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CELLULAR AND MOLECULAR MECHANISMS OF TUMOR IMMUNITY

JAPANESE CANCER ASSOCIATION

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PREFACE

One of the aims of research in tumor immunology is to successfully apply sophisticated immunological tactics to cancer diagnosis and therapy. Numerous investigations have attempted to delineate the consequences of malignant transformation of cells as the appearance of new cell surface structures that could be identified by specific antibody or by their ability to induce a specific cellular immune response.

In the course of the development of modern tumor immunology, it has become clear that many of the tumor antigens that can be recognized are apparently the products of genes involved in cell growth and malignant transformation. Furthermore, changes in the cell surface of malignant cells have often been found to include alteration of non-protein constitutes. These cell-surface markers are tremendously useful for tumor diagnosis.

The most compelling evidence for the existence of tumor resistance-inducing antigens comes from the study of experimentally induced tumors of inbred rodents. These tumors express neoantigens capable of immunizing hosts against subsequent challenge with the same tumor. Despite such evidence for the expression of tumor antigens on neoplastic cell surfaces, tumor-bearing hosts fail to reject these malignant cells. In order to combat cancer by immunological maneuvers, we must therefore know which types of a host's immune responses are generated against tumor antigens and which effector mechanism(s) is primarily responsible for eradicating tumor cells *in vivo*.

To cover these issues, a symposium entitled "Cellular and Molecular Aspects of Tumor Immunology" was held in Tokyo on September 22, 1986, under the auspices of the Japanese Cancer Association. This volume of "Gann Monograph on Cancer Research" summarizes the topics discussed at this symposium and also includes some additional articles recently released by major individual laboratories in Japan. The following three major issues are elucidated: 1) tumor-associated antigens, 2) cellular mechanisms of tumor immunity, and 3) lymphokines involved in the induction and implementation of tumor immunity. It is hoped that the information provided herein will contribute to the establishment of tumor specific diagnosis and immunotherapy as well as to the promotion of provocative research on tumor immunology in future.

March 15, 1988

Y. HASHIMOTO
T. HAMAOKA

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ALTERED GLYCOSYLATION OF GLYCOPROTEINS IN TUMOR CELLS

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There is a history of more than two decades involved in changes in the plasma membrane associated with the malignant transformation of cells. One of the interesting phenomena elucidated through these studies is the altered glycosylation of complex carbohydrates. Actually, almost all experimental and human cancers are characterized by aberrant glycosylation of glycoproteins and glycolipids. Although some of these alterations were related to growth rather than malignancy of cells, quite a few have been confirmed to be truly tumor-associated. This review will cover our current knowledge of the alteration found in glycoproteins.

Most of the secretory and membrane bound proteins produced by cells contain covalently bound sugars and are called glycoproteins. Recently, much evidence suggests that the sugar moieties of glycoproteins play an important role in intercellular recognition. This function is now considered to be essential for the maintenance of the ordered social life of each cell comprising the multicellular organisms.

Accordingly, alterations in the carbohydrate structures of glycoproteins found in various tumors are considered to be the basis of the abnormal social behaviors of tumor cells, such as invasion into the surrounding tissues and metastasis. Comparative studies of the sugar chains of glycoproteins produced by transformed *versus* normal cells therefore provide useful information for the diagnosis, prognosis as well as immunotherapy of tumors.

Because most of the glycoproteins produced by normal and malignant cells were obtained in limited amount, studies on their sugar moieties have been performed by indirect methods, such as monosaccharide analysis, comparison of the sizes of the glycopeptides obtained after exhaustive pronase digestion, and reactivities with several lectins. However, with the recent establishment of various sensitive methods to analyze the structures of sugar chains, elucidation of the exact structural alterations of the sugar chains of many tumor glycoproteins has begun.

In this chapter, I will present information obtained in our studies during the past five years in the hope of helping readers to consider the importance of this rather new field of cancer research. Due to limited space, this review will cover only the changes of asparagine-linked sugar chains during malignant transformation. Current information on the structural changes of mucin-type sugar chains of glycoproteins induced during malignant transformation is sparse compared to the asparagine-linked sugar chains. However, this field will expand in the near future, due to the many monoclonal antibodies which have been found to detect cancer-associated large molecular weight mucins in he serum of cancer patients.

