Identification of various types of α2-HS glycoprotein in sera of patients with pancreatic cancer: Possible implication in resistance to protease treatment

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Abstract. α2-Heremans-Schmid glycoprotein (human fetuin) is one of numerous serum proteins produced in the liver. Recently, the biological functions of fetuin, such as calcification and insulin resistance, have been clarified. However, these effects appear to be indirect, occurring through binding to other molecules. When equal amounts of fetuin in sera were treated with chymotrypsin, resistance to the protease treatment was observed in patients with pancreatic cancer, but not in normal volunteers. To investigate the molecular mechanism behind this resistance, gel-filtration chromatography was performed. The results revealed that high molecular types of fetuin showed a resistance to protease treatment. When fetuin was purified from sera of patients with pancreatic cancer and normal volunteers, certain types of proteins, including haptoglobin (which binds to fetuin derived from pancreatic cancer patients), were identified using mass spectrometry. Furthermore, the oligosaccharide structures of fetuin analyzed with lectin microarray differed between pancreatic cancer patients and normal volunteers. This macro/micro heterogeneity of fetuin might contribute to pancreatic cancer resistance to chymotrypsin treatment.

Introduction

α2-Heremans-Schmid glycoprotein (AHSG, human fetuin) is one of the plasma proteins that occur in high concentrations during fetal life and gradually decrease towards adulthood. However, fetuin remains abundant in adult serum, and is mostly produced in the liver (1,2). Previous studies have demonstrated that fetuin isolated from Cohn's fraction VI of human plasma is a two-chain molecule consisting of an A-chain of 282 amino acid residues and a B-chain of 27 residues (3,4). It is thought that the circulating form of fetuin in serum is a two-chain protein with a heavy chain of 321 residues, which indicates the A chain with the connecting peptide, and a light chain of 27 residues, which indicates the B chain (5). Jahnen Dechent W *et al* reported that chymotrypsin cleaved the critical Leu-Leu bond flanking the NH_2-terminal portion of the connecting peptide region, and that this proteolysis was accelerated in sera of patients with sepsis (6).

Fetuin is a glycoprotein with both N- and O-linked carbohydrate side chains whose structures vary in several biological aspects (7,8). Fetuin occurs in large amounts in blood and cerebrospinal fluid, accumulates to high concentrations in calcified bone, and is involved in the regulation of mineral concentration (9). Since fetuin belongs to one of the negative acute-phase proteins, levels of fetuin in serum are decreased in individuals with infections, malignancies, malnutrition and liver and renal diseases at the end stage (10-13). Its high affinity for bone mineral and ability to prevent the precipitation of basic calcium phosphates from supersaturated solutions suggest that fetuin is a potent systemic inhibitor of soft tissue calcification (14-17). As there is a similarity in anion acid structures between fetuin and TGF-β receptor, fetuin acts as an antagonist of TGF-β. By contrast, it was reported that fetuin acted as a natural inhibitor of the insulin receptor tyrosine kinase in liver and skeletal muscle (18-22), and as an inhibitor for TNF-α during inflammation (23). Recent studies demonstrated that human plasma fetuin-A levels were correlated with fatty liver, impaired glucose tolerance and insulin resistance (24,25). In order to study a variety of biological functions of fetuin in detail, fetuin-deficient mice were developed. Unexpectedly, these mice showed normal growth. When fed a high-fat diet, they were resistant to weight gain, demonstrated significantly decreased body fat and remained insulin sensitive (26). However, several studies have examined the role of fetuin as a calcification inhibitor, since

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Abbreviations: α2-Heremans-Schmid glycoprotein, human fetuin

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the mice developed microcalcifications in soft tissue. Fetuin is highly effective in the formation and stabilization of protein-mineral colloids, referred to as calciprotein particles (CPPs). CPPs may act as mineral chaperones mediating the stabilization, safe transport and clearance of calcium and phosphate as colloidal complexes in the body, thus preventing ectopic calcification (24,27). All these reports on fetuin suggest that fetuin may react biologically with other molecules. Therefore, it would be useful to know the heterogenic conditions of fetuin in serum, such as complex formation and binding with other proteins. In the present study, we found that the limited proteolysis of serum fetuin differed between patients with pancreatic cancer and normal volunteers. The mechanisms for the difference were investigated in terms of differences in the heterogeneity of fetuin in serum, complex formation with other molecules, and oligosaccharide structures.

