confirmed by MS/MS analyses.
Fig. 3. Purification of the 70-kDa gp and MALDI-TOF MS analyses of its tryptic digest. (a) Sephacryl S-100 chromatography of 50% (NH₄)₂SO₄ precipitate of inflamed mouse serum. Mouse serum was subjected to ammonium sulfate precipitation as described in Materials and Methods. The 50% (NH₄)₂SO₄ precipitate was applied to the column (1.3 x 75 cm) and eluted with 0.05 M NaCl in 50 mM Tris–HCl (pH 8.0). The elution profile was monitored by the absorbance at 280 nm and SDS–PAGE CBB and western blot using mAb.2-4B (V₀, void volume; Vₜ, total volume). Major 70-kDa gp-containing fractions (fractions 61–80) indicated by the bars were pooled. (b) DEAE-Toyopearl 650 M chromatography of the major 70-kDa gp-containing fraction in (a). The pooled fraction was applied to a DEAE-Toyopearl 650 M (Cl⁻ form; column size 1.0 x 24 cm) and eluted with discontinuous NaCl gradients in 0.05 M Tris–HCl (pH 8.0) as described in Materials and Methods. The NaCl concentration is shown by the line without a symbol. The elution profile was monitored by the absorbance at 280 nm (left panel). The 70-kDa gp-containing fractions (fractions 42–44 at 1.0 M NaCl) were pooled as indicated by the bars, and the pooled fraction was examined by SDS–PAGE/CBB staining (denoted as CBB) and western blotting using mAb.2-4B (2-4B). (c) Positions of the molecular mass markers are indicated on the left. (d) MS spectrum (reflectron mode) of a tryptic digest of the 70-kDa gp. The 70-kDa gp band observed in (b) was excised, digested in gel with trypsin, and analyzed by MALDI-TOF MS as described in Materials and Methods. (d) Vitronectin sequence (AAA40558). The putative sequences of observed peptides are summarized in Table I and are given in boldface type.
Affinity purification of the mouse plasminogen and demonstration of the presence of disialoglycopeptide

The 120-kDa gp that was specifically immunostained with mAb.2-4B was strongly indicated to be plasminogen by MALDI-TOF MS analyses (Figure 2e and Table I). A lysine-coupled column can be used to purify plasminogen (Edelberg et al., 1990). Thus, normal mouse serum was applied to a lysine-coupled column. After washing, plasminogen was eluted with 300 mM phosphate buffer and 0.1 M 6-aminohexanoic acid in 100 mM phosphate buffer (pH 7.4). SDS–PAGE/CBB staining indicated that the plasminogen was highly purified (Figure 5, lanes 4 and 5), and western blot analysis of each fraction using mAb.2-4B demonstrated that the purified plasminogen contained the di/oligoNeu5Gc structure (Figure 5a, IB: 2-4B). To confirm the presence of diSia chemically, we analyzed plasminogen by mild acid hydrolysis-fluorometric anion-exchange chromatography. The diNeu5Gc structure was observed and higher oligomeric structures were not observed (Figure 6a, middle panel). Furthermore, the fluorometric C7/C9 analysis of mouse vitronectin also showed the presence of C7-Neu5Gc-DMB (Table II). The ratio of C9- to C7-Neu5Gc of 0.2 indicated that 20% of the terminal end of the sialyl glycan chain in mouse vitronectin is the diNeu5Gc structure. These immunochemical and chemical results clearly demonstrated that mouse vitronectin is modified by di/oligoNeu5Gc. The sialic acid detected in mouse vitronectin was exclusively Neu5Gc (Table II). To determine the difference between species, we also analyzed rat vitronectin. Purified rat vitronectin from serum was also immunostained with mAb.S2-566, which specifically recognizes the Neu5Ac2,8Neu5Ac2,3Gal structure (Figure 6b). The acid hydrolysis-anion exchange fluorometric high-performance liquid chromatography analyses showed that rat vitronectin has the diNeu5Ac structure, and higher oligoNeu5Ac structures were not observed (Figure 6c). These findings indicate that the diSia structure is present in rat vitronectin. Vitronectin is a multifunctional glycoprotein (McKeown-Longo and Panetti, 1996), and sialylation and N-glycosylation of vitronectin change markedly in partially hepatocyte-mixed rats (Uchibori-Iwaki et al., 2000). Thus, we examined whether disialylation is altered by partial hepatocyte in rat. The purified vitronectin derived from the sera of normal, sham-operated, and partially hepatectomized rats were analyzed to determine the amount of protein by silver staining or the amount of diSia by immunostaining with mAb.S2-566 (Figure 6d), and the ratio of the amounts of diNeu5Ac to that of protein was quantified densitometrically (Figure 6e). Compared with normal rat vitronectin, the amount of diSia in sham-operated rat vitronectin decreased to 65% and that in partially hepatocyte-mixed rat vitronectin dramatically decreased to 30% (Figure 6e). These results demonstrate that the diSia structure in vitronectin changes following hepatectomy.

