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Eur J Cancer, Vol. 27, No. 3, pp. 277–280, 1991.
Printed in Great Britain

0277–5379/91 $3.00 + 0.00

Structures of the Asparagine-linked Sugar Chains of Human Chorionic Gonadotropin from a Patient with Extragonadal Germ Cell Tumour

Tamao Endo, Ryuichiro Nishimura, Shin-Ichi Teshima, Hisanao Ohkura, Shigeaki Baba and Akira Kobata

Human chorionic gonadotropin (hCG) purified from the urine of a male patient with extragonadal germ cell tumour contained four asparagine-linked sugar chains in one molecule. The sugar chains were quantitatively released from the polypeptide moiety by hydrazinolysis and recovered as oligosaccharides after *N*-acetylation. The oligosaccharide mixture was separated into a neutral (N) and three acidic (A1, A2 and A3) fractions by anion-exchange column chromatography. By sequential exoglycosidase digestion, methylation analysis and lectin column chromatography, the structures of these oligosaccharides were found to be the same as those of female gestational choriocarcinoma hCGs. Both contain eight kinds of sugar chains: triantennary, abnormal and normal biantennary, and monoantennary complex-type sugar chains with or without a fucosylated core portion.
Eur J Cancer, Vol. 27, No. 3, pp. 277–280, 1991.

INTRODUCTION

HUMAN CHORIONIC GONADOTROPIN (hCG), a glycoprotein produced by the trophoblast, is excreted into serum and urine. Urinary hCG is found not only in normal pregnant women but also in patients with trophoblastic diseases. In hCGs purified from the urine of normal pregnant women [1, 2] or patients with trophoblastic diseases including hydatidiform mole [3], invasive mole [4] and choriocarcinoma [3, 5], structural changes of the asparagine-linked sugar chains are induced in hCG produced by malignant tissues. Although hCG from the hydatidiform mole patient has the same sets of oligosaccharides as normal hCG, that from the invasive mole patient has 2,4-branched triantennary oligosaccharides together with the sugar chains found in normal hCG. In hCGs from the choriocarcinoma patient, abnormal biantennary oligosaccharides are also detected. With this altered glycosylation of hCG, we could successfully discriminate invasive mole or choriocarcinoma hCG from normal pregnancy or hydatidiform mole hCG in an immobilised *Datura stramonium* agglutinin (DSA) column [6].

Although rare, hCG is produced in non-gestational choriocarcinoma-like teratomatous choriocarcinoma. It is important to know whether structural changes also occur in the sugar chains of non-gestational choriocarcinoma hCG. We have therefore studied the sugar chains of hCG purified from the urine of a male patient with a primary extragonadal germ cell tumour of mediastinum.

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Revised 20 Nov. 1990; accepted 23 Nov. 1990.

MATERIALS AND METHODS

Purification of hCG

hCG was purified from the urine of a patient with extragonadal germ cell tumour [7]. This hCG will be called germ hCG.

Case history

A 24-year-old man was admitted to the National Cancer Center Hospital because of cough and dyspnoea. Multiple and massive tumours were noted in the bilateral lungs, mediastinum, liver and para-aortic lymph nodes with a stenosis of the colon and bloody fluid in bilateral pleural and pericardial spaces. He had soft skin and gynaecomastia with normal genital organs, and was diagnosed as having an extragonadal germ cell carcinoma at late stage. His urinary hCG, assayed by anti-whole hCG antibody, was 1024 000 IU/ml and that in the peripheral blood was 456 900 IU/ml. After continuous pleural and pericardial aspirations, four courses of chemotherapy with cisplatin, etoposide and human recombinant granulocyte colony-stimulating factor and thoracotomy, he received intensive chemotherapy with cisplatin, etoposide and cyclophosphamide followed by whole-body irradiation and autologous bone marrow transplantation. A complete remission was obtained and has lasted for 14 months. Histology of the excised pulmonary and mediastinal tumours showed no viable tumour cells but the tissue was positively stained with anti-hCG β-subunit antibody. 10 litres of urine were collected at the sixth week of admission, when urinary hCG was about 100 000 IU/ml.

