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Measurement of the Fucosylated Sugar Chains of Serum α_1-Antitrypsin: The Relationship between Reactivity with *Lens Culinaris* Agglutinin and HPLC Analysis of the Pyridylaminated Sugar Chains

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Summary. **Twelve samples of α_1-antitrypsin (AAT) were purified from six patients with hepatocellular carcinoma, one with pancreatic cancer and five healthy individuals, and subjected to the measurement of the fucosylated sugar chain. We compared different results from crossed immunoaffinoelectrophoresis (CIAE) with *Lens culinaris* agglutinin (LCA) and by the carbohydrate analysis of high-performance liquid chromatography (HPLC) with the pylidylaminated (PA-) oligosaccharides obtained from each AAT. A significantly good correlation was obtained between the percentage of PA-fucosylated biantennary sugar chains in whole PA-oligosaccharides of each AAT sample by HPLC analysis and the percentage of LCA-reactive species of AAT examined by CIAE. Thus, the percentages of LCA-reactive species of AAT examined by CIAE can be used as a substitute for the amount of fucosylated sugar chain of AAT.**

Key words—α_1-antitrypsin, *Lens culinaris* agglutinin, fucosylated sugar chain, crossed immuno-affinoelectrophoresis.

INTRODUCTION

We have recently shown a significant increase in the fucosylated biantennary sugar chain in α_1-antitrypsin (AAT) from patients with hepatocellular carcinoma (HCC) as compared with that from normal individuals.[1] However, the measurement of a fucosylated sugar chain by pyridylamination with high-performance liquid chromatography (HPLC) is both complex and in appropriate for clinical routine work. On the other hand, *Lens culinaris* agglutinin (LCA) specifically binds the biantennary sugar chain with a fucose residue at the innermost *N*-acetylglucosamine residue of the trimannosyl core.[2] Thus, crossed immuno-affinoelectrophoresis (CIAE) provides useful information to investigate the fucosylated carbohydrate structure of glycoproteins in the presence of LCA. The quantitative relationship, however, between the results by CIAE and HPLC analysis using PA-oligosaccharide chain of AAT remains to be solved.[2,3] The present study aims to show whether the percentages of LCA-reactive species of AAT examined by CIAE can be used as a substitute for the amount of fucosylated sugar chain of AAT.

MATERIALS AND METHODS

Materials

Neuraminidase from *Clostridium perfringens* (type X) and LCA were purchased from Sigma Chemical Company, St. Louis, MO, USA. Authentic pyridylaminated (PA)-oligosaccharide standards were from Takara Shuzo Co., Ltd. Kyoto, Japan. Anhydrous hydrazine was from Pierce Chemical Company, Rockford, Ill, USA and 2-aminopyridine was from Nacalai Tesque, Kyoto, Japan. Sodium cyanoborohydride was from Aldrich Chem. Co., Milw., USA. The other reagents were of analytical grade.

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Purification of AAT samples

Purified 12 AAT samples were prepared from the sera of six patients with HCC, one patient with pancreatic cancer and five healthy individuals, by affinity chromatography with anti-AAT antibody-coupled Sepharose 4B column as described previously[1]. Each sample was further purified by HPLC on a column of TSK-GEL G3000SW (0.75×60 cm, Tosoh Corp. Tokyo, Japan), equilibrated with a 10 mM phosphate buffer of pH 7.0 containing 5M guanidine hydrochloride. The purity of the AAT preparations was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the methods by Laemmli.[4]

Crossed immunoaffinoelectrophoresis

Affinoelectrophoresis of AAT was performed with 1% agarose in a 0.02 M barbital buffer of pH 8.6, containing 0.2 mg/ml of soluble LCA as described previously.[5] Briefly, serum samples were diluted with 0.02 M barbital buffer of pH 8.6 to a final concentration of Tf of 20 mg/ml. Five μl of a sample was run at 10 V/cm for 2.5 h in the first dimension with cooling. Second electrophoresis was then performed at 2 V/cm for 17 h in the gel containing anti-human serum AAT. The area under the peak of immunoprecipitation after the second dimension run was quantified as described previously.[6]

Preparation of PA-sugar chains

Sugar chains of AAT were released by hydrazinolysis at 100°C for 10 h and free amino groups were *N*-acetylated. Then the free oligosaccharides were reductively aminated with a fluorescent reagent, 2-aminopyridine, by use of sodium cyanoborohydride, and PA-derivatives of each oligosaccharide preparation were fractionated by Sephadex G-15 gel-filtration (1×50 cm) according to the method described previously.[7,8]

Neuraminidase digestion of PA-sugar chains

Digestion with neuraminidase from *Clostridium perfringens*(type X) was performed in 0.1 M sodium acetate buffer of pH 5.0 at 37°C for 20 h, and reaction was stopped by heating the solution at 100°C for 2 min.[7]

