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Structures, Function, and Transformational Changes of the Sugar Chains of Glycohormones

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Human chorionic gonadotropin (hCG), human luteinizing hormone, human thyroid-stimulating hormone, and human follicle-stimulating hormone are closely related family of proteins which share a common α-subunit. However, their sugar moieties are quite different.

hCG contains five acidic asparagine-linked sugar chains. These five sugar chains are derived by sialylation from three neutral oligosaccharides: two biantennary (N-1 and N-2) and one monoantennary (N-3) complex-type oligosaccharides. Although hCG purified from the urine of pregnant women is more enriched in sialylated sugar chains than that purified from placenta, the molar ratio of N-1, N-2, and N-3 of these two hCGs are the same (1:2:1). Comparative study of the sugar moieties of the α- and β-subunits of hCG revealed that α contains 1 mol each of N-2 and N-3, while β contains 1 mol each of N-1 and N-2. This specific distribution of oligosaccharides at the four asparagine loci of the hCG molecule is now helping us to consider the functional role of the sugar moiety of glycohormones.

hCG is produced not only by the trophoblast but also by various trophoblastic diseases. The hCGs purified from the urine of patients with hydatidiform mole contain the same oligosaccharides as normal hCG. However, those from the urine of choriocarcinoma patients contain five additional neutral oligosaccharides. In contrast, hCGs from invasive-mole patients contain three of the five oligosaccharides, specifically found in choriocarcinoma hCGs.

The malignant transformational change of the sugar moiety of hCG can be explained by an increase of a fucosyltransferase, which forms the Fucα1→6GlcNAc group and by ectopic expression and subsequent modification of *N*-acetylglucosaminyltransferase IV. The appearance of tumor-specific sugar chains of hCG has been used to develop a new diagnostic method for invasive mole and choriocarcinoma.

Abbreviations used: hCG, human chorionic gonadotropin; hFSH, human follicle-stimulating hormone; hLH, human luteinizing hormone; hTSH, human thyroid stimulating hormone; NeuAc, *N*-acetylneuraminic acid; Gal, galactose; Man, mannose; Fuc, fucose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine.

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Human chorionic gonadotropin (hCG) derived from human placenta and three human pituitary hormones—luteinizing hormone (hLH), thyroid-stimulating hormone (hTSH), and follicle-stimulating hormone (hFSH)—are all composed of two noncovalently linked subunits, designated α and β. Studies of amino acid sequences of these hormones have revealed that they are a closely related family of proteins which share a common α-subunit [1]. Although they contain large amount (18–30%) of carbohydrates, information regarding the structure of their sugar moieties was delayed because of the limited number of samples available. Structural information on the sugar moiety was first obtained for hCG. Elucidations of the entire structure of its sugar chains and of the unique distribution of each oligosaccharide on the polypeptide chains of its two subunits are now leading us to consider how the sugar moiety of glycoproteins plays a role as a recognition signal. Furthermore, recent findings of the cancerous change induced in the sugar chains of hCG is opening a new field for the diagnosis and prognosis of choriocarcinoma.

This review paper will summarize the miscellaneous findings on the sugar chains of hCG in order to help readers to consider the functional role of the sugar chains of glycoproteins and also the clinical application of the malignant transformational change of the sugar moieties of glycoproteins.

STRUCTURES OF THE SUGAR CHAINS OF hCG

The molecular weights of α- and β-subunits of hCG are 16,000 and 30,000, respectively. Each subunit contains two asparagine-linked sugar chains [2,3]. In addition, the β-subunit contains four mucin-type sugar chains. Establishment of controlled hydrazinolysis [4], a method for the quantitative release of asparagine-linked sugar chains as oligosaccharides, has opened the way for fingerprinting the asparagine-linked sugar chains of glycoproteins. This novel technique was applied for the structural study of the asparagine-linked sugar chains of hCG purified from pooled urine of pregnant women [5]. In Figure 1, structures of the whole asparagine-linked sugar chains of urinary hCG are summarized together with the structures of mucin-type sugar chains elucidated by Kessler et al. [6]. Recently, Cole et al. [7] reported that sialyl derivatives of the Galβ1→4GlcNAcβ1→?(Galβ1→3)GalNAc occur as minor mucin-type sugar chains of hCG. Occurrence of oligosaccharide A-1 in hCG was also confirmed by Kessler et al. [8].