Discussion

In mouse serum, four components, a 30-, 32-, 70-, and 120-kDa gp, were immunoreactive with mAb.2-4B, which structure was clearly observed, and higher oligomeric structures were not observed (Figure 6a, middle panel). Furthermore, the fluorometric C7/C9 analysis of mouse vitronectin also showed the presence of C9-Neu5Gc-DMB (Table II). The ratio of C9- to C7-Neu5Gc of 0.2 indicated that 20% of the terminal end of the sialyl glycan chain in mouse vitronectin is the diNeu5Gc structure. These immunochemical and chemical results clearly demonstrated that mouse vitronectin is modified by di/oligoNeu5Gc. The sialic acid detected in mouse vitronectin was exclusively Neu5Gc (Table II). To determine the difference between species, we also analyzed rat vitronectin. Purified rat vitronectin from serum was also immunostained with mAb.S2-566, which specifically recognizes the Neu5Ac2,8Neu5Ac2,3Gal structure (Figure 6b). The acid hydrolysis-anion exchange fluorometric high-performance liquid chromatography analyses showed that rat vitronectin has the diNeu5Ac structure, and higher oligoNeu5Ac structures were not observed (Figure 6c). These findings indicate that the diSia structure is present in rat vitronectin. Vitronectin is a multifunctional glycoprotein (McKeown-Longo and Panetti, 1996), and sialylation and N-glycosylation of vitronectin change markedly in partially hepatocyte-mixed rats (Uchibori-Iwaki et al., 2000). Thus, we examined whether disialylation is altered by partial hepatocyte in rat. The purified vitronectin derived from the sera of normal, sham-operated, and partially hepatectomized rats were analyzed to determine the amount of protein by silver staining or the amount of diSia by immunostaining with mAb.S2-566 (Figure 6d), and the ratio of the amounts of diNeu5Ac to that of protein was quantified densitometrically (Figure 6e). Compared with normal rat vitronectin, the amount of diSia in sham-operated rat vitronectin decreased to 65% and that in partially hepatocyte-mixed rat vitronectin dramatically decreased to 30% (Figure 6e). These results demonstrate that the diSia structure in vitronectin changes following hepatectomy.
specifically recognizes the di/oligoNeu5Gc structure (Yasukawa et al., 2005). Our ultimate goal is to determine the function of disialylation in these molecules, and as a first step, we identified the protein components of these molecules in this study. Recently, we identified the 32-kDa gp as carbonic anhydrase II and demonstrated that it is an acute-phase inflammatory protein (Yasukawa Z et al., in preparation). We purified three other mAb.2-4B-immunoreactive components from mouse serum using several chromatographic steps. MALDI-TOF MS analyses demonstrated that the 30-, 70-, and 120-kDa gp were an Ig light, vitronectin, and plasminogen, respectively.

Ig light chains were purified from mouse serum, and this glycoprotein was confirmed by three methods to have diSia. One method was immunoblotting using the anti-di/oligoNeu5Gc antibody, mAb.2-4B. The other two were chemical methods. We observed diNeu5Gc residues by anion-exchange chromatography after 1,2-diamino-4,5-methylenedioxybenzene (DMB) labeling of mild acid hydrolysates of Ig (Figure 4b). The fluorometric C7/C9 analyses also showed that ∼9% of the terminal sialylglycan chains were disialylated based on the ratio of C9 to C7 of Neu5Gc (Table II). The amount of Neu5Ac was 3% that of Neu5Gc, and Neu5Ac was also present as diSia according to the ratio of C9 to C7 Neu5Ac (Table II). The presence of sialic acid residues, especially in the mouse light-chain Ig fraction, but not in the heavy-chain fraction, and the findings that IgM and IgE as well as IgG light chains were modified with diSia (Figure 4c) suggest that disialylation is important for the biologic processes of IgS.