Materials and methods

Western blot analysis of fetuin. A rabbit polyclonal antibody for fetuin was established as follows: briefly, a rabbit was immunized with commercial fetuin every 2 weeks. After 3 months, the total serum of the rabbit was collected. Availability of this antibody for Western blot analysis was verified using sera or conditioned medium from a hepatoma cell line. To purify the fetuin-specific antibody, the rabbit serum was applied to an affinity column that was coupled with 3 μg of fetuin after the depletion of immunoglobulin G (IgG) (Cell Signaling Technology) using the Protein G column (GE Healthcare, Amersham, UK). An 180 ng aliquot of fetuin was electrophoresed on a 10% SDS polyacrylamide gel and transferred onto a nitrocellulose membrane (Whatman, Germany). The membranes were incubated with 5% skim milk in phosphate buffered saline (PBS) for 1 h and then incubated with 1/5,000 diluted anti-human fetuin antibody, which was established by immunizing rabbits with purified human fetuin (Sigma, St. Louis, MO, USA) overnight. After washing three times with Tris-buffered saline containing 0.05% Tween 20 (TBST) (pH 7.4) for 10 min each, the membranes were incubated with peroxidase conjugated anti rabbit IgG for 40 min. After washing the membranes three times with TBS-T for 10 min each, development was performed using an Immobilon™ Western Chemiluminescent HRP Substrate (Millipore Corp., Billerica, MA, USA) according to the standard protocol.

Column chromatography. Serum (0.8 ml) was diluted with 0.1 M Tris-HCl pH 7.4 containing 7-aminoheptanoic acid at 2.4 times and was applied to a Superdex 200 pg (1.6x60 cm) column at 4˚C using FPLC systems (GE Healthcare). The elution was performed with 0.1 M Tris-HCl pH 7.4 at 0.5 ml/min conditions, and 1.0 ml of the elution was collected in each tube. The protein concentration was detected under UV light at 280 nm. The aliquot of 66-75 fractions (Superdex 200 pg chromatography) was applied to a MonoQ column (HR 5/5: equilibrated 0.1 M Tris-HCl pH 7.4) at 25˚C, then the column was washed with the buffer for 5 min at a flow rate of 1.0 ml/min and eluted for 40 min with a linear gradient of A buffer (0.1 M Tris-HCl pH 7.4) and B buffer (0.1 M Tris-HCl pH 7.4 with 1 M NaCl). The elution (1 ml) was collected in each tube and the protein concentration was detected as above.

Enzyme linked immunosorbent assay of fetuin. Fetuin levels were determined by a competitive enzyme linked immunosorbent assay (ELISA) system, which was established using polyclonal anti-human fetuin antibodies as described above. Briefly, anti human fetuin polyclonal antibody was coated as a solid phase (96-well plate). Diluted serum (1/200) and peroxidase-labeled fetuin were applied to each well and then incubated for 30 min at room temperature. After washing three times in washing buffer (0.03% Triton X-405, 0.05% Tween 80, 0.9% NaCl, 0.005 M sodium phosphate, pH 7), TMB (DAKO, Tokyo, Japan) was added to each well. After incubation for 30 min, the reaction was stopped by the addition of 5% H_2SO_4. Fetuin captured with coated antibodies in each well was recognized by the specific fetuin antibody on a solid phase, followed by the detection of the reaction signal using TMB peroxidase substrate. The signal was measured in a spectrophotometric microplate reader (Bio-Rad, Tokyo, Japan).

Limited proteolysis of fetuin by chymotrypsin and tyrpsin. Proteolysis of fetuin was carried out in PBS pH 7.2, and sera adjusted to fetuin (180 ng concentration) were incubated with various concentrations of bovine pancreatic chymotrypsin (Calbiochem) for 30 min at 37˚C. After 30 min of incubation at 37˚C, the reaction was stopped by the addition of 5 μl SDS sample buffer and boiled for 5 min at 100˚C. The samples were electrophoresed on a 10% SDS polyacrylamide gel, and then Western blot analysis was performed.

