Carbohydrate structures of human α-fetoprotein of patients with hepatocellular carcinoma: presence of fucosylated and non-fucosylated triantennary glycans

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Summary

Chemical structures of the sugar chains of various human α-fetoprotein (AFP) species with different affinity for Concanavalin A (Con A) and Lens culinaris agglutinin (LCA) were examined by pyridylation of their oligosaccharides and stepwise exoglycosidase digestion. Using reversed-phase and size-fractionation high performance liquid chromatography systems we identified six pyridylamino-sugar chains. The Con A-reactive and LCA-nonreactive species of AFP from patients with hepatocellular carcinoma contained a bi-antennary sugar chain, and the Con A-reactive and LCA-reactive species of AFP from patients with a biantennary one with a fucose residue at the innermost N-acetylglucosamine residue. The Con A-nonreactive and LCA-reactive species contained a bi-antennary sugar chain both with a bisecting-N-acetylgalcosamine residue at the trimannosyl core and with a fucose residue at the innermost N-acetylglucosamine residue. The Con A-nonreactive and LCA-nonreactive species contained a fucosylated triantennary sugar chain as a major component, and two minor components: a biantennary sugar chain and a biantennary sugar chain with a bisecting-N-acetylgalcosamine residue at the trimannosyl core. Thus, the fucosylated and non-fucosylated triantennary sugar chains were newly identified in human AFP. Essentially identical results were obtained for AFP from the patient with gallbladder carcinoma which metastasises to the liver. These results indicate that the increment in fucosylation and branching to form new antennae is a characteristic feature of the carbohydrate chains of AFP from patients with neoplastic diseases of the liver.

The measurement of the serum concentration of α-fetoprotein (AFP) has been used extensively for the early diagnosis of hepatocellular carcinoma (HCC) during a follow-up process of chronic liver diseases (Abelev, 1986; O'Conor et al., 1970; Nishi & Hirai, 1973). However, the serum concentration of AFP also increases in patients with carcinomas of digestive organs which metastasises to the liver (Alpert et al., 1971; Mcintire et al., 1975) and with nonneoplastic diseases of the liver (Karvountzis & Redeker, 1974; Alpert & Feller, 1978).

Several investigators and we have reported that the reactivity of AFP with Lens culinaris agglutinin (LCA) and Concanavalin A (Con A) is a good measure for distinction between HCC, nonneoplastic diseases of the liver and carcinomas of digestive organs which metastasise to the liver (Ruoslaiti et al., 1978; Breborowicz et al., 1981; Ishiguro et al., 1985; Taketa & Hirai, 1989; Aoyagi et al., 1984, 1991). The molecular basis for the different affinity of AFP for LCA and Con A is presumed to be the difference in fucosylation at the innermost N-acetylglucosamine residue and bisecting-glucosaminylation in the biantennary carbohydrate chain (Aoyagi et al., 1985). However, precise chemical structures of sugar chains in these molecular species of AFP have not been fully determined.

In this manuscript, we report the chemical structures of sugar chains of human AFP molecular species with special reference to lectins and the disease category.

Materials and methods

Materials

AFP specimens were prepared from serum samples of two patients with HCC and a patient with gallbladder carcinoma which metastasises to the liver by affinity chromatography as described previously (Aoyagi et al., 1977). Con A- and LCA-Sepharose 4B beads were obtained from Pharmacia Fine Chemicals, Upsalsa, Sweden. Lyophilised Con A and LCA, bovine epididymal α-L-fucosidase, Jack beans β-N-acetylgalcosaminidase, and neuraminidase from Clostridium perfringens, type X, were from Sigma Chemical Co., St. Louis, MO, USA. β-Galactosidase from E. coli was purchased from Boehringer Mannheim, Germany. Authentic pyridylamino (PA)-oligosaccharide standards were from Takara Shuzo Co., Ltd. Kyoto, Japan, and their structures were verified by 1H-nuclear magnetic resonance by the manufacturer. 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., WI, USA. The other reagents were of analytical grade.

Methods

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The purity of the AFP prepared by affinity chromatography was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method of Laemmli (1970).
Isolation of AFP species with different affinity for Con A and LCA

Four AFP species, i.e., Con A-nonreactive and LCA-nonreactive (Con A(-)/LCA(-)), Con A-nonreactive and LCA-reactive (Con A(-)/LCA(+)), Con A-reactive and LCA-nonreactive (Con A(+)/LCA(-)), Con A-reactive and LCA-reactive (Con A(+)/LCA(+)) species were obtained from each AFP sample with Con A- and LCA-Sepharose 4B as described previously (Aoyagi et al., 1985).