It is widely reported that IgG contains 2.3 N-linked biantennary oligosaccharides per molecule in mice (Mizuochi et al., 1987; Raju et al., 2000), and two of these represent the conserved glycosylation sites at Asn-297 in each of the two heavy-chain CH2 domains of the Fc portion, and the remainder is found in the hypervariable regions of the Fab section with the frequency and position dependent on the chance occurrence of the N-glycosylation consensus sequence (Asn-Xaa-Thr/Ser). (Parekh et al., 1985; Mizuochi et al., 1987). Variations in the N-glycosylation of IgG molecules at one conserved site, Asn-297, in the Fc region can directly modulate Fc-receptor recognition and effector functions (Dwek et al., 1995; Lund et al., 1995, 1996; Wright and Morrison, 1998). O-glycosylation in the Fc portion has also been detected in two mouse monoclonal IgG2a antibodies in dot blot experiments using lectins (Coco-Martin et al., 1992). Approximately 40% of the heavy chains of the mouse IgG2b antibodies are O-glycosylated at

detector (excitation 373 nm, emission 448 nm). The number at each peak represents the degree of polymerization (DP). Middle panel: IgG fraction, 30-kDa gp, (22 μg as BSA) was treated with 0.01 N trifluoroacetic acid at 50°C for 1 h, and released sialyloligomer was labeled with DMB. Labeled sialyloligomer was applied to a high-performance liquid chromatograph as described above. The peak labeled 'x' indicates an unidentified peak that was also detected in the background (bottom panel). (c) Western blot analyses of various Igs. Monoclonal Igs (0.3–5 μg as BSA/lane) were subjected to SDS–PAGE/CBB staining (denoted as CBB) and western blotting with mAb.2-4B (IB: 2-4B) or without primary antibody [1st antibody (-)]. mo, monoclonal; po, polyclonal. IgG fraction obtained from mouse serum was also analyzed (IgG fraction).
Identification of disialic acid-containing glycoproteins in mouse serum

Table II. Fluorescent C7/C9 analyses of the 30-, 70-, and 120-kDa gp

<table>
<thead>
<tr>
<th>Sia species</th>
<th>Neu5Gc</th>
<th>Neu5Ac</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C9 (mol/mol)</td>
<td>C7 (mol/mol)</td>
</tr>
<tr>
<td>30-kDa gp</td>
<td>0.011</td>
<td>0.13</td>
</tr>
<tr>
<td>70-kDa gp</td>
<td>0.38</td>
<td>2.2</td>
</tr>
<tr>
<td>120-kDa gp</td>
<td>0.010</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Neu5Gc, N-glycolylneuraminic acid.

* C9 indicates internal sialic acid via the α2,8-linkage.

Thr-221A in the hinge region, and O-glycosylation might be involved in protease resistance (Kim et al., 1994). O-glycosylation of the IgG1 light chain is reported on a chimeric antibody (cMu-9–1) (Krishnan et al., 1999). In the present study, we first found that mouse Ig light chains contained diNeu5Gc residues. Because of the ubiquitous presence of diSia in Ig light chains, it might be useful to examine the presence of diSia in Bence-Jones protein, which is secreted into urine due to an imbalance in the amounts of heavy and light chains in plasma cells (Bradwell et al., 2003; Miller et al., 2004). It is important to examine the presence of diSia on such Iggs derived during disease states.