Liberation of the asparagine-linked sugar chains of hCG as oligosaccharides

Purified hCG (2.1 mg) was subjected to hydrazinolysis and the liberated oligosaccharides were *N*-acetylated [4]. To one-fifth of the oligosaccharide fraction, 10 nmol/xylose was added as an internal standard and the mixture was reduced with NaB^3H_4 (15 GBq/mmol, New England Nuclear) to obtain tritium-labelled oligosaccharide. The remaining four-fifths of the oligosaccharide fraction was reduced with NaB^2H_4 (2 mg) to obtain deuterium-labelled oligosaccharide mixture for methylation analysis. [^{3}H]xytitol and [^{3}H]oligosaccharide fraction in the radioactive oligosaccharide mixture were separated by paper chromatography with butanol-1:ethanol:water (4:1:1) as solvent. On the basis of the radioactivities incorporated into xytitol and the oligosaccharide mixture and the molecular weight of hCG (48 000), it was estimated that four sugar chains are included in one molecule of hCG.

Analytical methods

Exoglycosidases were as described [4, 5]. DSA-Sepharose and *Aleuria aurantia* lectin (AAL)-Sepharose column chromatography were done [4, 8, 9]. Methylation analysis, sequential exoglycosidase digestion, Bio-Gel P-4 column chromatography, and other experimental procedures have been described [4, 5, 10].

RESULTS

Fractionation of oligosaccharides by anion-exchange column chromatography

Tritium-labelled oligosaccharide fraction was subjected to anion-exchange column chromatography with a mono Q HR5/5 column [10]. As shown in Fig. 1A, the oligosaccharide mixture was separated into a neutral (N) and three acidic (A1, A2 and A3) fractions. The percent molar ratio of N, A1, A2 and A3 on the basis of their radioactivities was 9, 49, 31 and 11. The

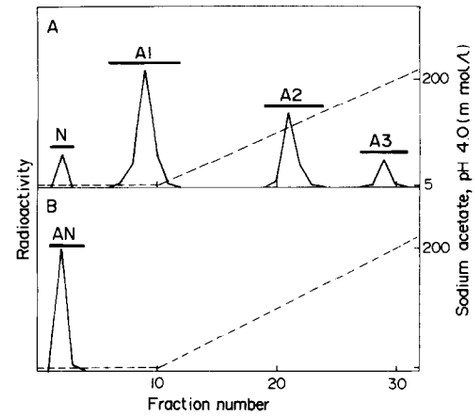

Fig. 1. Anion-exchange column chromatography of radioactive oligosaccharides. A = radioactive oligosaccharides liberated from germ hCG; B = acidic components (A1, A2, and A3 in [A]) digested with NDV sialidase.

pooled acidic components were completely converted to neutral components by exhaustive Newcastle disease virus (NDV) sialidase digestion (AN in Fig. 1B). Since NDV sialidase cleaves the Siaα2→3Gal linkage but not the Siaα2→6Gal linkage [11], the acidic oligosaccharides of germ hCG should contain only the Siaα2→3Gal linkage. That fractions A1, A2 and A3 contain monosialyl, disialyl and trisialyl oligosaccharides was confirmed by analysing the numbers of radioactive oligosaccharides obtained after mild acid hydrolysis (0.01 mol/l HCl at 100°C for 3 min) [3, 4].

Fractionation of the desialylated oligosaccharides by AAL-Sepharose and gel permeation chromatography

Methylation analysis of the neutral oligosaccharide mixtures obtained by sialidase treatment of the deuterium-labelled oligosaccharide fraction of the germ hCG sample gave 2,3,4-tri-O-methylfucitol, 2,3,4,6-tetra-O-methylgalactitol, 2,3,4,6-tetra-O-methylmannitol, 3,4,6-tri-O-methylmannitol, 3,6-di-O-methylmannitol, 2,4-di-O-methylmannitol, 3,6-di-O-methyl-2-N-methylacetamido-2-deoxyglucitol, 1,3,5,6-tetra-O-methyl-2-N-methylacetamido-2-deoxyglucitol and 1,3,5-tri-O-methyl-2-N-methylacetamido-2-deoxyglucitol. The data coincided with that of the neutral fractions from choriocarcinoma hCGs [5]. When the neutral radioactive oligosaccharide mixture (N plus AN) was subjected to AAL-Sepharose column chromatography, 60% of the fraction passed through the column without interaction. The remainder was retained and eluted with buffer containing 1 mmol/l L-fucose. The fractions retained and not retained by the column were named N-AN (+F) and N-AN (−F), respectively. When these two fractions were subjected to Bio-Gel P-4 column chromatography, both were separated into three fractions: N-AN (−F), *a*, *b* and *c* in Fig. 2A and N-AN (+F), *d*, *e* and *f* in Fig. 2B. Previously, Yamashita *et al.* [9] reported that an AAL-Sepharose column is useful for the group separation of mixtures of oligosaccharides, because all asparagine-linked sugar chains containing an α-fucosyl residue linked at the C-6 position of the proximal *N*-acetylglucosamine residue of their trimannosyl core bind to such a column, but those without the fucose residue do not. Because of this binding specificity of an AAL-Sepharose column, the oligosaccharides in fractions *a*, *b* and *c* were assumed to contain the non-fucosylated trimannosyl core, while those in fractions *d*, *e* and *f* the fucosylated trimannosyl core.

