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Abnormal biantennary sugar chains are expressed in human chorionic gonadotropin produced in the choriocarcinoma cell line, JEG-3

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Over the past two decades, sugar chain structures of human chorionic gonadotropin (hCG) produced in healthy people, three types of trophoblastic disease, and some types of cell lines have been analyzed. The abnormal biantennary structure of hCG is a good marker for the diagnosis of malignant choriocarcinoma. In spite of much research, hCG with an abnormal biantennary structure is only detected in the urine of choriocarcinoma or pregnant diabetic patients. We hypothesized that the formation mechanism of the abnormal biantennary sugar chain structure is mainly caused by high GnT-IV activity. To confirm this, we measured the *N*-acetylglucosaminyltransferase (GnT)-IV activity and hCG productivity in three choriocarcinoma cell lines, and selected JEG-3 cells. hCG samples were purified from medium conditioned by JEG-3 cells, and their sugar chain structures were analyzed. We detected an abnormal biantennary structure, and the proportions were different from those previously reported in the urine samples of choriocarcinoma patients. These findings proved our hypothesis and suggest the usefulness of JEG-3 cells for further analyses of abnormal biantennary structure formation.
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Keywords: **human chorionic gonadotropin (hCG), abnormal biantennary sugar chain, choriocarcinoma, *N*-acethylglucosaminyltransferase (GnT)-IV**

Abbreviations: **hCG: human chorionic gonadotropin; GnT, *N*-acetylglucosaminyltransferase: 2-AB: 2-aminobenzamide; MALDI-TOF/MS: matrix-assisted laser desorption ionization time-of-flight mass spectrometry; JB: jack bean; AAL: *Aluria aurantia* lectin; Con A: Concanavalin A; DHB: 2,5-dihydroxybenzoic acid; PA: 2-aminopyridine; Asn: asparagines; Man: D-mannose; GlcNAc: *N*-acetylglucosamine; GnT-II: UDP-*N*-acetylglucosamine : α-1,6-D-mannoside β-1,2-GnT; GnT-III: UDP-*N*-acetylglucosamine: β-D-mannoside β-1,4-GnT; GnT-IV: UDP-N-acetylglucosamine : α-1,3-D-mannoside β-1,4-GnT; α-Man'ase II: α-mannosidase II.**

Introduction

hCG is a glycoprotein hormone produced by trophoblasts of the human placenta [1–3], and is involved in the maintenance of the corpus luteum during early pregnancy. hCGs are also present in the blood and urine of patients with a variety of trophoblastic diseases [4–6]. Therefore, urinary hCG levels are used as useful markers for the diagnosis and prognosis of trophoblastic diseases as well as normal pregnancy [7–10].

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The sugar chain structures of urinary hCG purified from pregnant women and patients with trophoblastic diseases have been examined and completely characterized for about two decades now. The N-linked sugar chains of hCG from pregnant women contain three types of structures; monoantennary, biantennary and fucosylated biantennary sugar chains [11]. However, the sugar chains of hCG from patients with choriocarcinoma possess triantennary and abnormal biantennary sugar chain structures [12,13]. The abnormal biantennary sugar chain structure is specific in choriocarcinoma hCG, and is a good marker for the diagnosis of malignant choriocarcinoma disease [14]. An abnormal biantennary structure is unusual, but is present in two human tumor glycoproteins; carcinoembryonic antigen obtained from liver metastases of primary colon cancers and

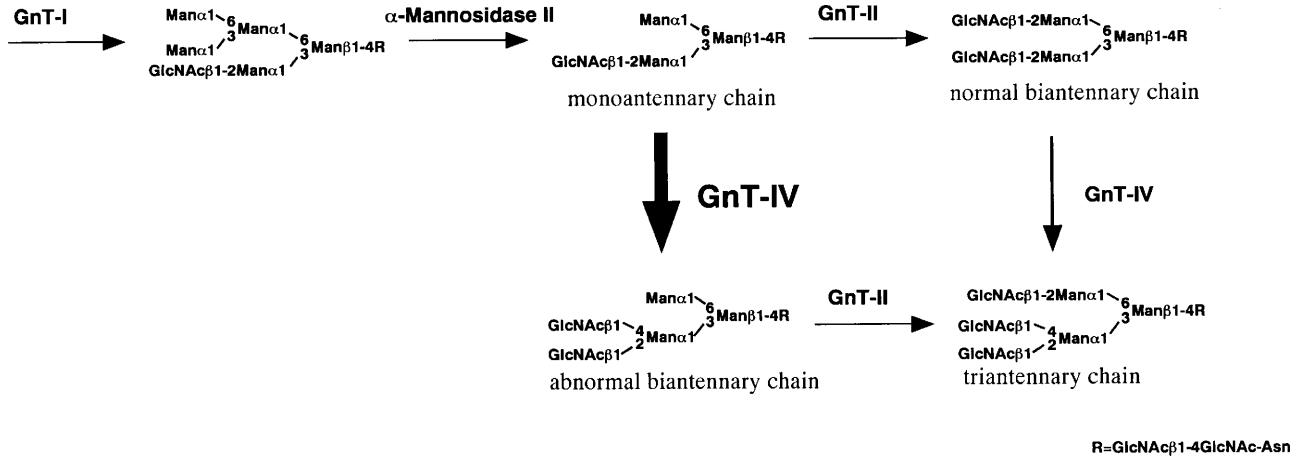

Figure 1. Synthetic pathway of abnormal biantennary sugar chain formation.