The 120-kDa gp was identified as plasminogen, and this glycoprotein also contained diSia in mouse serum. In human serum, plasminogen is present in two major glycoforms, plasminogen 1, which possesses an N-linked high mannose-type carbohydrate chain at Asp-289 and an O-linked carbohydrate chain at Thr-345, and plasminogen 2, which contains an O-linked glycan at Thr-345 (Hayes and Castellino, 1979a; Davidson and Castellino, 1991; Pirie-Shepherd et al., 1997). The O-linked carbohydrate chain at Thr-345 is Neu5Acβ2→3Galβ1→3GalNAc (Hayes and Castellino, 1979b; Davidson and Castellino, 1991). Plasminogen 2 can be separated into at least six glycoforms according to the Neu5Ac content by isoelectric focusing electrophoresis (Siefring and Castellino, 1974; Gonzalez-Gronow et al., 1990). The Neu5Ac content of plasminogen 2 varies from 1.3 to 13.7 mol/mol protein (Pirie-Shepherd et al., 1995), suggesting the presence of a sialyloligo/polymer. Each glycoform isolated by two-dimensional-PAGE of plasminogen 2 displays markedly different kinetic behaviors when activated with tissue-plasminogen activator, urinary-type plasminogen activator, and streptokinase (Pirie-Shepherd et al., 1995, 1996). In this study, mouse plasminogen was shown to have diSia, and this glycoform of plasminogen might regulate tissue plasminogen activator, urinary-type plasminogen activator, and streptokinase.

Vitronectin in mouse and rat also has diSia (Figure 6a and c). Vitronectin is a multifunctional adhesive glycoprotein that originates mainly in hepatocytes and circulates in the blood stream at high concentrations (0.2 mg/mL in humans) (Uchibori-Iwaki et al., 2000). Vitronectin regulates the blood systems related to protease cascades, such as cell lysis, by complement, coagulation, and fibrinolysis (Tomasini and Mosher, 1990; Preissner, 1991). Vitronectin is also found in the extracellular matrix of most tissues and is considered to have a role in cell adhesion, cellular motility, and matrix remodeling. Tissue vitronectin is considered to be present as an active multimeric form, and interactions with various matrix ligands, such as various types of integrins on the cell surfaces, Type-1 plasminogen activator inhibitor, and urokinase receptors, to regulate pericellular proteolysis (McKewon-Longo and Panetti, 1996; Seiffert, 1997; Preissner and Seiffert, 1998), are responsible for these functions. Vitronectin can also bind to various types of collagen through its conformational transition from the native inactive form to an active form (Gebb et al., 1986; Ishikawa-Sakurai and Hayashi, 1993; Izumi et al., 1998).

Vitronectins from various animals (human, horse, porcine, bovine, rabbit, and chicken) react with several lectins (Canavalia ensiformis, Triticum vulgare, Allomyrina dichotoma, Ulex europeaeus, Lens culinaris agglutinin, and Phaseolus vulgaris leukoagglutinin) (Kitagaki-Ogawa et al., 1990). In addition, the N-linked oligosaccharide structures of porcine (Yoneda et al., 1993) and human plasma vitronectins (Ogawa et al., 1995) have been elucidated. Carbohydrate composition and lectin reactivity indicate that N-glycosylation and sialylation of vitronectin change markedly after partial hepatectomy. Vitronectins from partially hepatectomized rats 24 h after surgery exhibit markedly enhanced binding to Type-I collagen, and equal enhancement of collagen binding was caused by desialylation of vitronectin with sialidase treatment (Uchibori-Iwaki et al., 2000). These results suggest that modulation of the biologic activity of vitronectin is achieved through altered glycosylation. In this study, we demonstrated that vitronectin was modified by diSia. In particular, the amounts of disialylation of vitronectin derived from hepatectomized rat dramatically decreased compared with that of normal rat (Figure 6e), suggesting that diSia residues regulate the function of vitronectin in sera to regenerate the liver. The fact that diSia in sham-operated rat vitronectin decreased compared to that of normal rat suggests that the inflammation caused by tissue injury reduces the disialylation state of vitronectin, although inflammation caused by turpentine oil, which produces the interleukin-1 and interleukin-6 cascade (Won et al., 1993), does not change the disialylation state in serum.