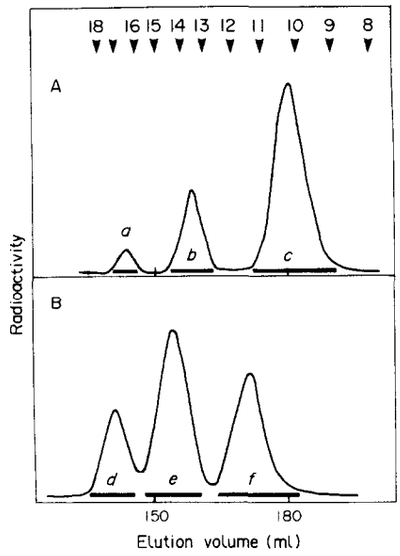

Fig. 2. Bio-Gel P-4 column chromatography of neutral oligosaccharide fractions separated by AAL-Sepharose column chromatography. Arrow = elution positions of glucose oligomers (numbers indicate glucose units). A and B = elution profiles of AAL-fraction that passed through or was retained (fractions N plus AN in Fig. 1), respectively.

Structures of neutral oligosaccharides

To elucidate the structures of neutral oligosaccharides in the fractions separated by Bio-Gel P-4 column chromatography, each radioactive oligosaccharide fraction was subjected to sequential exoglycosidase digestion. Since the results were the same as those described in the previous papers that reported the sugar chain structures of choriocarcinoma hCGs [3–5], details will not be repeated here. The results indicated that fractions *a* and *d* contained triantennary, *b* and *e* biantennary and *c* and *f* monoantennary complex-type sugar chains. The molar ratio of these sugar chains is summarised in Fig. 3.

DISCUSSION

We found that all of the abnormalities of the desialyated asparagine-linked sugar chains, which were found in choriocarcinoma hCGs, were also induced in the sugar chains of germ hCG (Fig. 3). Both tumour hCGs contained triantennary (oligosaccharides *a* and *b*), abnormal biantennary (oligosaccharides *b*-II and *e*-II) and fucosylated monoantennary (oligosaccharide *f*) sugar chains, which were not found in the hCGs purified from the urine of normal pregnant women [1, 2]. This result is interesting because both hCGs originate from a different organ. hCGs used in the previous studies were purified from the urine of gestational choriocarcinoma patients [3, 5]. The hCG used in this study was purified from the urine of a male patient with a primary extragonadal germ cell tumour of mediastinum. The presence of abnormal biantennary sugar chains in tumour hCGs indicated that the newly expressed *N*-acetylglucosaminyltransferase IV, which is responsible for the formation of the GlcNAcβ1→4Manα1→group, can transfer an *N*-acetylglucosamine residue to monoantennary sugar chains. Such a transfer does not occur in the glycosyltransferase system of normal tissues because the presence of abnormal biantennary sugar chains in normal human glycoproteins has not been reported. Although Gleeson and Shachter [12] reported that monoantennary sugar chains can be a poor substrate for solubilised *N*-acetylglucosaminyltransferase IV, the enzyme in the Golgi membrane of normal cells might be controlled not to work on monoantennary sugar chains by unknown mechanisms. The abnormal biantennary sugar chains were also found in two other human tumour glycoproteins: carcinoembryonic antigen samples purified from liver metastases of primary colon cancer [13] and γ-glutamyltranspeptidases purified from hepatocellular carcinoma [14]. These results indicated that the abnormal biantennary sugar chains could be a good marker for the diagnosis of several different tumours.