γ-glutamyltranspeptidases purified from hepatocellular carcinoma, and the hCG of urine from diabetic pregnant women except for choriocarcinoma hCG [15–17]. GnT-IV, which is composed of branches of GlcNAcβ1-4Manα1-3, is a key enzyme required for the synthesis of an abnormal biantennary sugar chain structure. In a recent study, we investigated the enzyme activities and expression levels of mRNAs of GnT-I to -V, β-1,4-galactosyltransferase (GalT), and α-mannosidase II in normal human placentae and three human choriocarcinoma cell lines [18]. From our findings, we proposed a mechanism for the formation of an abnormal biantennary sugar chain as follows (Figure 1). GnT-II and GnT-IV competitively use a monoantennary sugar chain as a donor substrate. In choriocarcinoma cell lines which synthesize the abnormal biantennary sugar chain, GnT-IV activity is enhanced, and GnT-II activity is almost equivalent with that found in normal placentae. Therefore, we concluded that an abnormal biantennary structure is synthesized via pathway IV (Figure 1) and accumulates owing to weak GnT-II activity in choriocarcinomas [18]. In spite of much research, it is not clear why an abnormal biantennary structure of hCG is detected only in samples purified from the urine of choriocarcinoma patients and individuals with diabetic pregnancy. To confirm our theory, we studied the following three choriocarcinoma cell lines, BeWo, JAR, and JEG-3 cells, to determine the source of hCG. First, we measured the enzyme activities of six glycosyltransferases (GalT, GnT-I, -II, -III, -IV, -V) and α-mannosidase, and the rate of formation of hCG in these cell lines. From results indicating abundant productivity and a balance of glycosyltransferase activities, we selected the JEG-3 cell line and purified hCG using an antibody column. In this study, the sugar chain structures of hCG produced by JEG-3 cells were analyzed by the 2-aminobenzamide (2-AB) labeling method along with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS), which enabled us to determine the precise sugar chain structure using limited samples. We successfully detected the abnormal biantennary structure, whose proportion was different compared to that previously found in urine samples from choriocarcinoma patients. This finding proved our hypothesis that high GnT-IV activity results in an abnormal biantennary structure in hCG responsible for and suggests the usefulness of JEG-3 cells for the further analysis of the mechanisms abnormal biantennary structure formation.

Experimental procedures

Materials

β-*N*-Acetylhexosaminidase and α-mannosidase from Jack bean (JB), an *Aluria aurantia* lectin column (LA-AAL-column) and a Concanavalin A column (LA-Con A-column), all these were purchased from Seikagaku Kogyo (Tokyo, Japan). β-galactosidase from *Diplococcus pneumoniae* (DP) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Sialidase from *Arthrobactor ureafaciens* (AU) and 2-aminobenzamide were purchased from Nacalai Tesque (Kyoto, Japan), and 2,5-Dihydroxybenzoic acid (DHB) was purchased from Aldrich Chemical (Milwaukee, WI, USA).

Cell lines

All cell lines used in this experiment were of human origin unless otherwise indicated. The choriocarcinoma cell line BeWo was obtained from the Health Science Research Resources Bank (Osaka, Japan). Choriocarcinoma cell lines JEG-3 and JAR were purchased from the American Type Culture Collection (Rockville, MD). BeWo cells were maintained in Ham's F-12 Kaighn's Modification Medium (GIBCO BRL) supplemented with 15% fetal bovine serum (FBS) (HyClone, Logan, UT). JEG-3 cells were maintained in minimum essential medium (GIBCO BRL) supplemented with 10% FBS. JAR cells were cultivated in RPMI-1640 (GIBCO BRL) supplemented with 10% FBS. All media were supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and all cell lines were incubated in a 5% CO_2 humidified atmosphere at 37°C.

Determination of the activities of GnTs, GalT and α-Man'ase II

The activities of GnT-I and GnT-II were measured by the method of Schachter *et al.* [19] except that Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA(core-PA) and Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA [Gn(2)core-PA] were used as substrates for GnT-I and GnT-II, respectively, at a concentration of 0.8 mM and incubation time of 1 h. Assays of GnT-III, -IV and -V activity were performed according to the method of Oguri *et al.* [20], which is a modification of the method of Nishikawa *et al.* [21]. The substrate Gn2(2′,2)core-PA [GlcNAcβ1-2 Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4-GlcNAc-PA] was prepared as previously reported [22]. GalT activity was assayed as follows. Thirty micrograms of enzyme solution was incubated at 37°C for 30 min with 10 mM HEPES buffer (pH 7.2) containing 0.8 mM Gn2(2′,2)core-PA, 10 mM UDP-Gal, 33 mM NaCl, 3 mM KCl, 1.5% Triton X-100, 5.5 mM D-Galactono-1,4-lactone, and 10 mM $MnCl_2$ in a total volume of 20 μl. α-mannosidase II activity was measured according to the method of Chui *et al.* [23]. The substrate [Manα1-6(Manα1-3)Manα1-6](Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA was kindly provided by Dr. Y. Chiba (KIRIN Brewery, Yokohama) and used at 10 μM; the incubation period was 3 h at 37°C.