Purification of fetuin from sera of patients with pancreatic cancer and normal volunteers. To purify fetuin, 100 μl of sera was applied to an anti-fetuin affinity column coupled with 0.5 mg of anti-human fetuin antibody according to the standard protocols of HiTrap NHS-activated HP (Amersham Bioscience). This antibody was established as previously described. Fetuin bound to the antibody column was eluted with 5 ml of elution buffer (100 mM glycine, 0.5 M NaCl pH 3.0) and then neutralized with Tris.

Mass spectrometry analysis of fetuin-binding proteins. Purified fetuin fractions in the affinity column were evaporated until dry, and 200 μl of a reducing solution containing 250 mM Tris-HCl pH 8.5, 6 M guanidine hydrochloride, 2 mM EDTA and 4 mg dithiothreitol were added to the residue. The mixture was incubated at 50˚C for 1 h to reduce Cys residues. Iodoacetamide (10 mg) was added to the mixture and the reaction was allowed to proceed for 30 min at room temperature in the dark. Water (300 μl) was added to the reaction solution and the mixture was passed through a Nap-5 column (GE Healthcare) equilibrated with water to remove salts from the reducing solution and excess iodoacetamide. The elute containing S-carbamidomethylated fetuin (1 ml, in water) was evaporated until dry. An enzyme mixture of lysylendopeptidase (40 ng) and trypsin (40 ng) in 50 mM ammonium bicarbonate (100 μl) was added to the dried residue and incubated for 16 h at 37˚C. After boiling, the solution was evaporated until dry. The residue was dissolved in 20 μl of water. A 10-μl aliquot of the tryptic peptides mixture was separated by RPLC (a Cadenza CD-C18 column, 150x2.0 mm i.d.; Imtakt, Kyoto, Japan) under the following gradient conditions: mobile phases of i) 0.1% formic acid and ii) 0.1% formic acid/80% acetonitrile. The gradient elution was performed from

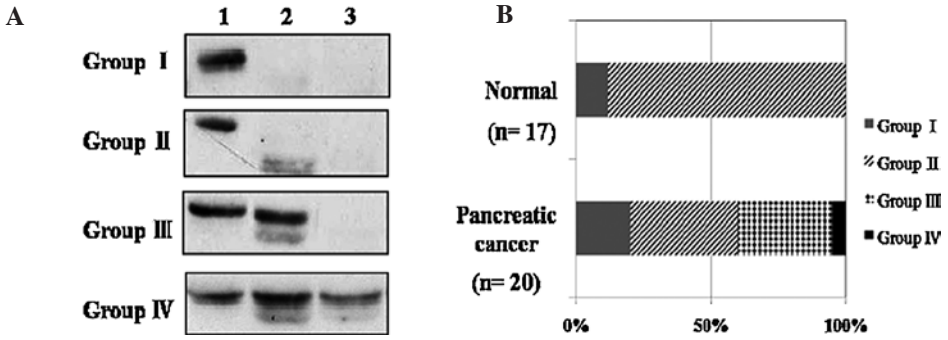


Figure 1. Proteolysis pattern of fetuin by treatment with chymotrypsin. (A) Proteolysis of fetuin was categorized into four groups (Groups I-IV). Sera adjusted by fetuin levels (180 ng) were incubated with various concentrations of bovine pancreatic chymotrypsin for 30 min at 37˚C. The chymotrypsin concentration in each lane was as follows: lane 1, 0 μg/μl; lane 2, 0.2 μg/μl; lane 3, 0.5 μg/μl. The samples were electrophoresed on a 10% SDS polyacrylamide gel, and then Western blot analysis was performed. (B) The proteolysis activity of fetuin differed between the sera of normal volunteers and pancreatic cancer patients. While normal volunteers belonged only to Groups I and II, 50% of patients with pancreatic cancer belonged to Groups III and IV, suggesting that certain cases of fetuin derived from sera of patients with pancreatic cancer were resistant to chymotrypsin treatment.