Sugar chains of glycoproteins are synthesized by sequential addition of monosaccharides from nucleotide sugars to the preformed sugar chain acceptors [9]. Accordingly, the structure of the final sugar chain produced is determined by the specificity of each glycosyltransferase for a particular nucleotide sugar and for a specific sugar chain acceptor, and by its ability to form a particular type of linkage, including an anomeric configuration. Since no template is included in this biosynthetic machinery, microheterogeneity has been considered as an inherent characteristic of the sugar moiety of glycoproteins. Oligosaccharides A-2 to A-5 in Figure 1 could be considered as incomplete biosynthetic products of A-1. However, comparative study of the sugar chains of α- and β-subunits of hCG [10] revealed that this is not the case.

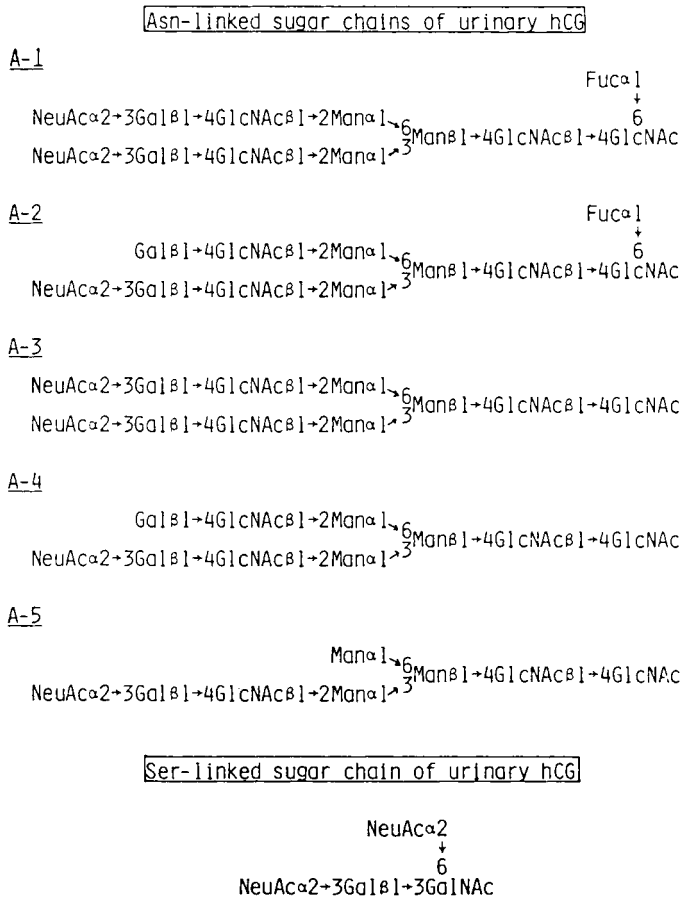

Fig. 1. Structures of the sugar chains of hCG purified from the urine of pregnant women.

By sialidase treatment, the five acidic oligosaccharides in Figure 1 are converted to the three oligosaccharides N-1, N-2, and N-3 shown in Figure 2. Comparative study of the structures of oligosaccharides released from α- and β-subunits of hCG by hydrazinolysis revealed that the α-subunit contains equal amounts of oligosaccharides N-2 and N-3 but no N-1, while the β-subunit contains oligosaccharides N-1 and N-2 in 1:1 molar ratio, but no N-3. Furthermore, hCGs purified from the urine of pregnant women and from the placenta were found to contain oligosaccharides N-1, N-2, and N-3 in 1:2:1 molar ratio, although the extent of sialylation of these three neutral oligosaccharides was different by the sample. These results indicate that oligosaccharides N-2 and N-3 should not be considered as incomplete biosynthetic products of oligosaccharide N-1, but are the final products at particular asparagine loci of hCG. In other words, the two asparagine-linked sugar chains of the α-subunit of hCG are never fucosylated, and one of them remains at the state of a monoantennary sugar chain, whereas one asparagine-linked sugar chain of the β-subunit is always fucosylated. The mechanism of the site-specific formation of different sugar chains cannot be explained by our current knowledge of sugar chain biosynthesis. An unknown control mechanism involving steric effects