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Changes Induced in the Membrane-bound Glycoproteins

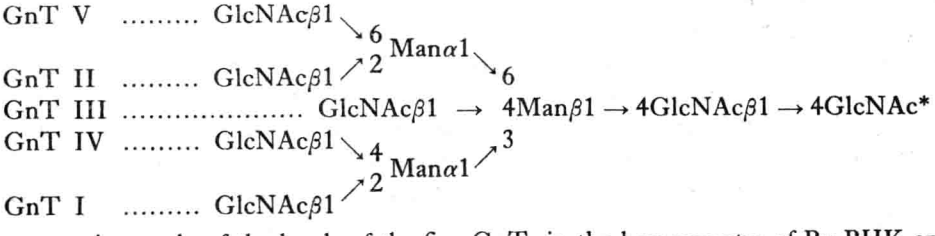
1. Glycoproteins in the plasma membrane of fibroblasts

In 1969, Meezan *et al.* (*9*) reported an interesting phenomenon obtained by the comparative studies of membrane glycoproteins of mouse 3T3 cells and their Simian virus (SV)-40 transformant. After metabolically labelling the glycoproteins of both cells with radioactive glucosamine, plasma membranes were isolated by conventional methods. The glycoproteins in the membrane preparations were then converted to glycopeptides by exhaustive pronase digestion. When the radioactive glycopeptides were analyzed by gel permeation column chromatography, those from transformed cells were larger than the major glycopeptides from non-transformed cells.

Soon after their work, Warren's group (*2*) confirmed that the same phenomenon was observed between Rous sarcoma virus (RSV)-transformed baby hamster kidney (BHK) cells and their normal counterpart. Further comparison of the surface glycopeptides of malignant and non-malignant lymphoblasts, and several different fibroblast cell lines and their malignant counterparts produced by viral, chemical and spontaneous transformation demonstrated that the larger glycopeptides are always found in malignant cells (*20*). Since the chick embryo cells, transformed by a *ts* mutant of RSV, produced the larger glycopeptides at the permissive temperature but not at the non-permissive temperature (*22*), the phenomenon was considered to be an expression of true transformed phenotype. It was also confirmed that the phenomenon is detected in the neoplastic epithelial cell lines but not in those of mesenchymal origin (*16*).

Based on the result that the earlier eluting glycopeptides from RSV-BHK or polyoma (Py)-BHK cells were shifted to the elution positions of desialylated glycopeptides from BHK cells by sialidase digestion, Warren *et al.* (*23*) suggested that the presence of extra sialic acid residues is responsible for the higher molecular weight of glycopeptides from malignant cells. However, comparative studies of the glycopeptides from Py-BHK and BHK cells by an immobilized concanavalin A column suggested that the larger glycopeptides have more branches composed of galactose and N-acetylglucosamine as well as sialic acid (*14*). Oligosaccharides chemically released from glycopeptides from Py-BHK cells were found to be more enriched in the complex-type sugar chains containing the Galβ1 → 4GlcNAcβ1 → 6(Galβ1 → 4GlcNAcβ1 → 2)Man group as well as the elongated outer chains than those from BHK cells (*27*).

So far, five different N-acetylglucosaminyltransferases (GnTs), as shown below, have been found to transfer β-N-acetylglucosaminyl residues at the different positions of the trimannosyl core commonly found in the complex-type sugar chains.



By comparative study of the levels of the five GnTs in the homogenates of Py-BHK and

* Abbreviations used are: Sia, sialic acid; NeuAc, *N*-acetylneuraminic acid; GlcNAc, N-acetylglucosamine.

BHK cells, we found that GnT V activity was increased 2-fold in Py-BHK cells compared to BHK cells (*29*). In contrast, the same levels of GnTs I, II, and IV were detected in both cells. GnT III was not detected in the two cells at all. The last evidence agrees with the fact that no bisected asparagine-linked sugar chain was detected in the surface glycoprotein of either cell (*27*). Recently, Van den Eijnden and Schiphorst (*21*) reported that another N-acetylglucosaminyltransferase, responsible for the outer chain elongation by adding β-N-acetylglucosaminyl residue to the Galβ1 → 4GlcNAc group, works most favorably on sugar chains with the Galβ1 → 4GlcNAcβ1 → 6(Galβ1 → 4GlcNAcβ1 → 2)Manα1 → 6 group. Therefore, the increase of GnT V in Py-BHK cells explains the enrichment of tri- and tetraantennary complex-type sugar chains as well as those with elongated outer chains in Py-BHK cells compared to BHK cells.