5-50% (i) over 30 min, and then from 50-100% (ii) over 10 min with a flow rate of 0.2 ml/min. The eluates were monitored at 280 nm, and continuously introduced into an electrospray ionization (ESI) source (Esquire HCT, Bruker Daltonics Inc,). The identification of the protein was performed against the NCBInr database with the MASCOT (Matrix Science Ltd.) database-searching algorithm.

Lectin microarray analysis. Lectin microarray analysis was performed as described previously (28). Briefly, ~10 ng of fetuin purified from sera of patients with pancreatic cancer and normal volunteers and conditioned medium from the human hepatoma cell line Hep3B were applied to the lectin microarray. This array is capable of comparative binding to 43 types of lectins (28). Levels of lectin binding were described as the relative intensity adjusted to the total fluorescence.

Results

Proteolysis pattern of fetuin after treatment with chymotrypsin. To investigate the limited proteolysis of fetuin in sera of patients with pancreatic cancer and normal volunteers, the serum samples were incubated with various concentrations of chymotrypsin (Fig. 1A). Certain samples were rapidly cleaved by chymotrypsin (Groups I and II). The proteolysis pattern of fetuin is categorized into four groups. While the majority of normal volunteers belonged to Groups I and II, 50% of patients with pancreatic cancer belonged to Groups III and IV, suggesting that certain patients with pancreatic cancer were resistant to chymotrypsin treatment (Fig. 1B).

Column chromatography analysis of fetuin in sera. Fetuin bands in the Western blot analysis were detected at 53 and 58 kDa, which are known as major fractions of fetuin A with and without the connecting peptide, respectively. To evaluate the dynamics of fetuin in sera, gel filtration column chromatography analysis of fetuin was performed using the serum from a patient with pancreatic cancer. Sera (0.8 ml) were applied to a Superdex (200 pg) column at 25˚C using FPLC systems. When using the sera of another pancreatic cancer patient or of normal volunteers, the fractionation pattern of fetuin was almost the same. The elution (1 ml) was collected in the tubes, and the concentration of fetuin in the fractions was determined by a competitive ELISA (Fig. 2A). The fractions of No. 65-76 peak were identified with a major band of fetuin. In contrast, except for this peak, quite low levels of fetuin were detected in a high molecular weight fraction, near No. 47-56. Western blot analysis using anti-fetuin antibody revealed that the expression levels of fetuin in each fraction of No. 54-75 were compatible with the fetuin concentration determined by a competitive ELISA (Fig. 2B).

Molecular mass of fetuin in high molecular fractions differed from that in low molecular fractions. As described previously, extremely low levels of fetuin were detected in a high molecular weight fraction. To investigate the difference between fetuin with a high molecular weight fraction and a major fraction of low molecular weight, Western blot analysis was performed under reducing conditions (Fig. 3). Notably, the molecular mass of fetuin in these two fractions was different under reducing conditions. To examine whether or not the difference in this molecular weight is dependent on glycosylation, each fraction was treated with N-endglycosidase F (PNGase). While the bands of fetuin were suppressed with PNGase treatment, there were no changes in the molecular mass of fetuin between high and low molecular fractions, suggesting that the molecular difference between the two types of fetuin was not dependent on glycosylation (data not shown).

Proteolytic pattern of the eluted fraction after treatment with chymotrypsin and trypsin. To investigate the limited proteolysis of fetuin in the high molecular weight fraction, both fractions were treated with various concentrations of chymotrypsin or trypsin. Fetuin in the low molecular fraction was cleaved by both chymotrypsin and trypsin. By contrast, the high molecular mass fetuin was resistant to chymotrypsin and trypsin treatment (Fig. 4).

Identification of fetuin-associated proteins. To examine whether or not fetuin specifically binds to other molecules in sera of patients with pancreatic cancer, we purified fetuin from sera of normal volunteers and patients with pancreatic cancer using an anti-fetuin antibody affinity column. Eluted proteins were electrophoresed on a 10% SDS polyacrylamide