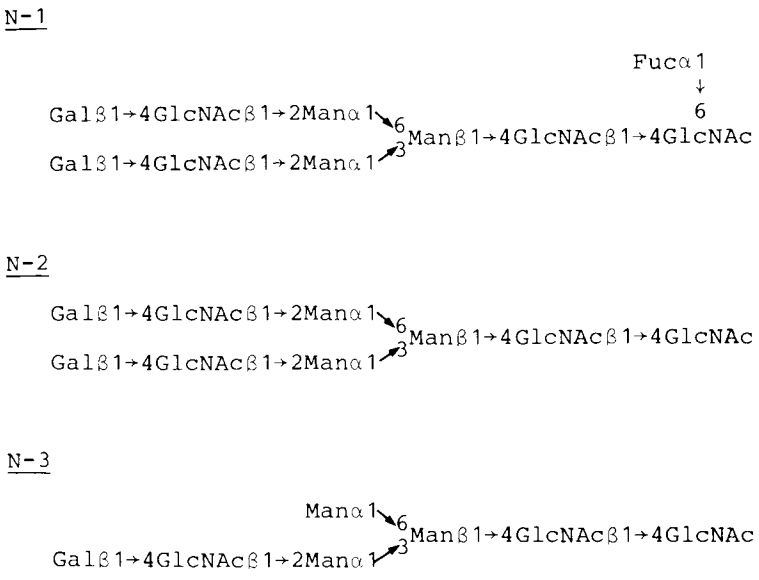

Fig. 2. Structures of three neutral core portions of the asparagine-linked sugar chains of normal hCG.

from the surrounding polypeptide moiety may also play a role in the formation of asparagine-linked sugar chains of hCG.

FUNCTIONAL ROLE OF THE SUGAR CHAINS OF hCG

In 1975, Moyle et al. reported data suggesting for the first time the possibly important role of the sugar moiety of hCG [11] for the expression of its hormonal action. It was reported by Dufau et al. [12] that sialic acid residues were not essential for hCG binding to gonadal target cells, or for stimulating testicular steroidogenesis and ovarian growth in vitro. However, desialylation deprived hCG of its in vivo biological effects on gonadal target cells, because asialo-hCG is cleared from circulation more than 50 times faster than intact hCG [13]. Moyle et al. found that further removal of monosaccharide residues from asialo-hCG by sequential exoglycosidase digestions reduced the ability of the hormone to stimulate the production of cAMP and testosterone by rat Leydig cells.

Their report was later supported by a series of experiments examining the effect of deglycosylation by enzymatic [14] and chemical [15–17] means on the molecular and biological characteristics of hCG. The results of these experiments can be summarized as follows: Removal of the sugar moiety from hCG does not affect the physicochemical properties, reassociation of the two subunits, and in vitro binding to the receptor of target cells. However, deglycosylation of hCG leads to a drastic drop in the biological activity of the hormone: the deglycosylated hCG fails to stimulate in vitro cellular responses such as cAMP production in Leydig cells and progesterone production in rat corpus luteum cells. By means in vivo experiments using the rat, the biological response of ovarian ascorbic acid depletion is impaired also by deglycosylation of hCG. The functional role of the carbohydrate moiety of hCG was more concretely pointed out by the work of Calvo and Ryan [18]. The adenylyl cyclase activity of rat corpora luteal

membrane is increased in linear fashion in response to the addition of hCG [19]. Calvo and Ryan found that addition of the glycopeptide fraction, obtained from hCG by exhaustive pronase digestion, to this reaction mixture inhibited dose-dependently the adenylyl cyclase activation by hCG. Inhibition was enhanced when the glycopeptide was pretreated with sialidase. This result indicated that a membrane lectin, which binds to desialylated sugar chains of hCG, may be involved in the regulation of luteal cell hCG-stimulated adenylyl cyclase. Although bovine IgG is known to have a variety of biantennary complex-type asparagine-linked sugar chains among which oligosaccharides N-1 and N-2 in Figure 2 are included, the glycopeptide fraction obtained from bovine IgG did not inhibit the activation of adenylyl cyclase of corpora lutea membrane by hCG. The most important finding was that the glycopeptide obtained from the α-subunit of hCG showed the inhibitory activity.