Later on, a similar increase of GnT V was found to occur in RSV-BHK cells compared to BHK cells by Pierce and Arango (*15*). Quite recently, Dennis and coworkers reported that enrichment of the complex-type sugar chains containing the -GlcNAcβ1 → 6(-GlcNAcβ1 → 2)Manα1 → 6 branch is correlated strongly with the acquisition of metastatic potential of murine mammary carcinoma (*3*). Therefore, elucidation of the mechanism that increases the level of GnT V in malignant cells may provide the key in understanding tumor progression caused by increased metastatic potential.

2. *γ-Glutamyltranspeptidase (γ-GT)*

The plasma membrane contains many glycoprotein enzymes. Some of these enzymes have been studied as important tumor markers. However, studies on the change of their sugar moieties during transformation have been neglected until recently.

We chose γ-GT to open this new field. γ-GT is a membrane bound glycoenzyme distributed widely in the plasma membrane of epithelial cells of various organs. The enzyme is composed of two subunits with different sizes (heavy and light), both of which con-

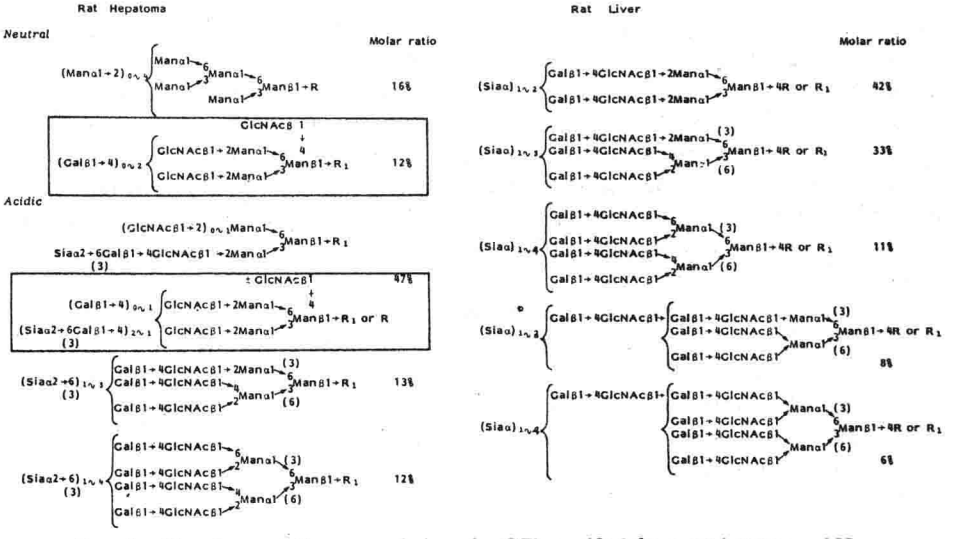


FIG. 1. Structures of the sugar chains of γ-GTs purified from rat hepatoma AH-66 cells and from normal rat liver

R = 4GlcNAcβ1 → 4GlcNAc, R_1 = 4GlcNAcβ1 → 4(Fucα1 → 6)GlcNAc.

tain sugars. The enzyme hydrolytically cleaves the γ-glutamyl residue of glutathione, and the catalytic site is included in the light subunit. The heavy subunit, which non-covalently binds the light subunit, is associated with the plasma membrane of cells by its hydrophobic amino acid cluster. One of the characteristic features of γ-GT is its extremely high molecular multiplicity. For example, rat kidney γ-GT can be separated by isoelectric focusing into more than ten different isozymic forms (*18*). These isozymic forms have the same polypeptide but contain different numbers of sialylated complex-type sugar chains. Although γ-GTs purified from various organs are immunologically identical, their heterogeneity varies according to tissues.

Fiala *et al.* (*6*) reported that γ-GT level in rat hepatoma is more than 50 times higher than that in normal rat liver. Since the elevation of the enzyme has been observed in the preneoplastic nodule of liver (*7*), and the γ-GT level in the serum reflects the enzyme level in the liver, the enzyme was expected to become a good marker for the diagnosis of hepatoma. However, clinical studies revealed that the level of γ-GT in serum is also elevated in many non-cancerous hepatic diseases.