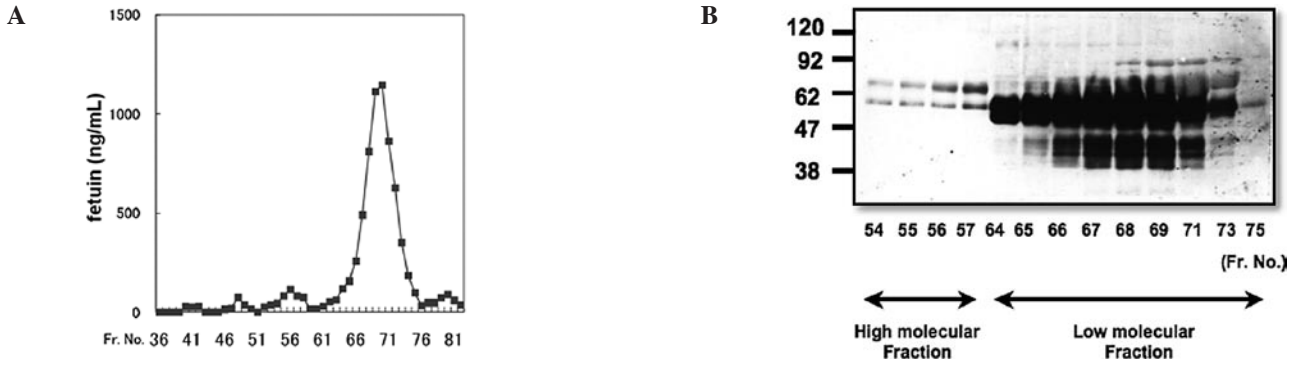


Figure 2. Column chromatography analysis of fetuin in sera. (A) Column chromatography analysis of fetuin in serum of a patient with pancreatic cancer. Sera (0.8 ml) was applied to a Superdex (200 pg) column at 25˚C using FPLC systems. The elution (1 ml) was collected in the tubes. The fetuin concentration in the fractions were determined by ELISA. (B) Fraction samples (10 μl) of Nos. 54-75 were electrophoresed on a 10% polyacrylamide gel under reducing conditions.

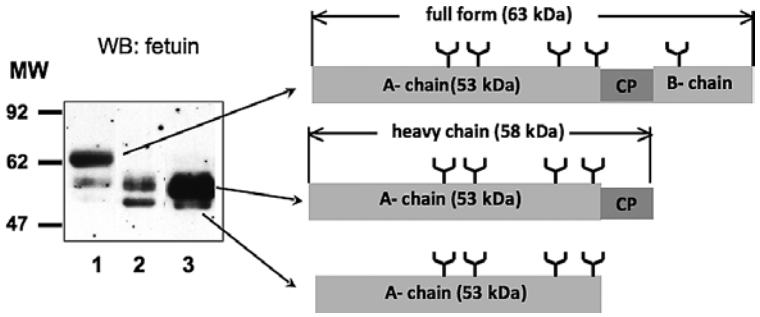


Figure 3. The molecular mass of fetuin in the high molecular fraction eluted on a Superdex column differed from fetuin in the low molecular fraction. The eluted fractions of the Superdex column (10 μl) were electrophoresed on a 10% SDS polyacrylamide gel under reducing conditions. Lane 1, high molecular fraction; lane 2, low molecular fraction; lane 3, total serum fetuin. High molecular fetuin was expected as a complete form of fetuin, which consists of an A-chain, a connecting peptide (CP) and a B-chain. The molecular mass of fetuin differed slightly between the high and low molecular fractions.

gel, confirmed by Western blot analysis (Fig. 5A), and visualized using silver staining (Fig. 5B). Co-purified molecules with fetuin were identified using mass spectro-metry. Major proteins identified by mass spectrometry are listed in Fig. 5C.

Lectin microarray. Since changes in oligosaccharide structures are linked to the susceptibility to certain cases of proteolysis, we analyzed the oligosaccharide structures on fetuin with a lectin microarray. While this system is limited to quantitation of the details of oligosaccharide structures, the array is capable of evaluating native oligosaccharides on glycoproteins. Increases in fucosylation and decreases in the sialylation of fetuin derived from sera of patients with pancreatic cancer were observed as expected (Fig. 6). The oligosaccharides of fetuin derived from sera of patients with pancreatic cancer showed a similar pattern to those in hepatoma cells.