HPLC

The separation of PA-oligosaccharides was carried out by HPLC using a Hitachi 655A chromatograph equipped with a Rheodyne Model 7125 injector and a Hitachi Model F-1050 fluorescence spectrophotometer on a reversed-phase column (PALPAK type R-MB, 0.21×15 cm, Takara Shuzo Co., Ltd. Kyoto, Japan). Elution was performed at a flow rate of 0.5 ml/min at 40°C using two solvents, A and B. Solvent A was a 0.1 M acetic acid triethylamine buffer of pH 4.0 and solvent B was solvent A with 0.5% 1-butanol. The column was equilibrated with a mixture of solvent A (95%) and B (5%). After injection of a sample, the ratio of solvent B to A was increased linearly to 55% solvent B in 80 min. Pyridylamino (PA)-oligosaccharides were detected by fluorescence using excitation and emission wavelengths of 320 and 400 nm, respectively.[7,9]

Statistical analysis

The correlation between percentages of LCA-reactive species and fucosylated biantennary sugar chain

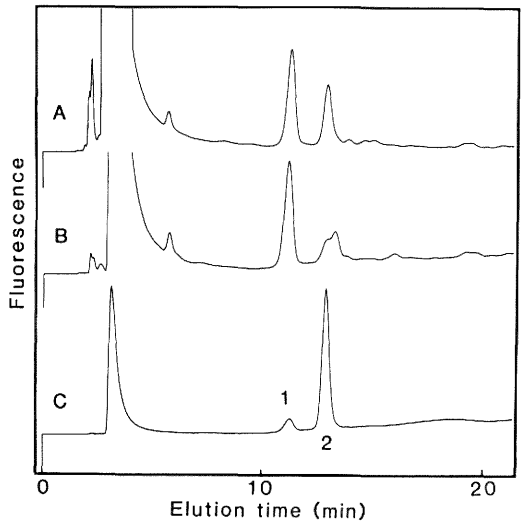

Fig. 1. Reversed-phase HPLC elution profiles of a PA-oligosaccharide of AAT from a patient with HCC (**A**) and a healthy individual (**B**). An increment of a fucosylated biantennary suger chain, Gal β1-4GlcNAc β1-2Man α1-6 (Gal β1-4GlcNAc β1-2Man α1-3) Man β1-4GlcNAc β1-4 (Fuc α1-6) GlcNAc-PA can be observed in HCC. However, no increase of a fucosylated biantennary sugar chain was revealed in the AAT from the normal individual shown in **B**. Authentic PA-oligosaccharide standards in **C** are: *1*, biantennary sugar chain, Gal β1-4GlcNAc β1-2Man α1-6 (Gal β1-4GlcNAc β1-2Man α1-3) Man β1-4GlcNAc β1-4GlcNAc-PA; *2*, fucosylated biantennary sugar chain, Gal β1-4GlcNAc β1-2Man α1-6 (Gal β1-4GlcNAc β1-2Man α1-3) Man β1-4GlcNAc β1-4 (Fuc α1-6) GlcNAc-PA.

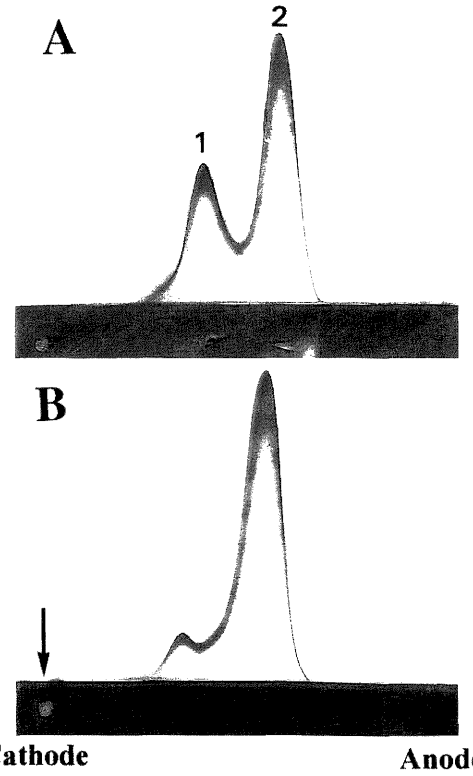

Fig. 2. CIAE patterns of AAT from a patient with HCC (**A**) and healthy individuals (**B**). Two species of AAT were detected on CIAE in a gel containing LCA. Migration of one of the two AAT species was retarded (LCA-reactive species, *1* in **A**) and that of the other remained unchanged (LCA-nonreactive species, *2* in **A**). An increased percentage of LCA-reactive species in total AAT in the patient with HCC is depicted in comparison with healthy individuals.