Preparation of conditioned medium and purification of hCG

Subconfluent JEG-3 cells were washed with PBS (-) three times and the medium was replaced to a serum-free synthetic S-clone CM-B (Sanko Pure Chemical). After 72 h of incubation, the medium was collected and stored at −20°C. The pooled conditioned medium (3,000 ml) was concentrated to 10 ml using an Amicon YM10 ultrafiltration membrane (MILLIPORE). hCG was purified from the concentrated medium using an anti-hCG antibody column, where anti-hCG polyclonal antibody (Biostride, Inc.) was linked to affi-gel 10 resin (BIO-RAD). The concentrated conditioned medium was applied to this column, and the fraction that bound to the column was subjected to a sep-pak C18 cartridge. The cartridge was eluted with 30% acetonitrile containing 0.1% TFA. hCG was confirmed by electrophoresis to be purified.

Preparation of 2-AB labeled oligosaccharides from JEG-3 cells hCG

The asparagine (Asn)-linked sugar chains of hCG were liberated by hydrazinolysis at 100°C for 16 h and then re-N-acetylated [24]. The oligosaccharides were reductively aminated with 2-AB in 20 μl of acetic acid/dimethylsulfoxide (3:7) solution containing 7 μmol 2-AB and 2 mg of sodium cyanoborohydrate at 37°C for 16 h [25,26]. The 2-AB-labeled Asn-linked oligosaccharides were purified by a series of paper and gel-permiation chromatographies. Upsending chromatography was performed on No. 514 paper (Advantec Toyo, Tokyo, Japan) with 1-butanol/ethanol/distilled water (4:1:1) as a solvent. Gel permiation chromatography was performed on a Superdex Peptide HR 10/30 column (Pharmacia, Uppsala, Sweden) equilibrated and eluted with 50 mM pyridine acetate buffer (pH 5.0). 2-AB labeled Asn-linked oligosaccharides were collected from the flow-through fraction and detected by fluorescence at an excitation of 330 nm and emission wavelength of 420 nm.

Charge analysis of oligosaccharides

Charge analysis of 2-AB-labeled oligosaccharides was performed by anion exchange chromatography on a HiTrap-Q column (1 ml, Pharmacia, Uppsala, Sweden) at 1 ml/min, using a linear gradient of sodium acetate buffer (pH 5.0) from 2 mM to 160 mM per 30 min as previously described [26].

Enzyme digestion

Glycosidase digestion of 2-AB labeled sugar chains was carried out under the following conditions: 200 mU of sialidase from AU [27] in 200 μl of 20 mM sodium acetate buffer (pH 5.0); 40 mU of β-galactosidase from DP [28] in 80 μl of 10 mM sodium cacodylate buffer (pH 6.0) containing 40 μg of bovine serum albumin; 1U of α-mannosidase from JB [29] in 50 μl of 100 mM citrate phosphate buffer (pH 4.0); 4 mU of β-*N*-acetylhexosaminidase from JB [30] in 50 μl of 100 mM citrate phosphate buffer (pH 5.0). All digestions were performed at 37°C for 24 h and the reactions were terminated by heating the solution at 100°C for 2 min.

Lectin affinity chromatography

Desialylated 2-AB-labeled oligosaccharides were analyzed by lectin affinity column chromatography using a LA-AAL-column [31] and LA-ConA-column [32] with 10 mM Tris-H_2SO_4 buffer (pH 7.5) as a loading buffer at 0.5 ml/min at room temperature. The bound fractions were eluted with the 1 mM fucose from the LA-AAL-column. Elution from the LA-ConA-column was performed using a linear gradient of increasing α-methylmannoside from 0 to 10 mM.

Size analysis of oligosaccharides by column chromatography

Bound and unbound AAL oligosaccharides were chromatographed on a TSK gel Amide-80 column (4.6 × 250 mm, Tosoh) by a linear gradient decrease in acetonitrile of 60–50% for 60 min in 50 mM ammonium acetate buffer (pH 4.0) at 1.0 ml/min at room temperature.

Reverse phase chromatography

2-AB-labeled oligosaccharides were chromatographed on a column of TSK gel ODS 80Ts (4.6 × 150 mm, Tosoh, Tokyo, Japan) by a linear gradient increase in acetonitrile of 0.5–7% for 40 or 50 min in 50 mM ammonium acetate buffer (pH 4.0) at 1.0 ml/min at room temperature.