Although these authors did not refer at all, our experiment (described above) indicated the presence of a rather rare monoantennary complex-type sugar chain (oligosaccharide N-3) in the α-subunit. Since this oligosaccharide is not included in the sugar chains of bovine IgG, it could be the most probable candidate to react with the postulated membrane lectin.

COMPARATIVE STUDY OF THE ASPARAGINE-LINKED SUGAR CHAINS OF hCG PURIFIED FROM THE URINE OF PATIENTS WITH A VARIETY OF TROPHOBLASTIC DISEASES

A number of structural differences have been reported in the sugar moieties of membrane-bound [20–28] as well as soluble [29–32] glycoproteins produced by normal and malignant cells. Choriocarcinoma is known to produce large amounts of hCG which are excreted in the urine. Because the asparagine-linked sugar chain patterns of hCGs from individuals are constant, it was of interest to investigate the oligosaccharide structure of hCG produced by choriocarcinoma. Nishimura et al. [33] purified hCG from the urine of a patient with choriocarcinoma and characterized its biochemical properties. This choriocarcinoma hCG was also composed of α- and β-subunits with the same amino acid composition as normal hCG. An interesting finding was that the tumor hormone gave a carbohydrate composition different from that of normal urinary hCG. It showed extremely lower biological activity in vivo but about three times higher receptor binding activity in vitro than normal hCG. Structural study of the asparagine-linked sugar chains of the choriocarcinoma hCG revealed many interesting evidences [34]. Although 4 mol of oligosaccharides were released from 1 mol of hCG by hydrazinolysis, none of the oligosaccharides were sialylated. When fractionated by Bio-Gel P-4 column chromatography, the neutral oligosaccharide fraction gave quite a different elution profile from that of desialylated oligosaccharide mixture obtained from normal hCG. Structural studies of oligosaccharides in each peak by sequential exoglycosidase digestion in combination with methylation analysis revealed that it was a mixture of eight oligosaccharides as summarized in Figure 3.

Among the eight oligosaccharides, E, F, and H were detected in normal hCG. The occurrence of the five new oligosaccharides in choriocarcinoma hCG can be explained by the changes of two glycosyltransferases. The sum total of the fucosylated oligosaccharides in Figure 3 amounts to almost 50%, which is twice as high as the fucosylated sugar chains of normal hCG. This evidence indicated that the fucosyltransferase responsible for the formation of the Fucα1→6GlcNAc group is prominently

enhanced in choriocarcinoma. Since oligosaccharide G is not found in normal hCG, the fucosyltransferase in choriocarcinoma may have a wider specificity toward the acceptor sugar chain than that in trophoblasts. Structures of oligosaccharides A, B, C, and D indicated that they are formed by the addition of the Galβ1→4GlcNAcβ1→ group at the C-4 position of Manα1→3 residues of oligosaccharides E, F, G, and H, respectively. Therefore, *N*-acetylglucosaminyltransferase IV [35], which is responsible for the formation of the GlcNAcβ1→4Manα1→ group, should be newly expressed in choriocarcinoma. Because oligosaccharides C and D are never found in normal human glycoproteins, the enzyme may not use Manα1→6 (GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc→Asn-peptide as an acceptor. Therefore, the *N*-acetylglucosaminyltransferase IV of choriocarcinoma may also have a wider specificity than regular enzyme towards the acceptor sugars. Since the structural change of the asparagine-linked sugar chains of choriocarcinoma hCG was so prominent, Mizuochi et al. investigated the sugar chains of four additional choriocarcinoma hCGs [36]. An interesting piece of evidence revealed