Based on the fact that diSia is present in plasminogen, vitronectin, and α2-macroglobulin (Kitajima et al., 1999), it might have an important role in the regulation of protease activity. It is noteworthy that some proteases have a basic cluster and a key basic amino acid near active sites that regulates protease activity (Cheng et al., 1986; Rezaie and Yang, 2003). Owing to the negative charge of diSia, it might bind to these basic amino acids to inhibit function. As plasminogen and vitronectin have major roles in fibrinolytic activity in
Fig. 5. Affinity-purified plasminogen contained diNeu5Gc residues. (a) SDS-PAGE/CBB and western blotting using mAb.2-4B (IB: 2-4B) of the fractions eluted from the lysine-Sepharose. Mouse serum were applied to a lysine-Sepharose and eluted as described in Materials and Methods. Mouse serum (15 μg, lane 1), flow-through from non-coupled Sepharose 4B (15 μg, lane 2), flow-through from lysine-Sepharose (15 μg, lane 3), 300 mM phosphate buffer (pH 7.4) eluate (0.2 μg, lane 4), and 0.1 M 6-aminohexanoic acid in 100 mM phosphate buffer (pH 7.4) eluate (0.2 μg, lane 5) were analyzed. Positions of the molecular mass markers are indicated on the left. (b) Resource Q anion-exchange chromatography of di/oligo/polyNeu5Gc–DBM. Upper panel: α2–8-linked di/oligo/polyNeu5Gc derived from mild acid hydrolysates of polysialoglycoprotein [(Neu5Gc)n, 2 μg as Neu5Gc] labeled with DMB were applied to a Resource Q anion-exchange column (1 mL, Cl– form). The conditions are described in Materials and Methods and in Figure 4b. Middle panel: Purified rat vitronectin (3.2 μg as BSA) was treated with 0.01 N trifluoroacetic acid at 50°C for 1 h, and released sialyloligomer was labeled with DMB. Labeled sialyloligomer was applied to the high-performance liquid chromatograph as described above. The peak labeled ‘x’ indicates an unidentified peak that was also detected in the background (bottom panel). (c) SDS-PAGE/silver staining (silver) and western blot analysis using mAb.S2-566 (IB: S2-566 [specific to Neu5Acα2→8Neu5Acα2→3Gal structure]) of vitronectin from normal rat serum. Positions of molecular mass markers are indicated on the left.

Fig. 6. Mouse and rat vitronectin contained diNeu5Gc and diNeu5Ac, respectively, and the diNeu5Ac residues on rat vitronectin decreased after partial hepatectomy. (a) Resource Q anion-exchange chromatography of di/oligo/polyNeu5Gc–DBM. Upper panel: α2→8-linked di/oligo/polyNeu5Gc derived from mild acid hydrolysates of polysialoglycoprotein [(Neu5Gc)n, 2 μg as Neu5Gc] labeled with DMB were applied to a Resource Q anion-exchange column (1 mL, Cl– form). The conditions are described in Materials and Methods and in Figure 4b. Middle panel: Purified rat vitronectin (3.2 μg as BSA) was treated with 0.01 N trifluoroacetic acid at 50°C for 1 h, and released sialyloligomer was labeled with DMB. Labeled sialyloligomer was applied to the high-performance liquid chromatograph as described above. The peak labeled ‘x’ indicates an unidentified peak that was also detected in the background (bottom panel). (b) SDS-PAGE/silver staining (silver) and western blot analysis using mAb.S2-566 (IB: S2-566 [specific to Neu5Acα2→8Neu5Acα2→3Gal structure]) of vitronectin from normal rat serum. Positions of molecular mass markers are indicated on the left. (c) Resource Q anion-exchange chromatography of di/oligo/polyNeu5Ac–DBM. Upper panel: α2→8-linked di/oligo/polyNeu5Ac derived from mild acid hydrolysates of colominic acid [(Neu5Ac)n, 10 μg as Neu5Ac] labeled with DMB were applied to a Resource Q anion-exchange column (1 mL, Cl– form). The conditions are described in Materials and Methods and in Figure 4b. Middle panel: Purified rat vitronectin (3.2 μg as BSA) was treated with 0.01 N trifluoroacetic acid at 50°C for 1 h, and released sialyloligomer was labeled with DMB. Labeled sialyloligomer was applied to the high-performance liquid chromatograph as described above. The peak labeled ‘x’ indicates an unidentified peak that was also detected in the background (bottom panel). (d) SDS-PAGE/silver staining (silver) and western blotting using mAb.S2-566 of vitronectin derived from non-operated rat (NO-VN), sham-operated rat (SH-VN), and partially hepatectomized rat (PH-VN). Vitronectin (0.1 μg/lane) was analyzed. (e) The ratio of the amount of diNeu5Ac on vitronectin to the protein amount of vitronectin decreased after partial hepatectomy. The amount of diNeu5Ac and the protein of vitronectin were densitometrically quantified, and the relative ratio of disialic acid to the protein amount of the vitronectin derived from normal serum was set equal to 1.0. The bars indicate standard deviation (n = 3).
sera, diSia might regulate fibrinolytic activity via inhibition of proteases in sera.