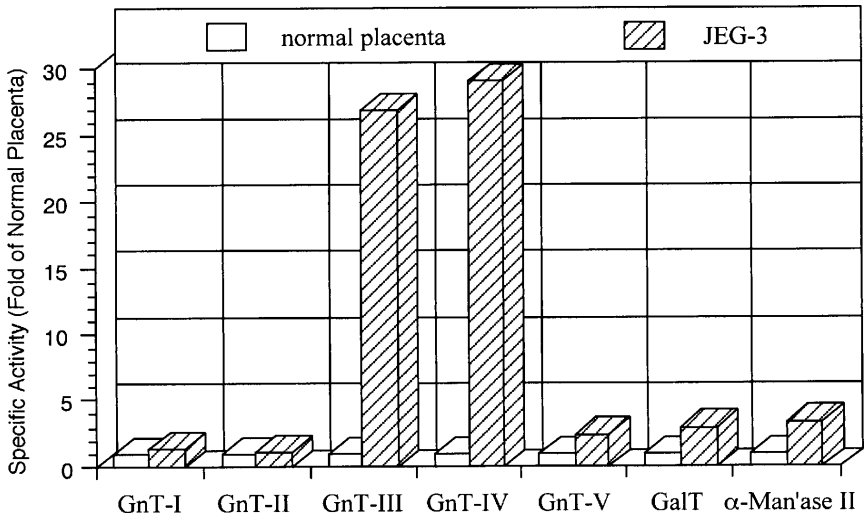

Figure 2. Glycosyltransferases and α-Man'ase II activities in choriocarcinoma cell line JEG-3. Five GnTs, GalT, and α-Man'ase II activities were assayed as described in "Materials and Methods." A representative experiment is shown as one of three replicates with similar results. The specific activity of each enzyme is expressed as moles of product per hour of incubation per mg of protein in the cell lysate.

MALDI-TOF/MS

MALDI-TOF/MS spectra were analyzed using a Lasermat 2000 (Finnigan MAT, Hempstead, UK). Neutral oligosaccharides were positively ionized with DHB solution and observed as [M+Na] +ions. The DHB solution consisted of acetonitrile/distilled water (3:7) containing 1% (w/v) DHB and 0.01% (w/v) sodium dihydrogenphosphate dihydrate.

Results

Comparison of enzyme activities related to sugar chain synthesis

Figure 2 shows six glycosyltransferases and α-mannosidase II activities for normal placentae and choriocarcinoma cell line JEG-3. The activities for BeWo and JAR cells were almost equivalent to JEG-3 cells except for the synthesis activity of hCG (data not shown). *N*-acetylglucosaminyltransferase-III (GnT-III) and GnT-IV activities were elevated in three choriocarcinoma cell lines compared to normal placentae. GnT-III activity in JEG-3 cells was twenty seven times higher than that found in normal placentae, and GnT-IV activity in JEG-3 cells was twenty nine times higher than that found in normal placentae. Elevated levels of GnT-III activity in the three choriocarcinoma cell lines suggested the formation of a bisecting structure on hCG. However, bisecting structures were not detected in purified hCG from urine samples of choriocarcinoma patients. In the present study, it was unclear whether this discrepancy was caused by specific type of cell lines used or not.

Purification of hCG

hCG samples were purified from the concentrated conditioned medium of JEG-3 cells using an anti-hCG antibody column (Figure 3A). As shown in Figure 3B, the size of purified hCG in the samples was slightly larger than standard hCG and showed a broadband in SDS-PAGE. This discrepancy may be due to differences in the sugar chain structures.

Charge analysis of 2-AB-labeled oligosaccharide

2-AB labeled Asn-linked oligosaccharides of hCG derived from a purchased standard sample and JEG-3 cells were resolved into three peaks (N, A1, A2) by anion-exchange column chromatography (Figure 4). N, A1 and A2 indicate oligosaccharide fractions with 0, 1 and 2 negative charges (sialic acid), respectively. The molar ratio of the neutral chain was 8.2% for standard hCG, and over 50% for JEG-3 hCG.

Structural analysis of 2-AB-labeled oligosaccharides in JEG-3 hCG

2-AB-labeled oligosaccharides neutralized by AU sialidase digestion were separated on an LA-AAL column. AAL lectin has specificity for fucose residues. Fucosylated sugar chains were digested with galactosidase and *N*-acetylhexosaminidase, and compared with standard sugar chain, Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-2AB prepared from recombinant human erythropoietin. Then, the fucosylated sugar chain has a fucose α1-6 residue at the reducing terminal of GlcNAc. Sugar chains from standard hCG were fucosylated by 40% and that from JEG-3 hCG was fucosylated by 25% (data not shown). Each type of LA-AAL bound (AAL+) and unbound oligosaccharide (AAL−) was subjected to Amide 80 column chromatography. Two peaks were observed for AAL− chromatography and a single peak was observed for AAL+ chromatography using standard hCG (data not shown). These results were consistent with previous observations [11,33]. For JEG-3 hCG,

