Structural Differences Found in the Asparagine-linked Sugar Chains of Human Chorionic Gonadotropins Purified from the Urine of Patients with Invasive Mole and with Choriocarcinoma

Tamao Endo, Ryuichiro Nishimura, Takehiro Kawano, Matsuto Mochizuki, and Akira Kobata

Department of Biochemistry, Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108 [T. E., T. K., A. K.], and Department of Obstetrics and Gynecology, Kobe University School of Medicine, Chuo-ku, Kobe 650 [R. N., M. M. J., Japan]

ABSTRACT

Paper electrophoresis of the oligosaccharide fraction obtained by hydrazinolysis of human chorionic gonadotropin (HCG), which was purified from the urine of two patients with invasive mole, gave fractionation patterns different from those of normal and hydatidiform mole HCGs. Structural study of the oligosaccharides in each fraction revealed that the triantennary complex-type asparagine-linked sugar chains, specifically found in choriocarcinoma HCGs, were included in the two HCG samples. However, the unusual biantennary complex-type sugar chains, which are also specific for the tumor HCGs, were not detected at all. This result indicated that abnormal expression of N-acetylgalactosaminyltransferase IV during the development of choriocarcinoma occurs in two steps: (a) ectopic expression of the regular N-acetylgalactosaminyltransferase IV; and (b) modification of the substrate specificity of the enzyme.

INTRODUCTION

HCG, a glycohormone produced by the trophoblast, is composed of α- and β-subunits and contains about 30% carbohydrate (1–3). Both subunits contain two asparagine-linked sugar chains, the structures of which were elucidated as follows (4, 5).

\[ \text{α-D-NeuAc} \rightarrow 3 \text{Galα1-4GlcNAcβ1-2Man} \]
\[ \text{±Fucα1} \]
\[ \text{±NeuAcα2-3Galβ1-4GlcNAcβ1-2Man} \]
\[ \text{α-D-NeuAc} \rightarrow 3 \text{Galβ1-4GlcNAcβ1-2Man} \]

Quite recently, the structures of the asparagine-linked sugar chains of HCG purified from the urine of patients with trophoblastic diseases hydatidiform mole and choriocarcinoma have also been elucidated (6, 7). Although the carbohydrate structures of hydatidiform mole HCG are the same as those of normal HCG, choriocarcinoma HCG contains triantennary and unusual biantennary complex-type sugar chains which are not found in normal HCG (4–7). Because the changes are specific and always found in the choriocarcinoma HCGs, they are expected to be useful for the diagnosis of this particular tumor.

It is important to determine at what stage of the pathological process leading to choriocarcinoma the alteration of sugar chains starts. Hydatidiform mole is considered to be a benign lesion. However, some of the moles show apparently more malignant characteristics such as invasion into the surrounding tissues and metastasis and are discriminated from most typical moles by the name of invasive mole. We have therefore performed the structural study of the sugar chain of HCGs purified from the urine of patients with invasive mole.

MATERIALS AND METHODS

Purification of HCG. HCG samples were purified from the urine of two patients with invasive mole by the same procedure as described in a previous paper (8). In this paper these HCG samples will be called invasive mole HCG. The case histories of the two patients were as follows.

Case 1 (Shi), a 34-year-old woman, gravida 4, para 3, was admitted to Kobe University Hospital with molar pregnancy on October 7, 1985. She underwent cervical dilation and evacuation of the uterine content, and total hydatidiform mole was diagnosed pathologically. At 20–30 days after the evacuation, the level of HCG stopped declining and began to increase gradually. Chest X-ray revealed a metastatic lesion in the right lung. At that time the patient was diagnosed to have clinical invasive mole by the criteria of Japan Society of Obstetrics and Gynecology. She was given etoposide (1 course, 100 mg/day for 5 days). Following 3 courses, the level of HCG decreased below 1 mlU/ml assayed by HCG β-CTP enzyme immunoassay and the metastatic lesion was no longer seen in chest X-rays. The HCG sample was extracted from the urine collected before evacuation of the mole.

Case 2 (Kam), a 48-year-old woman, gravida 5, para 2, was admitted to Kobe University Hospital with genital bleeding on August 29, 1985. The concentration of urinary HCG was about 4,500,000 units/liter. Trophoblastic disease was suspected from the findings of the pelvic angiography and the ultrasonography. She underwent a simple hysterectomy and the pathological diagnosis was invasive mole. The HCG titer rapidly decreased to below 1 mlU/ml assayed by HCG β-CTP enzyme immunoassay 40 days after the operation. The HCG sample was extracted from urine collected before hysterectomy.