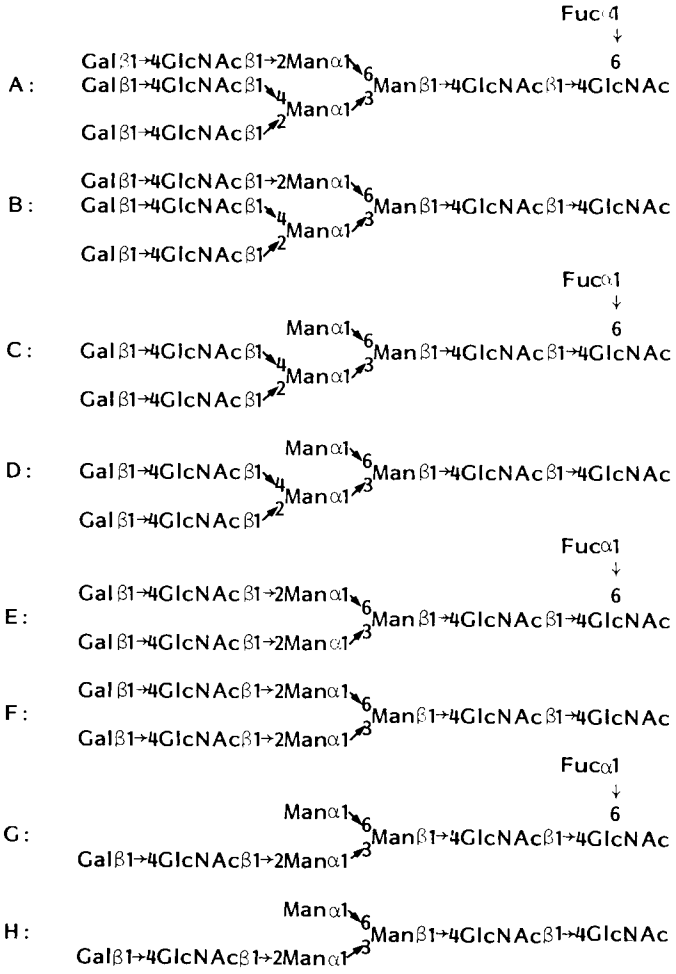

Fig. 3. Structures of the asparagine-linked sugar chains of choriocarcinoma hCG.

by this study was that two choriocarcinoma hCG contained fully sialylated asparagine-linked sugar chains, although the remaining two lack sialic acid in their asparagine-linked sugar chains. Therefore, the deletion of sialic acid residues in the sugar chains is not a common phenomenon of the hCG produced by choriocarcinoma. Unlike the change in sialic acid content, abnormality of the neutral portion of the asparagine-linked sugar chains as depicted in Figure 3 was found in all four choriocarcinoma hCGs, although the molar ratio of each oligosaccharide was different in the four tumor glycoproteins. Another interesting finding is that the three hCG samples purified from the urine of patients with hydatidiform mole have exactly the same neutral portion of the asparagine-linked oligosaccharides as normal hCG [36]. Furthermore, the molar ratio of oligosaccharides E, F, and H in these hCG samples was also 1:2:1. Therefore, the appearance of the five oligosaccharides, A, B, C, D, and G might well be considered as a specific characteristic of choriocarcinoma hCG.

Hydatidiform mole is considered to be essentially benign lesion, although the rate of incidence of choriocarcinoma from this disease is much higher than normal pregnancy. Therefore, it is important to find out at what stage of the pathological process leading to choriocarcinoma the alteration of sugar chains starts. Some of the moles apparently show more malignant characteristics than others, such as invasion into the surrounding tissues and metastasis, and are discriminated from most typical moles by the name "invasive" mole.