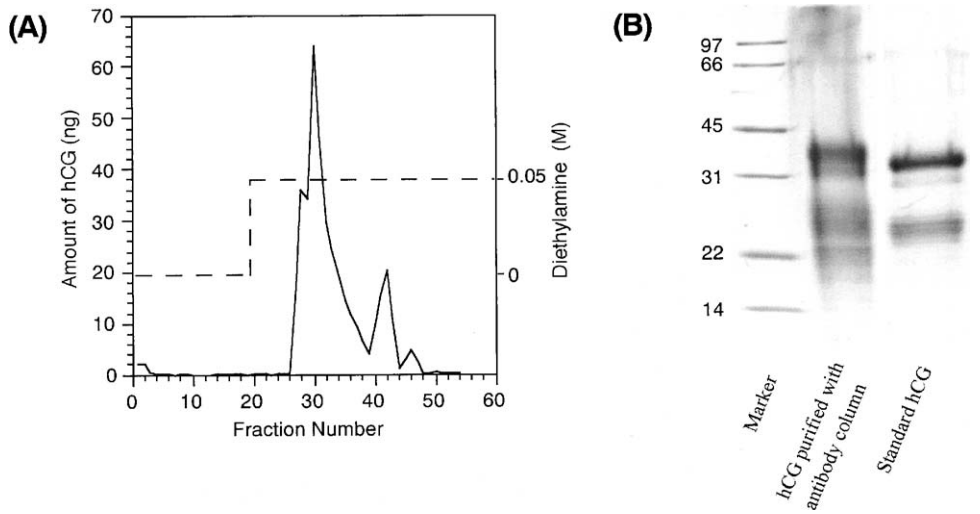

Figure 3. Elution pattern of anti-hCG polyclonal antibody column chromatography and polyacrylamide gel electrophoresis of purified hCG produced by choriocarcinoma JEG-3. (A) Anti-hCG polyclonal antibody column chromatography; (B) SDS-PAGE of purified hCG produced by JEG-3. The purification procedure is described in "Materials and Methods." Three micrograms of purified hCG was analyzed by electrophoresis on a 10% polyacrylamide gel followed by CBB staining.

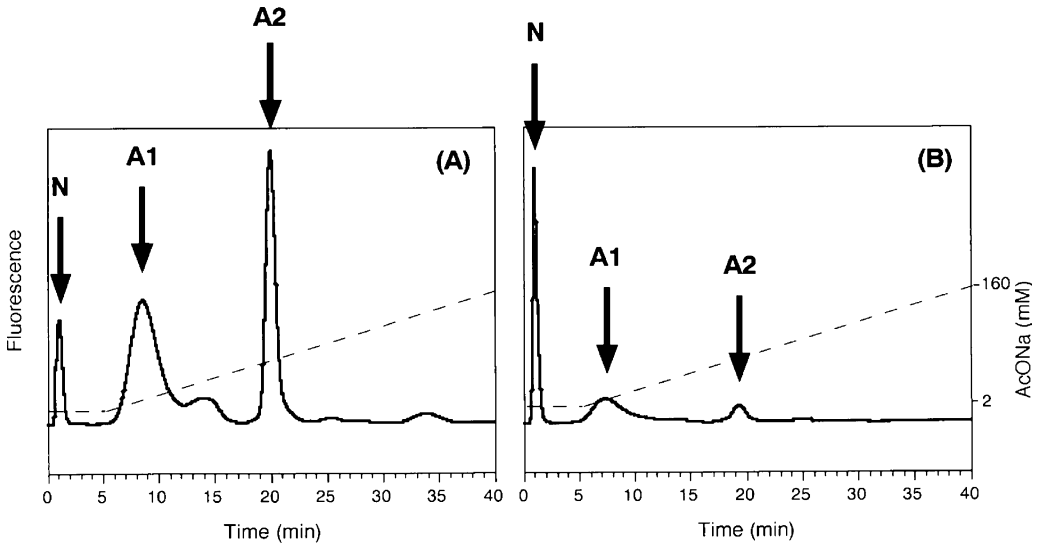

Figure 4. Anion-exchange chromatography of Asn-linked sugar chains. 2-AB-labeled Asn-linked sugar chains of normal or JEG-3 hCG were applied to a HiTrap-Q column. (A) normal hCG; (B) JEG-3 hCG. N, A1 and A2 with arrows indicate the elution positions of 2-AB-labeled Asn-linked sugar chains obtained from normal or JEG-3 hCG containing 0,1 and 2 negative charges, respectively.

three peaks were observed by AAL− chromatography and two peaks were found by AAL+ chromatography. We designated these peaks as 1, 2 and 3 for AAL− and F1 and F2 for AAL+, respectively (Figure 5). Next, the five separated fractions (1, 2, 3, F1 and F2) were subjected to reverse phase chromatography. The following four fractions except for fraction 1 were resolved into two peaks, and we named the peaks 2-1and 2-2 in fraction 2, 3-1 and 3-2 in fraction 3, F1-1 and F1-2 in fraction F1, and F2-1 and F2-2 in fraction F2 (Figure 6). The oligosaccharides (2-2, 3-2, F1-2, F2-2) in JEG-3 hCG passed through the LA-ConA lectin affinity column, and their MALDI-TOF/MS spectra showed that the oligosaccharides (2-2, 3-2, F1-2, F2-2) in JEG-3 hCG were oligosaccharides that possessed bisecting GlcNAc. Fractions 2-1 and F1-1 were further analyzed by LA-ConA lectin affinity column chromatography to examine their abnormal biantennary sugar chains. Twenty-five percent of fraction 2-1 oligosaccharides passed through the LA-ConA-column and 14% of fraction F1-1 oligosaccharides passed through (data not shown). Each eluted fraction (2-1a and F1-1a) suggested the existance of abnormal biantennary sugar chains. The structures for fraction 2-1a and F1-1a were confirmed by successive glycosidase digestion and MALDI-TOF/MS spectra. For