Hepatoma γ-GTs have more acidic isoelectric points than normal liver enzymes. Since the difference disappears after sialidase treatment, it can be ascribed to the transformational change in the sugar moiety of γ-GTs. Comparative study of the sugar chains of γ-GTs purified from rat hepatoma and from normal rat liver revealed a variety of differences (*25*). The average number of sugar chains of hepatoma γ-GT was 4 times that of the normal liver enzyme. Furthermore, the sugar chains were found to be of a different set than the normal liver enzyme (Fig. 1).

Among the various structural differences shown in Fig. 1, we focused on the appearance of the bisected sugar chains (enclosed by solid lines) in the hepatoma γ-GT. Comparative study of the sugar chains of γ-GTs purified from the liver and the kidney of many mammals revealed that extensive organ-specific and species-specific differences occur in the sugar chains of this enzyme (*26*). However, the bisecting GlcNAc residue is never found in the sugar chains of liver enzyme, while it is always detected in those of kidney γ-GT. Not only the sugar chains of γ-GT, but those of all other glycoproteins of liver origin lack the bisecting GlcNAc residue. Therefore, it is most probable that GnT III, which adds the bisecting GlcNAc residue to sugar chains, is not manifested in the liver of mammals including human. For that reason, detection of the bisecting GlcNAc residue in more than half of the total sugar chains of hepatoma γ-GT can be considered as one of the dedifferation phenomena characteristic of malignant transformation. This hypothesis has recently been proven enzymatically by Schachter's group (*13*), who found that normal rat liver totally lacks GnT III, whereas rat hepatoma cells contain a significant amount of this enzyme.

E-PHA, one of the isolectins purified from *Phaseolus vulgaris*, binds many but not all of the bisected complex-type sugar chains (*24*). An immobilized E-PHA column was successfully used to discriminate human serum γ-GT associated with primary hepatoma from that of non-hepatoma patients (*8*). More studies including the structural investigation of the sugar chains of normal human liver and human hepatoma γ-GTs will be needed to prove the usefulness of this new diagnostic method.

Changes Induced in the Glycoproteins Secreted from Tumor Cells

1. α-Amylase

Transformational changes of three glycoproteins have been studied in detail to date. α-Amylase together with alkaline phosphatase has been attracting the interest of researchers in the cancer field because both are ectopically formed in many tumors. α-Amylases purified from various tumors behave like the α-amylase from human parotid gland but not like the pancreatic enzyme on agar gel electrophoresis and on ion exchange column chromatography (*1*).

Study of the sugar chains of human parotid α-amylase revealed that they are biantennary complex-type sugar chains containing either Galβ1 → 4GlcNAcβ1 →, Galβ1 → 4(Fucα1 → 3)GlcNAcβ1 → or NeuAcα2 → 6Galβ1 → 4GlcNAcβ1 → group at their outer chain moieties (*28*) (Fig. 2A). An interesting fact is that the acidic sugar chain occurs only as a monosialyl derivative, and the sialic acid is exclusively located on the Manα1 → 3 arm of the trimannosyl core. This characteristic is very unusual, because at least a part of the biantennary complex-type sugar chains of most other glycoproteins contain two sialic acid residues.

Structures of the sugar chains of two α-amylases purified from lung cancer and ovarian cancer were elucidated as shown in Fig. 2B (*30*). Although the fucose residue, found in the outer chain moieties of the sugar chains of parotid α-amylase, is completely missing in these sugar chains, some characteristic features of the sugar chains of the former are included in the tumor enzymes. The biassed distribution of sialic acid residue on the Manα1 → 3 arm is also found to occur in these sugar chains. A common characteristic feature, detected in the sugar chains of the tumor α-amylases and not of the parotid enzyme, is the incompleteness in their outer chain moieties: many of their outer chains ended with β-N-acetylglucosamine and not galactose. Such incomplete outer sugar chains were also found in hepatoma γ-GT (Fig. 1).