Liberation of the Asparagine-linked Sugar Chains of HCG as Oligosaccharides. Purified HCG samples (4 mg each) were subjected to hydrazinolysis and the liberated oligosaccharides were N-acetylated according to the method described previously (9). This procedure is well confirmed to release the asparagine-linked sugar chains of glycoproteins quantitatively as oligosaccharides. To one-fifth of the oligosaccharide fraction, 25 nmol of lactose were added as an internal standard and each mixture was reduced with NaBH₄ (360 mM/C/mmol; New England Nuclear, Boston, MA) to obtain tritium-labeled oligosaccharide mixture. The remaining four-fifths of the oligosaccharide fraction was reduced with NaBH₄ (2 mg) to obtain deuterium-labeled oligosaccharide mixture for methylation analysis. [3H]Lactitol and [3H]oligosaccharide fractions in the radioactive oligosaccharide mixture were separated by paper chromatography using butanol:1:ethanol:water (4:1:1) as a solvent. The radioactivities incorporated into the oligosaccharide fractions were approximately 2.3 × 10⁸ cpm/mg of the two HCG samples. Based on the specific activity of NaBH₄ calculated from the radioactivities...
RESULTS AND DISCUSSION

Analysis of the Oligosaccharides Liberated from Invasive Mole HCGs by Paper Electrophoresis. The 3H-labeled oligosaccharide fractions were subjected to paper electrophoresis at 73 V/cm for 90 min with a buffer system of pyridine:acetic acid:water (3:1:387), pH 5.4. As shown in Fig. 1, the samples from invasive mole HCGs gave fractionation profiles that were different from that of normal HCG. Both invasive mole HCGs gave three acidic fractions in contrast to normal HCG, which has only two acidic fractions. The molar percentage distributions of radioactive components from each sample were calculated on the basis of their radioactivities as: Kam, N (5.1%), A1 (30.6%), A2 (57.2%), and A3 (7.1%); Shi, N (11.2%), A1 (45.0%), A2 (37.7%), and A3 (6.1%).

All acidic components from the two invasive mole HCGs were completely converted to neutral components by sialidase digestion indicating that acidic nature of the oligosaccharides in fractions A1, A2, and A3 can be ascribed to sialic acid residues. All the sialic acids liberated from invasive mole HCGs by sialidase treatment were N-acetyneuraminic acid (data not shown). On treatment with mild acid hydrolysis (0.01 N HCl at 100°C for 3 min) for partial removal of sialic acid residues, only a neutral oligosaccharide fraction was formed from A1 in addition to the original acidic fraction. In contrast, A2 gave another acidic fraction and A3 gave two acidic fractions together with a neutral fraction (data not shown). These results indicated that components in fractions A1, A2, and A3 contain mono-, di-, and trisialyl oligosaccharides, respectively.

Analysis of the Desialylated Oligosaccharide Fractions by Gel Permeation Chromatography. The results thus far described indicate that both invasive mole HCGs contain different sets of oligosaccharides from normal HCG. Methylation analysis of the neutral oligosaccharide mixtures obtained by sialidase treatment of the deuterium-labeled oligosaccharide fractions of the two invasive mole HCG samples gave 2,3,4,6-tetra-O-methylfucitol, 2,3,4,6-tetra-0-methylgalactitol, 2,3,4,6-tetra-O-methylmannitol, 3,4,6-tri-O-methylmannitol, 3,4,6-di-O-methylmannitol, 2,4-di-O-methylmannitol, 3,6-di-O-methyl-2-N-acetylamino-2-deoxyglucitol, 1,3,5,6-tetra-O-methyl-2-N-acetylamino-2-deoxyglucitol, and 1,3,5-tri-O-methyl-2-N-acetylamino-2-deoxyglucitol. These results coincided with the methylation analysis data for the neutral fractions from choriocarcinoma HCGs (6, 7) but not with those from normal HCGs in which 3,6-di-O-methyl mannitol was not detected (4). In order to clarify the differences in more detail, the neutral oligosaccharide mixtures were analyzed by Bio-Gel P-4 column chromatography. As shown in Fig. 2, each separated into three fractions, NI, NII, and NIII, in molar ratios calculated on the basis of their radioactivities as: Kam, NI (12.0%), NII (68.0%), and NIII (20.0%); Shi, NI (9.0%), NII (51.0%), and NIII (40.0%).