Structural study of the asparagine-linked sugar chains of hCGs purified from the urine of patients with invasive moles revealed that oligosaccharides C and D were missing but the other six oligosaccharides in Figure 3 were detected in these hormones [37]. Therefore, a part, but not all, of the abnormalities of the neutral portion of the asparagine-linked sugar chains, which were found in choriocarcinoma hCGs, is also induced in the sugar chains of invasive-mole hCGs. Detection of oligosaccharide G and the increased ratio of fucosylated sugar chains (65 ~ 66%) indicated that enhancement of fucosyltransferase, which forms the Fucα1→6GlcNAc group, already takes place in an invasive mole. Detection of triantennary sugar chains, A and B, in invasive-mole hCGs indicated that ectopic expression of *N*-acetylglucosaminyltransferase IV also occurs in this lesion. However, the absence of oligosaccharides C and D indicated that the newly expressed *N*-acetylglucosaminyltransferase IV can transfer *N*-acetylglucosamine to biantennary complex-type sugar chains but not to monoantennary sugar chains (Fig. 4). This substrate specificity is considered to be the same as that of *N*-acetylglucosaminyltransferase IV in normal tissues, because oligosaccharides C and D are not detected in the glycoproteins produced by normal cells. Accordingly, transformational change induced in the *N*-acetylglucosaminyltransferase IV in choriocarcinoma might take place in two steps. The first step is the ectopic expression of the regular *N*-acetylglucosaminyltransferase IV and the second step is the modification of the substrate specificity of the enzyme. Structures of the sugar chains of many glycoproteins as well as glycolipids produced by tumor cells suggested that glycosyltransferases of tumor cells may have wider specificity than those in normal cells. However, the mechanism of this phenomenon is not known. Expression and modification of *N*-acetylglucosaminyltransferase IV during the process leading to choriocarcinoma might become a useful target to solve this interesting problem.

As already described, the asparagine-linked sugar chains of a part but not all of choriocarcinoma hCGs lack sialic acid. The sugar chains of invasive mole hCGs were highly sialylated like in the case of normal and hydatidiform mole hCGs. Therefore, it is possible that the aberration of sialytransferase occurs only at the advanced stage of

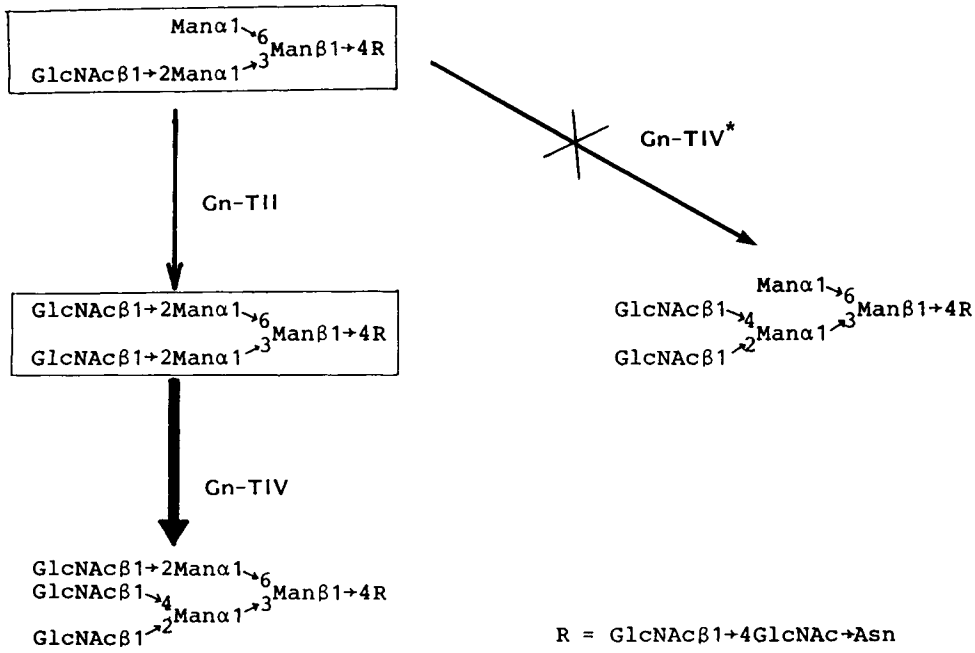

Fig. 4. Biosynthetic pathway of complex-type asparagine-linked sugar chains. Gn-T, *N*-acetylglucosaminyltransferase.

choriocarcinoma. Preliminary data to support this assumption were obtained from study of hCG of a choriocarcinoma patient. The asparagine-linked sugar chains of the hCG obtained from the urine of this patient were almost completely sialylated, while those of the hCG obtained from the same patient at later stage were mostly free from sialic acid residues (Mizuochi, T., Nishimura, R., and Kobata, A., unpublished data).