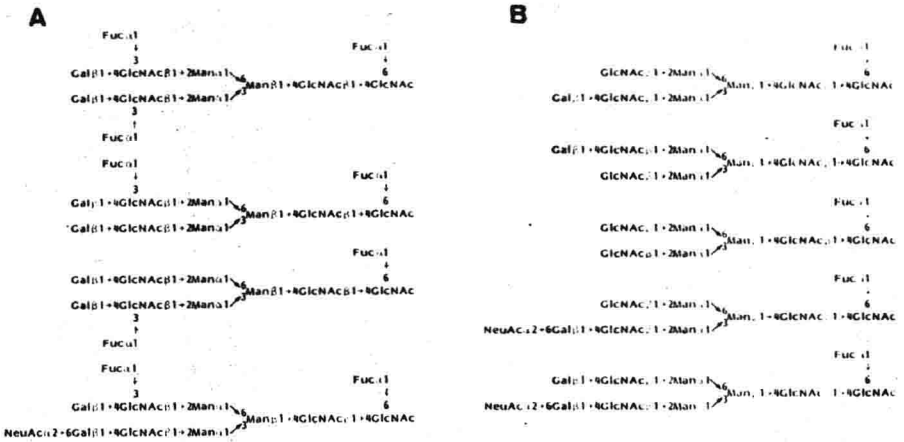


FIG. 2. Structures of the sugar chains of α-amylases purified from human parotid gland (A) and from two tumors, a serous papillary cystadenocarcinoma of the ovarium and a bronchioalveolar adenocarcinoma of the lung (B)

2. α-Fetoprotein

α-Fetoprotein is a glycoprotein synthesized by mammalian embryonic liver and yolk sac. It has been known for more than 20 years as a tumor marker. The concentration of α-fetoprotein is extremely low in the sera and the ascites fluid of adult mammals except during pregnancy, but is elevated in those of patients with hepatoma and yolk sac tumor (*17*). α-Fetoproteins produced by liver cancer and yolk sac tumor are immunologically indistinguishable, however, they contain different sugar chains as listed in Fig. 3. The difference can be discriminated by either a concanavalin A-Sepharose column (*19*) or an E-PHA-agarose column (Yamashita, K. and Kobata, A., unpublished result). Almost none of the α-fetoprotein from yolk sac tumor bound to a concanavalin A-Sepharose column, but was retarded in an E-PHA-agarose column. In contrast, the α-fetoprotein from primary hepatocellular cancer binds to a concanavalin A column but not to an E-PHA column.

One might wonder why the bisecting GlcNAc residue appears in the sugar chains of γ-GT but not in the α-fetoprotein of hepatoma. It is known that the levels of γ-GT and α-fetoprotein do not rise in parallel. This may indicate the complex nature of hepatocellular cancer.

3. Human Chorionic Gonadotropin (hCG)

HCG is a glycoprotein hormone produced by trophoblasts in placenta. It is composed of two subunits with molecular weights of 16,000 (α) and 30,000 (β). Both subunits contain two asparagine-linked sugar chains, the structures of which were determined by Endo *et al.* (*4*) as shown in Fig. 4. These five sugar chains are derived by sialylation from

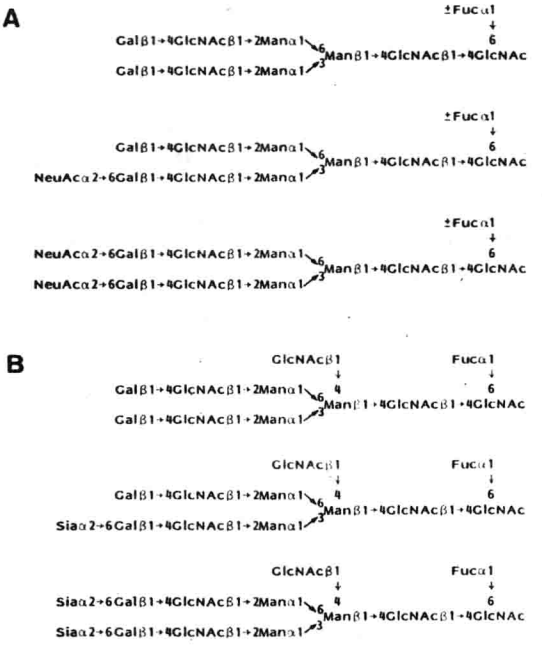


FIG. 3. Structures of the sugar chains of α-fetoproteins purified from hepatoma (A) and from yolk sac tumor (B)