Crossed immunoaffinoelectrophoresis

Purity of each molecular species of AFP with different affinity for Con A and LCA was examined by crossed immunoaffinoelectrophoresis as described previously (Aoyagi et al., 1984).

Preparation of PA-sugar chains

Sugar chains of AFP samples 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-aminoypyridine, by use of sodium cyanoacrylate, and PA-derivatives of each oligosaccharide preparation were fractionated by Sephadex G-15 gel filtration (1 x 50 cm) according to the method described previously (Hase et al., 1984; Yamamoto et al., 1989).

Glycosidase digestion of PA-sugar chains

Digestion with neuraminidase was performed in 0.1 M sodium acetate buffer, pH 5.0; with α-fucosidase in 0.1 M sodium citrate buffer, pH 6.0; and with β-galactosidase and β-N-acetylgalactosaminidase in 0.01 M sodium phosphate buffer, pH 7.0, containing 1 mM MgCl₂. All digestions were done with 50–100 pmoles of each PA-sugar chain at 37°C for 20 h, and reactions were stopped by heating the solution at 100°C for 2 min.

High-performance liquid chromatography (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 with reversed-phase HPLC (Cosmosil SC18-PM, 0.46 x 15 cm, Nacalai Tesque) and size-fractionation HPLC (TSK-GEL Amide-80, 0.46 x 25 cm, Tosoh Corp.) essentially according to the methods of Tomiya et al. (1988) and Yamamoto et al. (1989). In both HPLC systems, PA-oligosaccharides were detected by fluorescence using excitation and emission wavelengths of 320 and 400 nm, respectively.

Results

Preparation of four AFP species different in lectin affinity

Purity of each patient AFP sample was established by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as shown in Figure 1. The yield of AFP purification by affinity chromatography from each patient was about 90%, and the relative yields of four AFP species separated by lectin affinity chromatography with two lectin columns are listed in Table 1. Purity and affinity for two lectins of each species were confirmed by crossed immunoaffinoelectrophoresis (results not shown).

Each of these four AFP species (30–100 nmoles) different in lectin affinity was subjected to hydrazinolysis to prepare PA-oligosaccharides, and subsequently PA-sugar chains from each species (50–300 pmoles) were applied to HPLC analysis. The yields of hydrazinolysis and reductive amination with 2-aminoypyridine were 60–70 and 85–90%, respectively. Thus, overall yield was around 60%.

Carbohydrate structure of Con A(+)/LCA(−) species of AFP

The PA-oligosaccharide of the Con A(+)/LCA(−) species of AFP from HCC patient 1 was eluted at the position of the authentic PA-biantennary chain, both on reversed-phase HPLC (Figure 2) and on size-fractionation HPLC. By β-galactosidase and subsequent β-N-acetylgalactosaminidase digestion, the elution positions were converted to that of the PA-agalactobiantennary chain, and that of the PA-trimannosyl sugar chain, respectively (results not shown). Essentially identical elution profiles of HPLC were obtained with the PA-oligosaccharide of the Con A(+)/LCA(−) species of AFP from HCC patient 2 and the patient with gallbladder carcinoma which metastasises to the liver (results not shown).

Carbohydrate structure of Con A(+)/LCA(+) species of AFP

The PA-oligosaccharide of the Con A(+)/LCA(+) species of AFP from HCC patient 1 was eluted at the position of the authentic PA-fucosylated biantennary chain, both on reversed-phase HPLC (Figure 3a) and on size-fractionation HPLC (Figure 4a). Upon digestion with fucosidase, the elution position was converted to that of the authentic PA-biantennary chain, as shown in Figure 3c (reversed-phase HPLC) and Figure 4c (size-fractionation HPLC). Essentially identical results were obtained for the PA-oligosaccharides of the Con A(+)/LCA(+) species of AFP from HCC patient 2 and the patient with gallbladder carcinoma which metastasises to the liver (results not shown).
Carbohydrate structure of \( \text{Con A}(-)/\text{LCA}(+) \) species of \( \text{AFP} \)

Figure 5a shows a reversed-phase HPLC elution pattern of the PA-oligosaccharide of the \( \text{Con A}(-)/\text{LCA}(+) \) species of \( \text{AFP} \) from HCC patient 1. The elution position was same as that of the authentic PA-fucosylated and N-acetylglucosaminylated biantennary chain. The elution position of Amide-80 size-fractionation HPLC was also same as that of the reference compound mentioned above (results not shown). Fucosidase digestion converted the elution position on reversed-phase HPLC of this PA-oligosaccharide to that of the PA-N-acetylglucosaminylated biantennary chain as shown in Figure 5b. Essentially identical results were obtained for the \( \text{Con A}(-)/\text{LCA}(+) \) species of \( \text{AFP} \) from HCC patient 2 and the patient with gallbladder carcinoma which metastasises to the liver (results not shown).