It has been shown that two sialyltransferases, ST8Sia III and VI, are responsible for the synthesis of diSia structures. ST8Sia III catalyzes the synthesis of disialic and oligosialic acids on glycolipids, glycoproteins, and oligosaccharides (Yoshida et al., 1995; Angata et al., 2000), whereas ST8Sia VI acts on O-glycans as well as oligosaccharides (Takashima et al., 2002; Teintenier-Leleivre et al., 2005). ST8Sia III is strongly expressed in fetal brain and testis (Tsuji, 1999). We reported that ST8Sia III was moderately up-regulated during neural differentiation of Neuro2A cells (Sato et al., 2002) and during adipogenesis of 3T3-L1 cells (Sato et al., 2001). The expression of ST8Sia VI is ubiquitous in various cells and tissues (Takashima et al., 2002). In mouse liver, we previously demonstrated that ST8Sia III and VI were constantly expressed before and after inflammation (Yasukawa et al., 2005). Therefore, these sialyltransferases might synthesize the diSia structure on vitronectin, plasminogen, and the light chain of antibodies.
In serum, natural antibodies are present, and sometimes, these antibodies are involved in diseases such as neuropathy (Kornberg and Pestrkon, 1995). The antigens of these antibodies are sometimes considered to be gangliosides (O’Hanlon et al., 2002; Schwerer, 2002), which are mimicked by some bacterial lipopolysaccharides or lipooligosaccharides. In this study, we demonstrated the presence of diSia in serum glycoproteins. Thus, diSia might be an immunogen that induces the production of anti-ganglioside antibodies that are sometimes present in sera as natural antibodies.

Materials and Methods

Materials

Silver Stain II Kit Wako was obtained from Wako Pure Chemical (Osaka, Japan). Sephacryl S-100, Protein G-Sepharose, CNBr-activated Sepharose resins, and enhanced chemiluminescence reagents were purchased from Amershamb Biosciences (Piscataway, NJ). DEAE-Toyopearl 650 M resin was purchased from Tosoh (Tokyo, Japan). Polyvinylidene difluoride (PVDF) membrane (Immobilon P) was a product obtained from Millipore (Bedford, MA). Prestained molecular weight markers were purchased from Bio-Rad (Hercules, CA), Daiichi Pure Chemicals (Tokyo, Japan), or Sigma Chemical (St. Louis, MO). Peroxidase-conjugated rat anti-mouse IgG was purchased from American Qualex (San Clemente, CA). Peroxidase-conjugated rat antimouse IgM was purchased from Zymed Laboratories, (San Francisco, CA). Mouse monoclonal IgM antibody 2-4B, which recognizes Neu5Gc2→(8Neu5Gc2→)n, n ≥ 2, was prepared as described previously (Sato et al., 1998). DMB was purchased from Dojindo (Kumamoto, Japan). Male, 8-week-old ddY mice were obtained from Japan SLC, (Hamamatsu, Japan). Turpentine oil was kindly provided by Dr. Hiroaki Oda (Nagoya University, Nagoya, Japan). Avidin–biotin-peroxidase complex was obtained from Vectastain ABC Kit (Vector Laboratories, Burlingame, CA). mAbs., mAb.4D4 (IgG1), mAb.5D4 (IgG1), mAb.5E9 (IgG1), mAb.6C6 (IgG1), mAb.L101 (IgG1), mAb.4A1 (IgE), and mAb.9E4 (IgE) were kindly provided by Dr. Tsukasa Matsuda (Nagoya University). mAb.AC1 (IgG3), mAb.9E10 (IgG1) and mAb.735 (IgG2a), and mAb.S2-566 (IgM) were kindly provided by Dr. Keiko Nohara (National Institute of Environmental Studies, Tsukuba, Japan), Dr. Rita Gerady-Schahn (Medizinische Hochschule, Hannover, Germany), and Dr. Koichi Furukawa (Nagoya University), respectively.