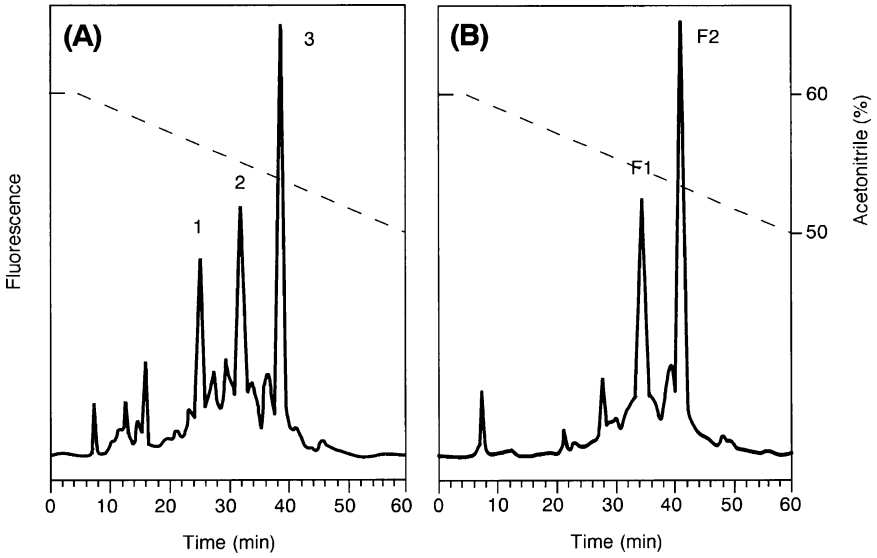

Figure 5. Size analysis of Asn-linked sugar chains by TSK gel Amide-80 column chromatography. Neutralized AAL-unbound or bound Asn-linked sugar chains of JEG-3 hCG were separated on a TSK gel Amide-80 column. (A) AAL-unbound fraction; (B) AAL-bound fraction.

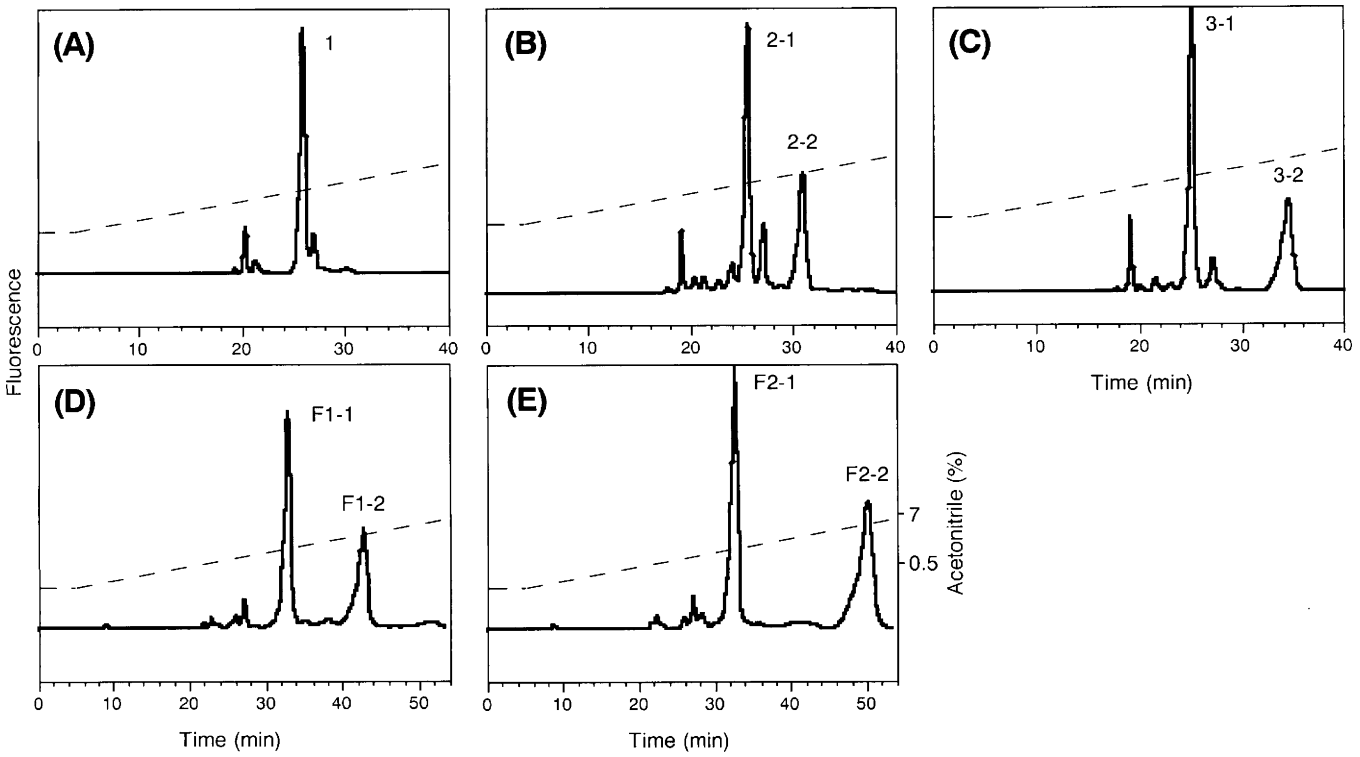

Figure 6. Reverse phase chromatography of Asn-linked sugar chains. A, B, and C, fraction 1, 2, and 3 of unbound AAL were separated on a TSK gel ODS-80 Ts column. Peaks were designated 2-1, 2-2 and 3-1, 3-2, respectively. D and E, fractions F1 and F2 of bound AAL were separated. Peaks were designated F1-1, F1-2 and F2-1, F2-2, respectively.