Figure 3  Reversed-phase HPLC elution profiles of native and fucosidase-digested PA-oligosaccharides from the \( \text{Con A}(+)/\text{LCA}(+) \) species of \( \text{AFP} \) of HCC patient 1. The native PA-oligosaccharide (1 in a) was eluted at the same position as that of the PA-fucosylated biantennary chain (1 in b). After fucosidase-digestion this PA-oligosaccharide gave a new major peak (2 in c) eluted at the position of the PA-biantennary chain (2 in d).

Figure 4  Size-fractionation HPLC elution profiles of native and fucosidase-digested PA-oligosaccharides from the \( \text{Con A}(+)/\text{LCA}(+) \) species of \( \text{AFP} \) of HCC of patient 1. The PA-oligosaccharide (1 in a) was eluted at the position of the PA-fucosylated biantennary chain (1 in b). The fucosidase-digested PA-oligosaccharide of the \( \text{Con A}(+)/\text{LCA}(+) \) species of \( \text{AFP} \) (2 in c) was eluted at the position of the PA-biantennary chain (2 in d).
P-N-acetylglucosaminidase

Carbohydrate structure of Con A(-)/LCA(-) species of AFP

Figure 5 Reversed-phase HPLC elution profiles of native and fucosidase-digested PA-oligosaccharides from the Con A(-)/LCA(+) species of AFP of HCC patient 1. The native PA-oligosaccharide (1 in a) was eluted at the position of the PA-fucosylated N-acetylgalactosaminylated biantennary chain (4 in c). Fucosidase-digestion gave a peak (2 in b) at the position of the N-acetylgalactosaminylated biantennary chain (peak 3 in e). Authentic PA-oligosaccharide standards in e are: 1, biantennary chain; 2, fucosylated biantennary chain; 3, N-acetylgalactosaminylated biantennary chain; and 4, fucosylated and N-acetylgalactosaminylated chain.

Figure 6 Size-fractionation HPLC elution profiles of native and fucosidase-digested PA-oligosaccharides of the Con A(-)/LCA(+) species of AFP of patient 1. The major peak (peak 1 in Figure 6a) representing 40 and 45% of the total peak areas in patient 1 and patient 2, respectively, was eluted at the position of the authentic PA-fucosylated triantennary chain (peak 4 in Figure 6c). The PA-triantennary oligosaccharide (peak 3 in Figure 6c) was also detected as a minor component (representing 14 and 22% of the total peak areas in the respective patient, peak 2 in Figure 6a). Upon fucosidase digestion, the major peak disappears, and an increase of the peak at the position of the authentic PA-triantennary chain, was observed (peak 2 in Figure 6b)

To confirm the chemical structure of the sugar chain, the main peak oligosaccharide was collected, and a portion was subjected to fucosidase digestion. Figure 7a shows an elution profile of the PA-oligosaccharide thus isolated on size-fractionation HPLC. The elution position (peak 1 in Figure 7a) was same as that of the PA-fucosylated triantennary chain (peak 6 in Figure 7c). Fucosidase digestion gave an oligosaccharide eluted at a position (peak 2 in Figure 7b) of the authentic PA-triantennary chain (peak 5 in Figure 7c). The fucosidase-treated oligosaccharide was collected and then subjected to β-galactosidase digestion and subsequently to β-N-acetylgalactosaminidase digestion. The elution positions of resultant oligosaccharides (peak 3 in Figure 7d and peak 4 in Figure 7e) were identical with those of authentic oligosaccharides, a PA-agalactotriantennary chain (peak 2 in Figure 7f), and a PA-trimannosyl chain (peak 1 in Figure 7f) standards, respectively.