CLINICAL APPLICATION OF THE STRUCTURAL CHANGE INDUCED IN THE ASPARAGINE-LINKED SUGAR CHAINS OF CHORIOCARCINOMA hCG

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. It has been confirmed that prophylactic chemotherapy reduces the development of persistent trophoblastic diseases. However, 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, development of any method to discriminate invasive mole from regular hydatidiform mole will help in avoiding indiscriminate prophylactic chemotherapy. Because invasive-mole hCG contains oligosaccharides A and B which are not found in hydatidiform-mole hCG, we tried to use this evidence to discriminate hCGs from the two trophoblastic diseases.

Crowley et al. [38] reported that Galβ1→4GlcNAcβ1→6(Galβ1→4GlcNAcβ1→2)Man interacts with *Datura stramonium* agglutinin (DSA) much more strongly than the isomeric Galβ1→4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Man. On the other hand,

Cummings and Kornfeld [39] reported that triantennary and tetraantennary complex-type sugar chains with C-2,6 outer chain branching bind to a DSA-Sepharose column. Being stimulated by these reports, Yamashita et al. [40] investigated the behaviors of 33 complex-type oligosaccharides in a DSA-Sepharose column. The results indicated that the complex-type oligosaccharides can be separated into three groups: a pass-through, a retarded, and a bound fraction. Oligosaccharides, which bound to a DSA-Sepharose column can be eluted with a buffer containing 1% of a mixture of equal amount of *N,N'*-diacetylchitobiose, *N,N',N''*-tri-*O*-acetylchitotriose and *N*, *N'*, *N''*, *N'''*-tetraoacetylchitotetraose. By comparing the structures of oligosaccharides in the three groups, it was confirmed that oligosaccharides in the retarded fraction contain in common the nonsubstituted Galβ1→4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Manα1→ group. In contrast, all oligosaccharides containing the nonsubstituted Galβ1→4GlcNAcβ1→6(Galβ1→4GlcNAcβ1→2)Manα1→ group or the Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→ group bound to the column.

The binding specificity of the immobilized DSA column was expected to be useful for the discrimination of invasive mole and choriocarcinoma hCGs from normal and hydatidiform mole hCGs, because the sugar chains of the former group contain the Galβ1→4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Manα1→ group, but those of the latter group do not. Therefore, the behavior of hCGs in the urine of pregnant woman, of a patient with invasive mole, and of a patient with choriocarcinoma after desialylation was investigated (Endo, T., Nozawa, S., Iizuka, R., and Kobata, A., manuscript in preparation). Urinary hCG of a pregnant woman passed through a DSA column without interaction. In contrast, urinary hCGs of choriocarcinoma and invasive-mole patients were retained by the column. An unexpected result was that the bound hormones were not eluted from the column by the mixture of *N*-acetylglucosamine oligomers but came out under much stronger elution condition. This strong interaction might be produced because a molecule of hCG contains more than one sugar chain interacting with DSA. Although the study of urinary hCGs from a large number of various trophoblastic diseases as well as pregnant women is necessary to confirm the usefulness of this newly developed method, the preliminary results indicated that urinary hCGs from various trophoblastic diseases can be discriminated by the difference of their sugar moieties.

CONCLUDING REMARKS

Recently, structures of the sugar chains of bovine LH were elucidated, as shown in Figure 5, by Green et al. [41]. They reported that these sugar chains are also included in hLH and hTSH. The whole structures of the sugar chains of hFSH were also elucidated, as shown in Figure 6, by Renwick et al. [42]. These data indicated that the sugar chains of the four glycohormones are quite different. In view of the evidence discussed for hCG already, the location of each oligosaccharide on the polypeptide chains of these glycohormones must be elucidated for the full understanding of the functional role of their sugar moieties. However, the currently available data indicated that the α-subunits of the four glycohormones should no more be considered the same. Since the α-subunits of hFSH and hLH are considered to be synthesized in the same cell type [43], it is also suggested that the sugar chains could be different even if the same polypeptides are produced in the same type of cells. The mechanism of this controlled glycosylation of human hormones remains to be resolved. Because the subunits of the glycoprotein hormones combine at the early stage of their biosynthesis [44,45], combination of β-