Discussion

Although fetuin is an abundant protein in serum, its biological functions remain controversial despite the establishment of fetuin-deficient mice. Recently, fetuin levels were found to be increased in patients with non-alcoholic fatty liver disease (29), as well as in patients with arteriosclerotic calcifications (30). These investigations were performed due to previous reports that fetuin inhibited the reaction of insulin and TGF-β. However, fetuin levels were changed less than 2-fold in these diseases. Fetuin is well known as a negative acute reactant, while its levels are up- or down-regulated in chronic diseases, including cancer and metabolic syndrome.

The data suggest that the biological reaction of fetuin is dependent on the regulation of other molecules. Therefore, the question of how fetuin exists in serum is critical. Nawaratil *et al* reported limited proteolysis of fetuin by chymotrypsin treatment (6). This prompted us to perform the experiment described in Fig. 1. Notably, in 50% of the patients with pancreatic cancer, fetuin was resistant to chymotrypsin treatment. Other serum proteins, such as antitrypsin, haptoglobin and transferrin, showed no changes in proteolysis by chymotrypsin between pancreatic cancer patients and normal volunteers (data not shown), suggesting that the heterogeneity of fetuin but not the existence of protease inhibitors is a key factor in the resistance to chymotrypsin treatment.

These heterogeneities include glycosylation and complex formation. Gel filtration chromatography of sera from a patient with pancreatic cancer followed by ELISA showed small amounts of fetuin at a high molecular weight (Figs. 2 and 3). The data suggest that fetuin forms homo/hetero

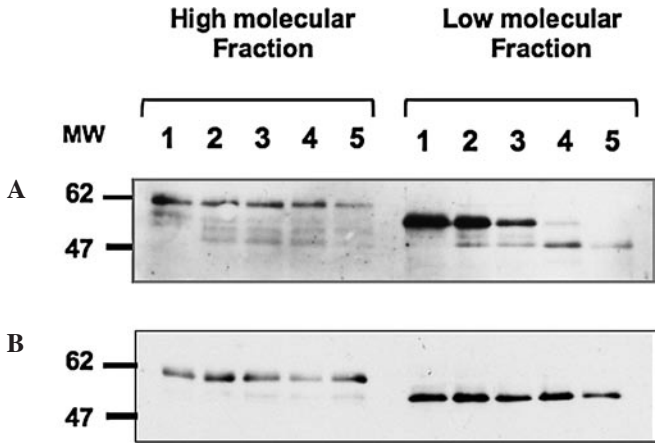
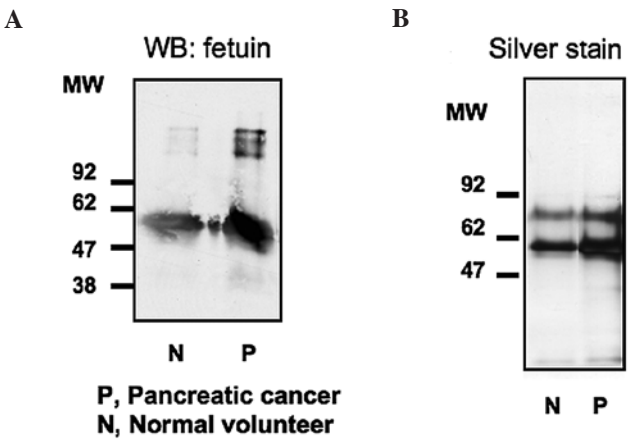


Figure 4. Fetuin in the high molecular fraction was resistant to treatment with chymotrypsin and trypsin. High and low molecular fractions eluted on a Superdex column were incubated with various concentrations of bovine pancreatic chymotrypsin for 30 min at 37˚C. (A) Chymotrypsin and (B) trypsin concentrations were each as follows: lane 1, 0 μg/μl; lane 2, 0.05 μg/μl, lane 3, 0.1 μg/μl; lane 4, 0.2 μg/μl, and lane 5, 0.5 μg/μl. Each sample was electrophoresed on a 10% SDS polyacrylamide gel, and then Western blot analysis was performed.