As reported previously (4), normal HCG gave only two fractions corresponding to NII and NIII (Fig. 2A). The elution patterns of the oligosaccharide fractions obtained from the two invasive mole HCGs are very similar to those of choriocarcinoma HCGs (6, 7). However, an interesting qualitative difference was found during the structural studies of oligosaccharides in each fraction as will be described in the following sections.

Structural Analysis of the Desialylated Oligosaccharides by Sequential Exoglycosidase Digestion and by Lectin Affinity Chromatographies. In order to elucidate whether the asparagine-linked sugar chains of invasive mole HCGs are exactly the same as those of choriocarcinoma HCGs or not, structures of the oligosaccharides in the three fractions separated by Bio-Gel
Fig. 2. Bio-Gel P-4 column chromatograms of the desialylated oligosaccharide mixtures. Black arrows, elution positions of glucose oligomers added as internal standards; arrow numbers, numbers of glucose residues. White arrows, elution positions of authentic oligosaccharides: a, Galβ1-GlcNAcβ1-Manβ1-GlcNAc-Fuc-GlcNAcOT; b, Galβ1-GlcNAcβ1-Manβ1-GlcNAc-GlcNAcOT; c, Galβ1-GlcNAc-Manβ1-GlcNAc-GlcNAcOT. A, the radioactive neutral oligosaccharide mixture obtained by sialidase digestion of the oligosaccharide fractions liberated from a normal HCG; B and C, those from invasive mole HCGs from Kam and Shi, respectively.

P-4 column chromatography were investigated. As in the case of choriocarcinoma HCGs, fractions NI and NIII were both found to contain the two oligosaccharides shown in Fig. 3. Since the experimental results that led to these structural conclusions were the same as reported in a preceding paper (6), they will not be repeated here.

Fraction NII from choriocarcinoma HCGs was shown to contain the following four oligosaccharides (6, 7).

When incubated with diplococcal β-galactosidase, the two radioactive peaks in fractions NII from invasive mole HCGs were converted to the two radioactive components with the same mobilities as authentic GlcNAc2-Man3-GlcNAc-Fuc-GlcNAcOT and GlcNAc2-Man3-GlcNAc-GlcNAcOT as in the case of fractions NII from choriocarcinoma HCGs (Fig. 4A). However, the degalactosylated oligosaccharides from invasive mole HCGs were completely converted to two radioactive components with the same mobilities as authentic Manβ1-GlcNAc-Fuc-GlcNAcOT and Manβ1-GlcNAc-GlcNAcOT by diplococcal β-galactosidase digestion; B, a mixture of the radioactive peaks in A incubated with diplococcal β-N-acetyl-hexosaminidase; C and D, elution profiles of fraction NII obtained from a choriocarcinoma HCG and fraction NII in Fig. 2C from a D. stramonium agglutinin-agarose column, respectively. Fraction NII from a choriocarcinoma HCG was obtained as reported previously (6). Although the data of Shi were shown here, fraction NII from another invasive mole HCG (Kam) gave the same results as shown here.

Fig. 3. Structures and percentage molar ratio of the asparagine-linked sugar chains of two invasive mole HCGs.

Fig. 4. Sequential exoglycosidase digestion of the fraction NII in Fig. 2C and its behavior on a D. stramonium agglutinin-Sepharose column. Black arrows are the same as in Fig. 2; white arrows, elution position of authentic oligosaccharides. A, GlcNAc2-Manβ1-GlcNAc-Fuc-GlcNAcOT; b, GlcNAc2-Manβ1-GlcNAc-GlcNAcOT; c, Manβ1-GlcNAc-Fuc-GlcNAcOT; d, Manβ1-GlcNAc-GlcNAcOT. A, Bio-Gel P-4 column chromatogram of fraction NII in Fig. 2C after diplococcal β-galactosidase digestion; B, a mixture of the radioactive peaks in A incubated with diplococcal β-N-acetyl-hexosaminidase; C and D, elution profiles of fraction NII obtained from a choriocarcinoma HCG and fraction NII in Fig. 2C from a D. stramonium agglutinin-agarose column, respectively. Fraction NII from a choriocarcinoma HCG was obtained as reported previously (6). Although the data of Shi were shown here, fraction NII from another invasive mole HCG (Kam) gave the same results as shown here.
\(\beta\)-N-acetylhexosaminidase treatment (Fig. 4B). In the case of the degalactosylated NII fraction from choriocarcinoma HCGs, four radioactive components were formed by the same treatment (6). Furthermore, the NII fractions from invasive mole HCGs bound completely to a concanavalin A-Sepharose column and eluted with 0.1 M \(\alpha\)-methylmannopyranoside solution, in contrast to those from choriocarcinoma HCGs which were separated into a bound and an unbound fraction (6). These results indicate that invasive mole HCGs contain oligosaccharides I but not II shown above.