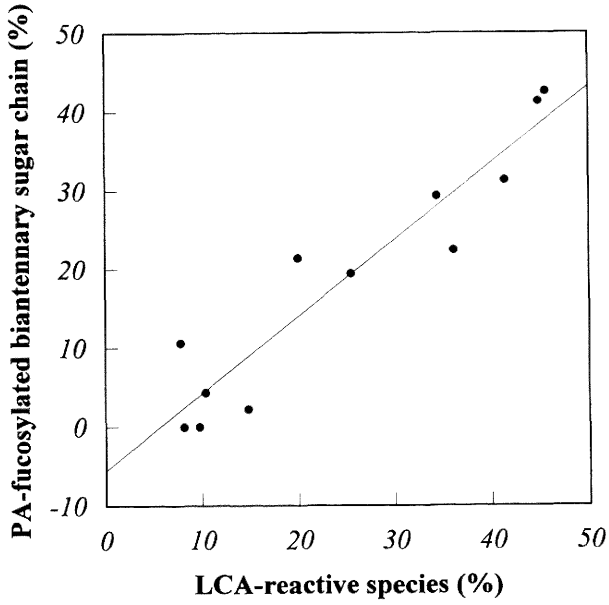

Fig. 3. Correlation between the percentages of LCA-reactive species of AAT examined by CIAE and of PA-fucosylated biantennary sugar chain obtained from HPLC analysis. There is a highly significant correlation ($r=0.9442$, $p<0.001$)between them. The formula of the regression line for calculating the fucosylated glycan of [percentages of the fucosylated biantennary sugar chain by HPLC] $=0.974\times$ [percentages of LCA-reactive species by CIAE]-5.503 was obtained by the least-squares regression analysis.

was tested by the least-squares regression analysis. The percentage of PA-fucosylated biantennary sugar chains in whole PA-oligosaccharides of each AAT sample was calculated from the peak areas of each PA-oligosaccharide on HPLC.

RESULTS

Fig. 1A shows an elution profile of the PA-oligosaccharides of AAT from a patient with HCC, representing an increment of a fucosylated biantennary sugar chain, Gal β1-4GlcNAc β1-2Man α1-6(Gal β1-4GlcNAc β1-2Man α1-3) Man β1-4GlcNAc β1-4(Fuc α1-6) GlcNAc-PA. The CIAE pattern of AAT from the same patient with HCC shown in Fig. 2A also indicated an increased percentage of LCA-reactive species in total AAT. On the other hand, no increment of a fucosylated biantennary sugar chain was revealed in AAT from the normal individual shown in Fig. 1B. No increase of LCA-reactive species was obtained by CIAE pattern of AAT from same nomal individual shown in Fig. 2B. Paired data from 12 samples indicate that there is a highly significant correlation ($R=0.9442$, $p<0.001$) between the percentage of LCA-reactive species of AAT by CIAE and the percentage of PA-fucosylated biantennary sugar chains by the measurement of PA-oligosaccharide with HPLC as shown in Fig. 3. The formula of the regression line for calculating the fucosylated glycan of [percentages of fucosylated biantennary sugar chain by HPLC] $=0.974\times$ [percentages of LCA-reactive species by CIAE]-5.503 was obtained by the least-squares regression analysis.

DISCUSSION

We have previously reported that the measurement of the LCA-reactive species of alpha-fetoprotein is

useful for the early detection of HCC, and that the fucosylation of the biantennary sugar chain at the innermost *N*-acetylglucosamine residue is the molecular basis for this variation of alpha-fetoprotein.[6,7,10-13] Alpha-fetoprotein has only one *N*-glycosylation site of 233rd asparagine from the aminoterminus.[14] Accordingly, the affinity for lectins reflects directly the structure of its sugar chain. However, the reactivity with LCA in AAT does not reflect the carbohydrate structures since an ATT molecule has 3 *N*-glycosylation sites.[15,16] Our previous results indicated that one or more, but not necessarily all three, carbohydrate chains of each LCA-reactive AAT carry fucose residues.[1] On the other hand, CIAE in the presence of LCA is the simpler method for a quantitative measurement of a fucosylated biantennary sugar chain although there is the possibility of overestimating the fucosylation of glycoproteins. The present study, however, clearly indicated that the percentage of LCA-reactive species of AAT examined by CIAE well correlates dose-dependently the percentage of PA-fucosylated biantennary sugar chains by HPLC analysis. The formula of representing the amount of fucosylated sugar chain in the AAT molecule by the data of CIAE was obtained. Thus, the measurement of LCA-reactive species in AAT can substitute for the content of fucosylated sugar chain, and so provide useful information for monitoring the fucosylated glycans.

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