The elution profile of the PA-oligosaccharide of the Con A(-)/LCA(-) species of AFP from HCC patient 1 by reversed-phase HPLC is shown in Figure 8. By this reversed-phase HPLC with Cosmosil 5C18-P, the PA-oligosaccharide of the N-acetylgalactosaminylated biantennary chain and that of the fucosylated triantennary chain were eluted at almost the same position (peak 4 in Figure 8c and peak 5 in Figure 8d), and therefore it was difficult to know their presence or absence. However, when digested with fucosidase, a major peak (2 in Figure 8b) was shifted to the position of the PA-triantennary chain (peak 3 in Figure 8c). A minor peak (1 in Figure 8b) remaining at the initial position after repeated fucosidase digestions was considered to represent N-acetylgalactosaminylated biantennary chain (peak 5 in Figure 8d). These results indicated that major components of PA-oligosaccharides of Con A(-)/LCA(-) species of AFP from the patient with HCC was the fucosylated triantennary chain. Although it was difficult to identify each peak, several minor components eluted at faster positions than that of the major...
component (Figure 6a), appeared to be fucosylated species, because after fucosidase digestion these minor peaks disappeared and new peaks became detectable at faster position than that of untreated minor components (Figure 6b).

Essentially the same elution profiles on HPLC were obtained for Con A(−)/LCA(−) species of AFP from HCC patient 2 and the patient with gallbladder carcinoma which metastasises to the liver. However, the ratio (10.1) of the fucosylated to non-fucosylated triantennary sugar chains of AFP from the patient with gallbladder carcinoma which metastasises to the liver was much higher than those (3.2 for patient 1 and 1.9 for patient 2) in patients with HCC. The peak areas due to the fucosylated and the nonfucosylated triantennary chains in this disease represented 32 and 3% of the total area, respectively.

**Discussion**

It is now accepted that there are several molecular species of AFP with different affinity for Con A and LCA. LCA binds specifically the biantennary chain with a fucose residue at the innermost N-acetylglucosamine residue at the trimannosyl core (fucosylated biantennary chain) and that both with a fucose and a bisecting-N-acetylglucosamine residue (fucosylated and N-acetylglucosaminylated biantennary chain) (Kornfeld et al., 1981). However, if these biantennary structures undergo modification of further branching leading to triantennary structures, they become nonreactive with LCA (Montreuil et al., 1983). On the other hand, Con A binds specifically the biantennary and the fucosylated biantennary chains. This binding does not occur if the biantennary sugar chain structure undergoes modification of bisecting-glucosaminylation and/or further branching leading to the formation of the triantennary and tetraantennary structures (Baenziger & Fiete, 1979).

On the basis of these facts, we have proposed the carbohydrate structures of various AFP species with difference in affinity for Con A and LCA (Aoyagi et al., 1985, 1991; Kornfeld et al., 1981; Montreuil et al., 1983; Baenziger et al., 1979), but they remain to be confirmed.

Recently, a very sensitive and convenient method has been developed to study the fine carbohydrate structures by combination of derivatization into fluorescent oligosaccharide and separation by HPLC (Hase et al., 1984; Tomiya et al., 1988; Yamamoto et al., 1989; Nishii et al., 1990). Since a number of PA-oligosaccharide, the structures of which have been established by 1H-nuclear magnetic resonance, have become commercially available, we applied this method to study the fine structures of various AFP species.

The present results are summarised in Table II. Here, we propose the classification of carbohydrate structures of human AFP according to the reactivity with lectins and the disease category (Table II). The first is the Con A(+) / LCA(−) sugar chain, and the structure is of the biantennary complex type, a basic carbohydrate structure of AFP. This structure is predominantly observed in AFP of nonneoplastic liver diseases. The second is the Con A(−) / LCA(+) chain which is the biantennary complex type with fucosylation and bisecting-N-acetylglucosaminylation, and this type is observed predominantly in AFP of carcinomas of digestive organs which metastasise to the liver and yolk sac tumour. The fourth is the Con A(−) / LCA(−) chain which includes three kinds of carbohydrate structure, namely, the biantennary complex type with bisecting-acetylglucosaminylation, the triantennary complex type and the triantennary complex type with fucosylation. The
N-acetyl-glucosaminylated biantennary chain is predominantly observed in AFP of carcinoma which metastasises to the liver and yolk sac tumour. The biantennary chain and its fucosylated form are present in AFP of HCC, carcinoma which metastasises to the liver and yolk sac tumour.

Recently we reported that the measurement of a fucosylation index of AFP is useful for distinction between HCC and nonneoplastic diseases of the liver (Aoyagi et al., 1984, 1991). The fucosylation index of AFP was defined as the percentage of the LCA-reactive species in total AFP (Aoyagi et al., 1991). The present study revealed that more than the 40% of Con A-nonreactive species of AFP were fucosylated, even if it is not reactive with LCA. In our previous study the percentage of the Con A-nonreactive species of AFP was 5 ± 7% in 351 patients with HCC (Aoyagi et al., 1991) and we may expect a little effect of this species on the fucosylation index. Special consideration, however, should be given to evaluate the fucosylation index determined by crossed immunoaffinoelctrophoresis with Con A and LCA, when the percentage of the Con A nonreactive species is high.