example, the MALDI/MS signal for the fraction 2-1a oligosaccharide was m/z 1785.0 which corresponds to the sodium adduct of 2-AB-labeled $Gal_2GlcNAc_4Man_3$. Sequential digestion with β-galactosidase from DP and α-mannosidase and β-*N*-acetylhexosaminidase from JB shifted the MALDI/MS signal to m/z 1460.6, 1298.4 and then to 892.0, respectively. These results suggest that the 2-1a oligosaccharide has a non-reducing Man(α1-6) end (Figure 7). The structures of asialo Asn-linked

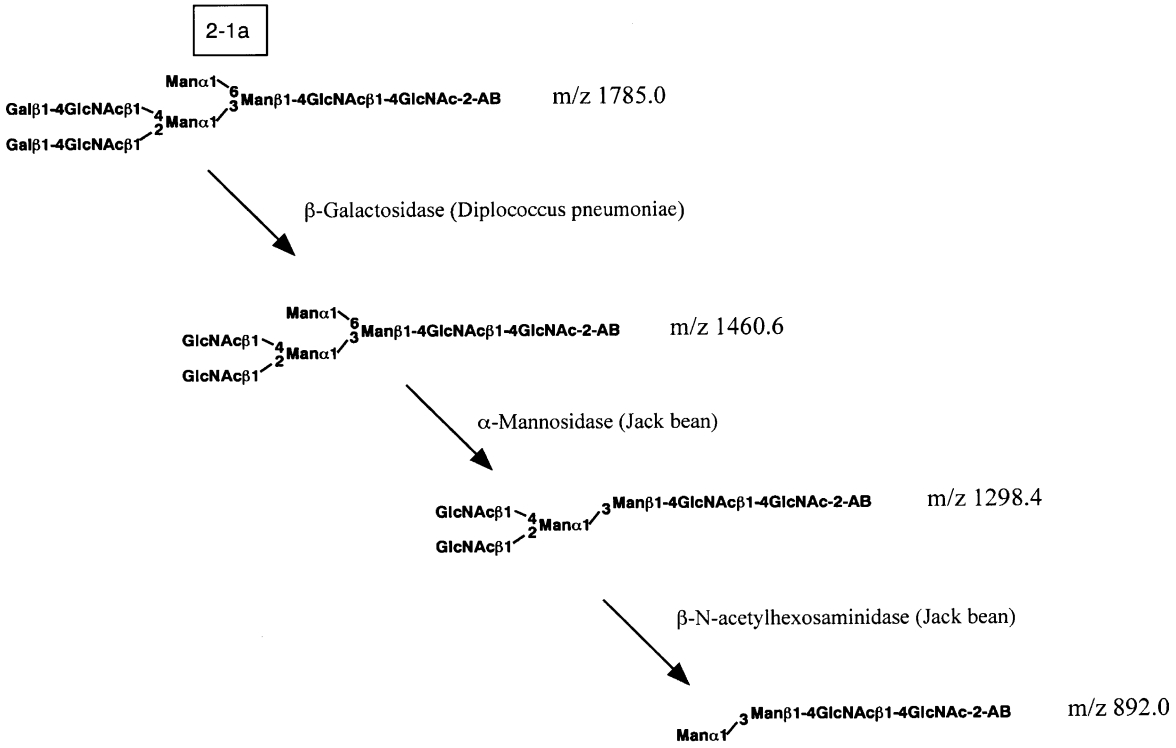

Figure 7. Confirmation of the presence of an abnormal biantennary chain using a combination of serial glycosidase digestion and MALDI-TOF/MS. The mass of fraction 2-1a was measured by MALDI-TOF MS and then digested by serial exoglycosidases; β-galactosidase (Diplococcus pneumoniae), α-mannosidase (Jack bean), and β-N-acetylhexosaminidase (Jack bean). Each digestion products was also mass measured to confirm the structure of the starting material (fraction 2-1a).

oligosaccharides of hCG produced by JEG-3 cells are summarized in Table 1. Abnormal biantennary sugar chains were found in hCG of this cell line. The molar ratio was 6% for α-1,6 fucose containg and not containing sugar chains. Sugar chains that have bisecting GlcNAc totaled 37%. These structures were not found in hCG obtained from the urine samples of choriocarcinoma patients. All structures shown in Table 1 were also confirmed by MALDI-TOF/MS spectra.