Experimental inflammation

Turpentine oil was injected subcutaneously into ddY mice to induce inflammation, and sera were prepared as described previously (Yasukawa et al., 2005).

Purification of the 30- and 120-kDa gp from mouse serum

Normal mouse sera (9.3 mL) was mixed with 9.3 mL of saturated (NH4)2SO4 (final concentration, 50%), stirred at 4°C for 1 h and centrifuged at 10,000 × g for 10 min. The pellet was dissolved in 10 mM Tris–HCl (pH 8.0) containing 0.1 M NaCl and applied to Sephacryl S-100 chromatography (1.3 × 75 cm, 100 mL, equilibrated with 10 mM Tris–HCl [pH 8.0] containing 0.1 M NaCl). The elution profile was monitored by absorbance at 280 nm and by SDS–PAGE followed by CBB staining. Glycoproteins were separated by SDS–PAGE and electrophorated onto a PVDF membrane and immunostained with mAb.2-4B. The fractions containing mAb.2-4B-immunoreactive 30 and 120-kDa gs were pooled, applied to DEAE-Toyopearl 650 M chromatography (column size: 1.2 × 1.7 cm, buffer: 10 mM Tris–HCl [pH 8.0] containing 10 mM NaCl), and eluted with a linear gradient of 10–70 mM NaCl in 10 mM Tris–HCl (pH 8.0). The elution profile was monitored by the absorbance at 280 nm and by SDS–PAGE followed by CBB staining and western blotting using mAb.2-4B.

Purification of the 70-kDa gp from mouse serum

Inflamed mouse sera (20 mL) was mixed with 20 mL of saturated (NH4)2SO4 (final concentration, 50%), stirred at 4°C for 3 h, and centrifuged at 10,000 × g for 15 min. The supernatant was mixed with 26.6 mL of saturated (NH4)2SO4 (final concentration, 70%), stirred at 4°C for 12 h, and centrifuged at 10,000 × g for 15 min. The pellet was dissolved in 50 mM Tris–HCl (pH 8.0) containing 50 mM NaCl and applied to Sephacryl S-100 chromatography (1.3 × 80.5 cm, equilibrated with 50 mM Tris–HCl [pH 8.0] containing 50 mM NaCl). Elution was monitored as described Purification of the 30-and 120-kDa mouse serum. The fractions containing the 70-kDa gp were pooled, applied to DEAE-Toyopearl 650 M chromatography (column size: 1.0 × 24 cm, buffer: 50 mM Tris–HCl [pH 8.0] containing 50 mM NaCl), and eluted in a step-wise manner with 0.05, 0.1, 0.15, 0.3, and 1.0 M NaCl containing 50 mM Tris–HCl (pH 8.0). The 70-kDa gp was purified in the 1.0 M NaCl-eluted fraction.

SDS–PAGE and western blotting

Samples were dissolved in Laemmli buffer with 5% mercaptoethanol and boiled at 100°C for 3 min. The samples were electrophoresed in 7 or 10% polyacrylamide gels and visualized by CBB or silver staining. Glycoproteins were electrophorated onto PVDF membranes using a semidry blotting apparatus. The membrane was blocked with 10 mM sodium phosphate buffer (pH 7.2) and 0.15 M NaCl with 0.05% Tween-20 containing 1% bovine serum albumin at 25°C for 1 h. The membrane was incubated with a primary antibody, mAb.2-4B (0.50 μg/mL) or mAb.S2-566 (1.0 μg/mL) at 4°C for 16 h. The secondary antibody was peroxidase-conjugated anti-mouse IgM (1/5000 diluted). Color development was performed as described earlier (Sato et al., 2000).