Thus, evidence presented here and our previous study indicate that increases in fucosylation and in branching to form new antennae are observed in carbohydrate chains of AFP from patients with neoplastic diseases of the liver. Other studies also indicated the tumour associated increase in fucosylation and branching. For example, Campion et al. (1989) reported the presence of fucosylated triantenary, tetraantennary and pentaantennary glycans, in transferrin synthesised by the human hepatocarcinoma cell line Hep G2. Dennis et al. (1987, 1989) reported that transfection of an activated ras gene into the non-metastatic line of SPI mouse mammary tumour cells resulted in the induction of both metastatic potential and elevated levels of β 1-6 branched oligosaccharides and that oncogenes conferring metastatic potential induced increased branching of asparagine-linked oligosaccharides in rat2 fibroblasts. However, the biological significance of this tumour-associated change remains to be clarified.

Some of chemical structures of sugar chains of human AFP have already been reported by Yoshima et al. (1980) and Yamashita et al. (1983). However, carbohydrate structures in the molecular species of human AFP with different affinity for Con A and LCA were not fully investigated. These authors presented the chemical structures of sugar chains of human AFP from ascites fluids as biantennary, fucosylated biantennary, N-acetylglicosaminylated biantennary and fucosylated, N-acetylglicosaminylated chains. These structures were confirmed to be present in our AFP preparations.

On the other hand, they reported that human AFP did not contain the triantennary chain. However, our present study clearly indicated the presence of the triantennary chains with and without a fucose residue at the innermost N-acetylglicosamine residue in AFP from patients with neoplastic diseases of the liver. Thus, this is the first report to establish the presence of these structures in detail in human AFP.

### Table II

| Lectin reactivity | Carbohydrate structure                                                                 | Disease category                          |
|-------------------|----------------------------------------------------------------------------------------|-------------------------------------------|
| Con A(+) | LCA(−)       | Biantennary sugar chain                                                              | Non-neoplastic liver diseases             |
| Con A(+) | LCA(+)       | Fucosylated biantennary sugar chain                                                  | Hepatocellular carcinoma                 |
| Con A(−) | LCA(+)       | Fucosylated and N-acetylglicosaminylated biantennary sugar chain                     | Carcinomas of digestive organs which metastasise to the liver and yolk sac tumour |
| Con A(−) | LCA(−)       | N-Acetylglicosaminylated biantennary sugar chain                                     | Carcinomas of digestive organs which metastasise to the liver and yolk sac tumour |
|                 |              | Triantennary and fucosylated                                                         | Hepatocellular carcinoma                 |
|                 |              | triantennary sugar chains                                                            | carcinomas of digestive organs which metastasise to the liver and yolk sac tumour |

*(+) and (−) represent 'reactive' and 'nonreactive', respectively. *Disease in which AFP with the relevant carbohydrate structure is found characteristically.

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**Figure 8** Reversed-phase HPLC elution profiles of native a, and fucosidase-digested PA-oligosaccharides b, from the Con A(−)/LCA(−) species of AFP of HCC patient 1. The native oligosaccharides gave a major peak (1 in a) at the position of the authentic fucosylated triantennary chain (4 in c). In this system, the N-acetylglicosaminylated biantennary chain (5 in d) was also eluted at the same position. Fucosidase digestion resulted in an increase of a peak (2 in b) at the position of triantennary chain (3 in e). A residual minor peak at the initial position (peak 1 in b) was considered to the N-acetylglicosaminylated biantennary chain (5 in d). Authentic PA-oligosaccharides in e and d are: 1, biantennary chain; 2, fucosylated biantennary chain; 3, triantennary chain; 4, fucosylated triantennary chain; 5, N-acetylglicosaminylated biantennary chain; and 6, fucosylated and N-acetylglicosaminylated biantennary chain.
In the image, there is a page from a scientific document discussing the presence of AFP (alpha-fetoprotein) in rat AFP, and its usefulness in diagnosing primary hepatocellular carcinoma. The text mentions the work of multiple authors and references studies on the molecular basis of AFP and its presence in various conditions, including the metastasis of gastric cancer and other diseases.

The text is a natural representation of the scientific content, discussing the use of AFP in diagnostic applications and its molecular structure. It concludes with the statement that the presence of AFP in a set of sugar transferases would lead to the qualitative and quantitative differences in each molecular species of AFP.

This study was supported in part by a Grant-in-Aid from The Niigata University Science Foundation.

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