Discussion

A number of studies on the carbohydrate structures and their functions in hCG have been performed over the past two decades. In particular, differences in sugar chain structures of hCG between healthy pregnant women and choriocarcinoma patients are unique and therefore were utilized for diagnosis [14]. Recently, we elucidated the mechanism of abnormal biantennary sugar chain formation in choriocarcinoma hCG [18]. We speculated that high GnT-IV activity causes the formation of abnormal biantennary sugar chains in hCG. The purpose of the present study was to confirm this theory in cell lines that have high GnT-IV activity. Abnormal biantennary structures from samples other than those found in urine hCG of choriocarcinoma patients and diabetic pregnant women have not been found [12,13,15]. In 1992, Hard and coworkers studied the sugar chain structure of the β-subunit of hCG produced in the choriocarcinoma cell line BeWo using NMR spectroscopy [34]. They confirmed 76% of the structures of N-glycans in hCG, but were unable to detect an abnormal biantennary structure. We previously measured the GnT-IV activity of BeWo cells that expressed elevated GnT-IV activity, and suggested the possibility of abnormal biantennary structure formation in hCG [18]. It is possible that an abnormal biantennary structure was present in the remaining population. In the case of urine hCG of choriocarcinoma patients, one report discovered an abnormal biantennary structure mainly associated with the α-subunit [15], and another found one on the α- and β-subunits [12]. The quantity of abnormal biantennary sugar chain on the β-subunit of hCG produced by BeWo cells may have been too small to detect. Therefore, in this study we used the α- and β-subunits of hCG produced in choriocarcinoma JEG-3 cell line to analyze the total carbohydrate population. Eleven sugar chain structures were detected and were found to be almost the same as those reported by Endo and Mizuochi except that they contained bisecting-GlcNAc [12,13]. A bisecting-GlcNAc structure was not detected in the urine hCG of healthy pregnant women [11,15,35,36]. These results are in agreement with our previous examinations which detected weak GnT-III activity in placentae. As seen by our present results, we detected bisecting GlcNAc structures in hCG from the conditioned medium of choriocarcinoma cells. Bisecting GlcNAc structures were also detected in urinary hCG of cervical cancers, but not in urine

Table 1. Structures of the Asparagine linked sugar chains of JEG-3 hCG

1	Manα1-6 Galβ1-4GlcNAcβ1-2Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc-2-AB	9.7%
2-1a	Manα1-6 Galβ1-4GlcNAcβ1-4Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc-2-AB Galβ1-4GlcNAcβ1-2	3.8%
2-1b	Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-2Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc-2-AB	11.6%
2-2	Galβ1-4GlcNAcβ1-2Manα1-6 GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc-2-AB Galβ1-4GlcNAcβ1-2Manα1-3	10.3%
3-1	Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-4Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc-2-AB Galβ1-4GlcNAcβ1-2	19.6%
3-2	Galβ1-4GlcNAcβ1-2Manα1-6 GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc-2-AB Galβ1-4GlcNAcβ1-4Manα1-3 Galβ1-4GlcNAcβ1-2	12.2%
F1-1a	Fucα1-6 Manα1-6 Galβ1-4GlcNAcβ1-4Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc-2-AB Galβ1-4GlcNAcβ1-2	1.2%
F1-1b	Fucα1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-2Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc-2-AB	7.5%
F1-2	Fucα1-6 Galβ1-4GlcNAcβ1-2Manα1-6 GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc-2-AB Galβ1-4GlcNAcβ1-2Manα1-3	6.0%
F2-1	Fucα1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-4Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc-2-AB Galβ1-4GlcNAcβ1-2	9.3%
F2-2	Fucα1-6 Galβ1-4GlcNAcβ1-2Manα1-6 GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc-2-AB Galβ1-4GlcNAcβ1-4Manα1-3 Galβ1-4GlcNAcβ1-2	8.6%

samples from choriocarcinoma patients [11–13,15,36]. On the other hand, hCG derived from a choriocarcinoma cell line possesses a large quantity of bisecting GlcNAc structures [34]. The reasons for these discrepancies are not clear. One possibility is that the GnT-III activity in choriocarcinoma tissue may be weak unlike that found in choriocarcinoma cell lines. The acceptor substrate specificity of GnT-III is broad, and the addition of bisecting GlcNAc eliminates the potential for α-mannosidase-II, GnT-II, GnT-IV, GnT-V, and core α1,6-fucosyltransferase to act subsequently [37,38]. Because of these findings, if the GnT-III activity is moderate or high in choriocarcinoma tissue, we suppose bisecting GlcNAc structures must be contains in choriocarcinoma hCG. In Figure 7, we confirmed the abnormal biantennary sugar chain structure using serial glycosidase digestion and MALDI-TOF/MS. Jacoby *et al*. determined the glycoforms of the human chorionic gonadotropin β-core fragment by MALDI-TOF/MS and assigned an inferred sugar mass of abnormal biantennary structure of 1299.2 [39]. The discrepancy between our mass data (m/z:1460.6) and theirs (m/z:1299.2) is due to the presence or absence of 2-AB and a sodium adduct. Since their method could not distinguish differences in structure between abnormal and normal biantenna, our procedure is advantageous from this stand point. We have successfully confirmed the existence of an abnormal biantennary structure in hCG produced by the choriocarcinoma cell line JEG-3. This supports our hypothesis that up-regulated GnT-IV activity is responsible for the formation of an abnormal biantennary sugar chain structure in hCG. Moreover, the JEG-3 cell line will be useful to further elucidate the mechanism of abnormal biantennary structure formation.

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