In order to confirm the complete absence of oligosaccharides II in invasive mole HCGs, the behavior of their NII fractions on a \(D.\) stramonium agglutinin-Sepharose column was investigated. As reported previously (11), oligosaccharides which contain the Gal\(\beta1\)\(+\)4GlcNAc\(\beta1\)\(+\)4(Gal\(\beta1\)\(+\)4GlcNAc\(\beta1\)\(+\)2)Man group are retarded by the column. Since oligosaccharides II contain the group and oligosaccharides I do not, they should be discriminated by the affinity column chromatography. The NII fraction from a choriocarcinoma HCG was actually separated into two fractions by a \(D.\) stramonium agglutinin-Sepharose column (Fig. 4C). In contrast, those from invasive mole HCGs completely passed through the lectin column without any interaction (Fig. 4D). Therefore, only two biantennary complex type asparagine-linked sugar chains shown in Fig. 3 should be included in invasive mole HCGs.

The data reported in this paper indicate that part but not all of the abnormalities of the neutral portion of the asparaginelinked sugar chains, which were found in choriocarcinoma HCGs, is also induced in the sugar chains of invasive mole HCGs. Detection of fucosylated monoantennary sugar chains (oligosaccharide e in Fig. 3) and the increased ratio of fucosylated sugar chains (Kam 66.0% and Shi 65.5% as compared to 25% in normal HCG) indicated that enhancement of fucosyltransferase which forms the Fuc\(\alpha1\)\(+\)6GlcNAc group already occurs in invasive mole. The presence of triantennary sugar chains in invasive mole HCGs indicated that expression of \(N\)-acytelyglucosaminyltransferase IV, which is responsible for the formation of the GlcNAc\(\beta1\)\(+\)4Man\(\alpha1\)\(+\)3 group, also occurs in this lesion. However, complete absence of unusual biantennary sugar chains in invasive mole HCGs indicates that the newly expressed \(N\)-acytelyglucosaminyltransferase IV can transfer an \(N\)-acytelyglucosamine residue to biantennary complex-type sugar chains but not to monoantennary sugar chains. This substrate specificity is considered to be the same as that of normal tissues (13). Therefore, transformational change induced in the \(N\)-acytelyglucosaminyltransferase in choriocarcinoma might be brought about in two steps: (a) ectopic expression of the regular \(N\)-acytelyglucosaminyltransferase IV; and (b) modification of the substrate specificity of the enzyme.

It has often been suggested from structural studies of the sugar chains of complex carbohydrates that glycosyltransferases of tumor cells might have wider substrate specificity than their normal counterparts. However, the mechanism of this deterioration is not yet known. Expression and modification of the structural gene of \(N\)-acytelyglucosaminyltransferase IV during the process leading to malignancy of trophoblasts might be a useful target to solve this interesting and important problem.

A comment should be added to the extent of sialylation of the sugar chains of HCG samples from various trophoblastic diseases. As reported previously (7), a large variation was found in the sialylation level of the sugar chains of choriocarcinoma HCGs. We have recently observed an interesting case in which the asparagine-linked sugar chains of the HCG obtained from urine of a choriocarcinoma patient were the most completely sialylated while the sample obtained from the same patient at a later stage contained nonsialylated sugars. The sugar chains of the two invasive mole HCGs, reported in this paper, are also highly sialylated. Therefore, it is probable that the aberration of sialyltransferase might occur only at the advanced stage of choriocarcinoma. These complicated changes in the structure of the sugar chains of HCG molecule thus far described might become a useful marker for the diagnosis and prognosis of choriocarcinoma, if proper methods to detect each structural change are devised.

Any method to detect the structural change induced in the invasive mole HCG will be useful for effective treatment of hydatidiform mole. Most of the patients with hydatidiform mole regress spontaneously. However, approximately 10% of the patients will develop persistent gestational trophoblastic diseases and need to be treated with therapeutic agents. Although many studies have demonstrated that prophylactic chemotherapy reduced the development of persistent trophoblastic diseases, use of prophylactic chemotherapy at the time of molar evacuation is still highly controversial because of the substantial drug toxicity and the danger of selectively growing resistant cells. Therefore, if the patient could be diagnosed to have an invasive mole by examination of the sugar chains of urinary HCG, indiscriminate prophylactic chemotherapy would be avoided.

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