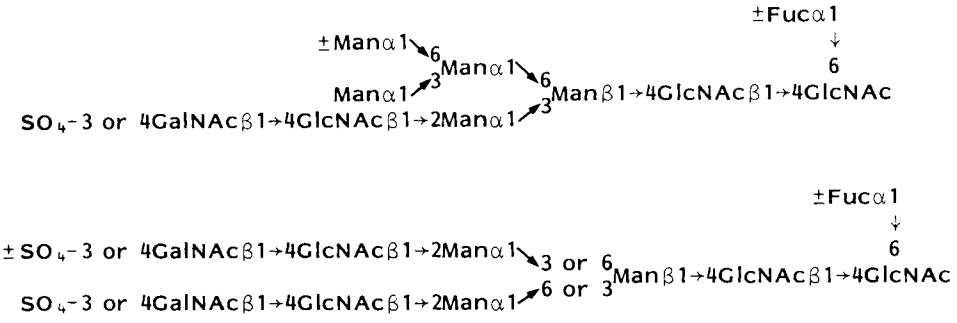

Fig. 5. Structures of the asparagine-linked sugar chains of bovine LH.

subunit may control the processing of the sugar chains linked to the entire dimer as suggested by Green et al. [41].

It is interesting that the sugar moiety of hCG, the formation of which is well controlled in normal trophoblasts, is extensively modified by malignant transformation of the producing cells. We have previously elucidated the transformational change of the sugar chains of hepatic γ-glutamyltranspeptidase and developed a new diagnostic method with use of a lectin column which binds specifically the modified sugar chain

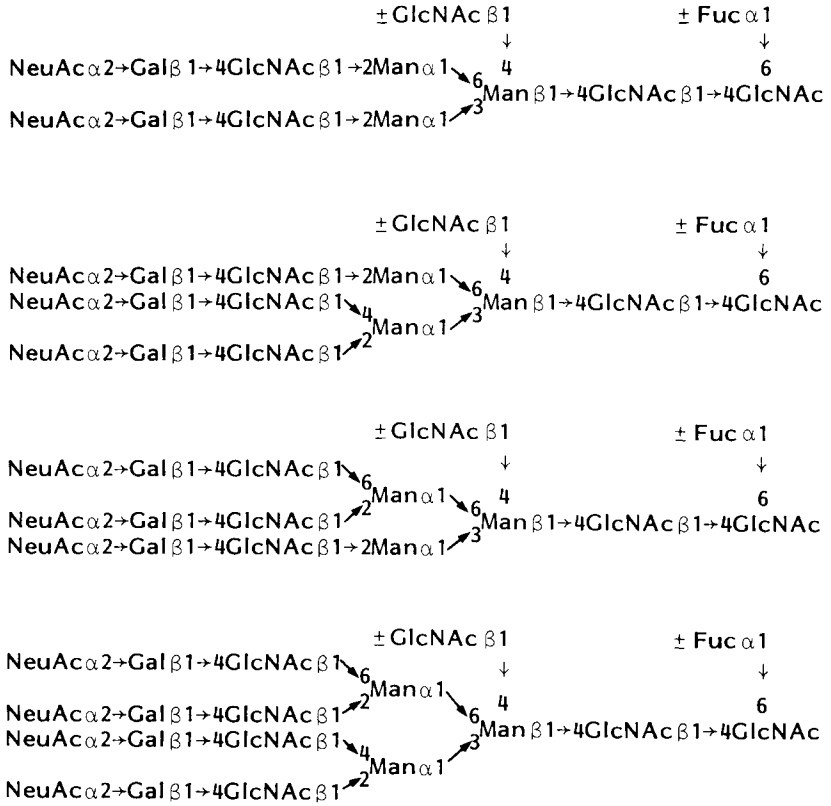

Fig. 6. Structures of the asparagine-linked sugar chains of hFSH.

[46]. In this case, the change is induced by ectopic expression of β-*N*-acetylglucosaminyltransferase III, which adds the bisecting *N*-acetylglucosamine residue to the complex-type sugar chains. In contrast, abnormal sugar chains produced by choriocarcinoma cells in hCG molecule include those which have never been detected in the normal glycoproteins. In such cases, the change can be used not only for the diagnosis and prognosis, but for the development of immunotherapy of tumors.

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