C

Normal & Pancreatic cancer	Pancreatic cancer
Centrosomal Protein 2	Ankyrin repeat domain protein21
Testis-specific Y-encoded-like protein 3	Protein C21 or rf
Ig Kappa chain C region	Haptoglobin
Zinc Finger Protein	Ig alpha-1 chain C region

Figure 5. Identification of fetuin-associated proteins. (A) Fetuin was purified from sera of patients with pancreatic cancer and normal volunteers using an anti-fetuin antibody affinity column, and confirmed by Western blot analysis. (B) Eluted proteins were visualized by silver stain. (C) Fetuin-associated proteins were identified by mass spectrometry. The results are described as common proteins for normal volunteers and pancreatic cancer patients, and as specific proteins for pancreatic cancer patients.

multivalent complexes. While it was unclear whether or not the complex formation of fetuin is involved in the resistance to chymotrypsin treatment, the molecular weight of fetuin differed between the complex and free forms of fetuin at 58 kDa in a Western blot analysis under reducing conditions. Fetuin at 65 kDa is a full form with an A chain, a connecting peptide and a B chain. While this form of fetuin was present at quite low levels, it was detected with mass spectrometry in normal sera (personal communication with Dr Yoshinao Wada, Osaka Medical Center and Research Institute for Maternal and Child Health, Japan).

Although the total levels of fetuin were decreased in patients with pancreatic cancer, a slight increase in the level of complex types of fetuin was observed (data not shown). However, this mass of fetuin was a minor fraction, possibly less than 1%. Haptoglobin was identified as a fetuin-binding protein using mass spectrometry analysis. This binding was also confirmed by co-purification experiments (data not shown). The binding capacity of fetuin with other serum proteins may reflect a resistance to various types of proteases. Notably, fetuin and haptoglobin are target proteins for fucosylation in cancer, since the level of fucosylated haptoglobin was increased in patients with pancreatic cancer (31) and fetuin was identified as a fucosylated protein in patients with hepatocellular carcinoma (32). A common lectin for fucosylated proteins might exist, which binds to fucosylated haptoglobin

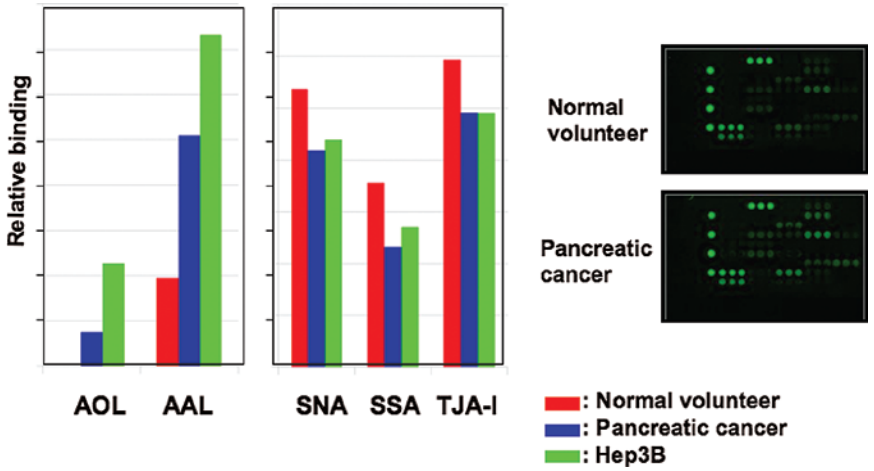


Figure 6. Lectin microarray analysis on fetuin derived from sera of patients with pancreatic cancer and from normal controls. Fetuin purified from sera of patients with pancreatic cancer (Group IV in Fig. 1) and normal volunteers and the conditioned medium of a human hepatoma cell line Hep3B were subjected to lectin microarray. AOL and AAL lectins recognized α1-3/α1-4/α1-6 fucosylation. SSA, SNA and TJA-1 lectins recognized α2-6 sialylation. Fetuin derived from Hep3B served as a positive control for fucosylated fetuin.

and fetuin. Fucosylation itself might regulate proteolysis with chymotrypsin treatment.

While fetuin was found to have varying heterogeneity in serum, we did not find direct evidence for the variation in the resistance to protease digestion by fetuin molecules. Further studies are required to determine the relationship between the heterogeneity of fetuin and various diseases as well for the functional analysis of various types of fetuins.

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