The asparagine-linked sugar chains of hCG are essential for the glycoprotein to express its hormonal activity [15, 16]. Study with site-directed mutagenesis revealed that the sugar chains linked to Asn^{52} of the α-subunit play a key role in signal transduction [17]. Choriocarcinoma hCG showed less hormonal activity than its normal counterpart. Because the polypeptide moieties of both hCGs are the same as those in normal hCG, the lowered hormonal activity of these hCGs should be induced by altered glycosylation [18]. In addition to the same altered glycosylation as in choriocarcinoma hCG, the germ hCG used for this study contained nicked β-subunit as found in choriocarcinoma hCG [19, 20] (data not shown). hCG with such a β-subunit was reported to have lower hormonal activity [21]. Therefore the germ hCG would be expected to have lower activity than normal hCG. However, the patient has large breasts and soft skin, probably due to the production of extremely large amounts of hCG by the tumour. Furthermore, we have previously found that altered glycosylation of choriocarcinoma hCG is induced both in α-subunit and β-subunit [22]. Because the four asparagine-linked sugar chains of normal hCG are site-specifically matured [2], the mechanism to induce the altered

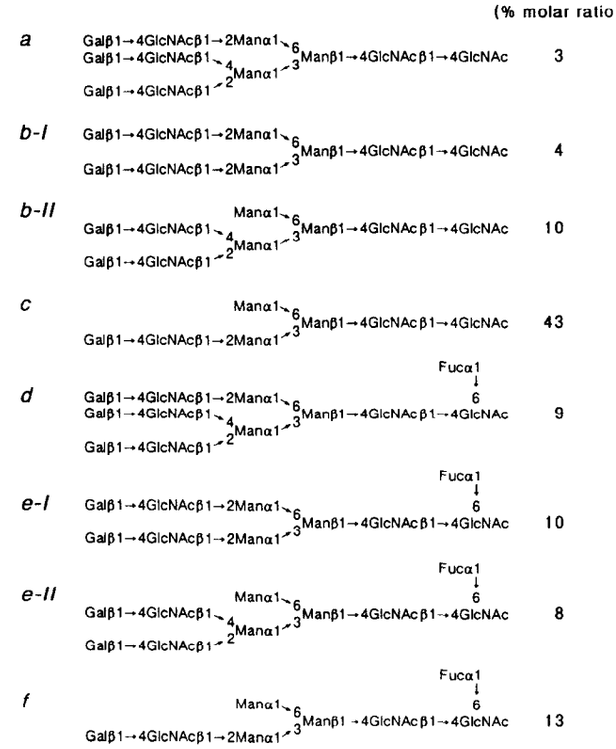

Fig. 3. Proposed structures for neutral oligosaccharides in fractions N and AN obtained from germ hCG and percent molar ratio calculated on basis of radioactivity incorporated in each oligosaccharide.

glycosylation at each asparagine site of hCG might be an interesting target for research.

It is well known that tumours produce ectopically whole hormone molecules or their subunit. The mechanism is unknown. As reported [23], a large structural difference was found between the sugar chains of eutopic α-subunit produced by trophoblast and ectopic α-subunit produced by adenocarcinoma. The eutopic α-subunit contained biantennary sugar chains only but the ectopic α-subunit contained 2,6-branched triantennary sugar chains in addition. This indicates that *N*-acetylglucosaminyltransferase V, which is responsible for the formation of the GlcNAcβ1→6Manα1→group, is highly expressed in this tumour. Enhanced expression of *N*-acetylglucosaminyltransferase V, is widely found in malignant cells [24, 25], and is considered to be correlated to the metastatic potential of tumours [26]. The alterations of the sugar chains of glycoproteins by malignant transformation are diverse. However, the changes induced in a particular glycoprotein produced in a particular tumour are constant. Since many glycosyltransferases have started to be cloned [27], expression and modification of the structural gene of glycosyltransferases during malignancy will become an important target.

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Acknowledgement—We thank Yumiko Kimizuka for secretarial assistance. This work was supported by grant-in-aid for special project research (cancer-bioscience) from the Ministry of Education, Science and Culture of Japan.