Two-dimensional gel electrophoresis

Two-dimensional PAGE was performed with a PROTEAN IEF Cell (Bio-Rad) using 7-cm pH 3–10 ReadyStrip IPG Strip (Bio-Rad). Protein samples were resuspended in rehydration buffer (9.8 M urea, 4% CHAPS, and 100 mM dithiothreitol [DTT]) at a ratio of 1 : 3. Isoelectric focusing was performed at 250 V for 15 min, 250–4000 V linear ramp for 2 h, and 4000 V for 5 h at 20°C. After the gel strips were rehydrated with 10 min in 6 M urea, 2% SDS, 0.375 M Tris–HCl (pH 8.8), 20% glycerol, and 130 mM DTT, and then re-equilibrated for 10 min in the same buffer containing 135 mM iodoacetamide in place of DTT. The proteins were separated by SDS–PAGE.
**In-gel digestion and MALDI-TOF MS**

In-gel trypsin digestion of CBB-stained bands and MALDI-TOF MS analyses were performed as described (Yasukawa et al., 2005).

**Affinity purification of IgG**

The 30-kDa gp-enriched fraction was applied to the Protein G-Sepharose column. The column was washed with 10 mM Tris–HCl (pH 8.0) containing 0.15 M NaCl and eluted with 50 mM diethylamine–HCl (pH 11.5). The eluted solution was quickly neutralized with 1 M Tris–HCl (pH 8.0).

**Affinity purification of plasminogen**

L-lysine monohydrochloride (15 mg; Wako Pure Chemical) was coupled with 5 mL of CNBr-activated Sepharose 4B, according to the manufacturer's instructions. Normal mouse sera (6.2 mL) were applied to the non-coupled Sepharose 4B (Amersham Biosciences, Piscataway, NJ), which was equilibrated with 100 mM phosphate buffer (PB; pH 7.4). The pass-through fraction was re-applied to a lysine-coupled Sepharose column, which was equilibrated with 100 mM PB (pH 7.4). The column was washed with 100 mM PB (pH 7.4), 300 mM PB (pH 7.4), and eluted with 100 mM PB (pH 7.4) containing 0.1 M 6-aminohexanoic acid (Sigma).

**Affinity purification of vitronectin**

Vitronectin was purified from mouse sera, according to the previously described method (Yatohgo et al., 1988; Kitagaki-Ogawa et al., 1990). In brief, serum was passed through a heparin-Sepharose column and supplemented with a final concentration of 8 M urea. The serum was charged again on a heparin-Sepharose affinity column in the presence of 8 M urea. Vitronectin was specifically bound to the column and eluted with 0.5 M NaCl in the presence of 8 M urea.

**Fluorescent C7/C9 analysis and mild acid hydrolysis–DMB derivatization followed by anion-exchange chromatography**

Fluorescent C7/C9 analyses were performed as described earlier (Sato, Inoue, et al., 1998). Mild acid hydrolysis–DMB derivatization followed by anion-exchange chromatography was conducted as described previously (Sato et al., 1999).

**Hepatectomy of rat and purification of vitronectin from serum**

Vitronectins from non-operated, sham-operated, and partially hepatectomized rat plasma were purified as described previously (Uchibori-Iwaki et al., 2000).

**Acknowledgements**

We thank Dr. Tsukasa Matsuda (Nagoya University) for his valuable discussion and providing us monoclonal antibodies. This research was supported in part by Grants-in-Aid for Scientific Research (C) (15570096) (to K.K.), CREST of Japan Science and Technology Corporation (to K.K.), the 21st Century COE Program (to K.K.), Young Scientists (B) (18770083) (to C.S.), Priority Areas and the Japan Society (17046006) (to C.S.) for the Promotion of Science Research Fellows (16005841) (to Z.Y.) from the Ministry of Education, Science, Sports and Culture, and Mizutani Foundation (to C.S.).

**Conflict of interest statement**

None declared.

**Abbreviations**

BSA, bovine serum albumin; diSia, disialic acid; DMB, 1,2-diamino-4,5-methylenedioxybenzene; DTT, dithiothreitol; Neu5Ac, N-acetylneuraminic acid; Neu5Gc, N-glycolylneuraminic acid; PB, phosphate buffer; PVDF, polyvinylidene difluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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