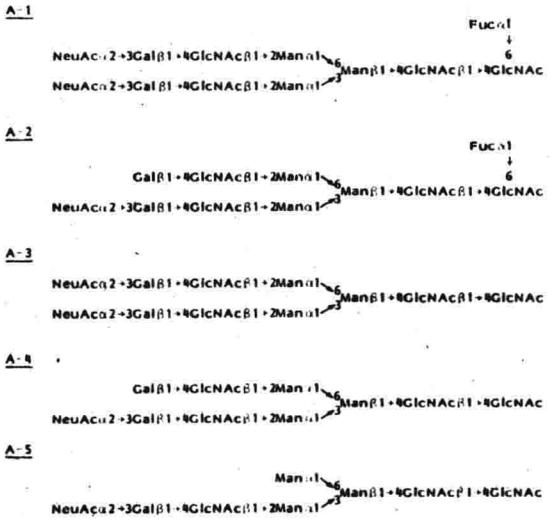


FIG. 4. Structures of the asparagine-linked sugar chains of hCG purified from the urine of pregnant women

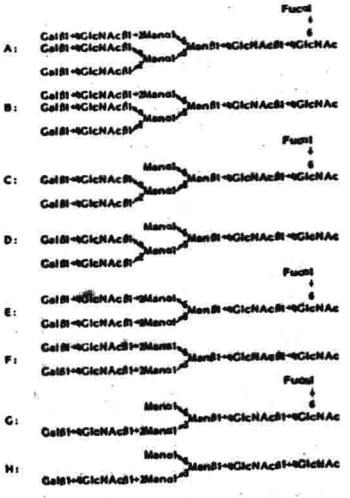


FIG. 5. Structures of the neutral core portions of the asparagine-linked sugar chains of choriocarcinoma hCG

the three neutral oligosaccharides (E, F, and H in Fig. 5). Although hCG purified from the urine of pregnant women is more enriched in sialylated sugar chains than that from placenta, the molar ratio of oligosaccharides E, F, and H of these hCGs is the same (1: 2: 1). Comparative study of the sugar moieties of the α- and β-subunits of hCG revealed that α contains one mole each of F and H, while β contains one mole each of E and F (*10*).

A high level of hCG is detected not only in the urine of pregnant women but in that of patients with hydatidiform mole and with choriocarcinoma. Accordingly, this

glycohormone is widely used in gynecology clinics as a diagnostic marker of various trophoblastic diseases as well as pregnancy. Because the formation of the four sugar chains of hCG molecule is highly controlled, it was of interest to see whether the sugar chains of hCG from choriocarcinoma showed any structural change. Study of an hCG sample purified from the urine of a choriocarcinoma patient gave several exciting results (*11*). None of the sugar chains was sialylated. Structural study of the neutral oligosaccharides revealed that the eight oligosaccharides shown in Fig. 5 were included in the choriocarcinoma hCG.

Further studies of the sugar chains of hCGs purified from the urine of three additional choriocarcinoma patients and three patients with hydatidiform mole revealed many new aspects of the altered glycosylation of hCG in choriocarcinoma (*12*). The deletion of sialic acid residues in the sugar chains is not commonly detected in the hCG produced by choriocarcinoma. However, eight neutral oligosaccharides in Fig. 5 were detected in all three choriocarcinoma hCGs, although the molar ratio of each oligosaccharide varied with the sample. In contrast, three hCG preparations from hydatidiform mole patients have exactly the same neutral portion of the asparagine-linked oligosaccharides as normal hCG, and the molar ratio of oligosaccharides E, F, and H was also 1: 2: 1. Therefore, the appearance of the five oligosaccharides, A, B, C, D, and G could be considered as a specific characteristic of choriocarcinoma hCG.

Hydatidiform mole is a benign lesion although the rate of incidence of choriocarcinoma in this disease is much higher than in normal pregnancy. Therefore, it was important to determine at what stage of the pathological process leading to choriocarcinoma the altered glycosylation of hCG starts. Some of the hydatidiform moles are further classified as invasive mole, since they apparently show more malignant characteristics such as invasion into the surrounding tissues and metastasis. It has recently been shown that the two hCGs purified from the urine of patients with invasive mole contain oligosaccharides A, B, E, F, G, and H in Fig. 5 but not C and D (*5*). Therefore, a part but not all of the structural abnormalities detected in the neutral portion of the asparagine-linked sugar chains of choriocarcinoma hCGs is also induced in the sugar chains of invasive mole hCGs.

Based on the results so far described, we can define the biochemical events leading to the altered glycosylation of hCG in choriocarcinoma as follows: because hCG produced by normal trophoblasts contains biantennary and monoantennary sugar chains, GnT IV may not be expressed in these cells. Detection of triantennary sugar chains A and B in invasive mole hCG indicated that ectopic expression of GnT IV occurs in this lesion. Therefore, invasive mole might well be considered as a precancerous state. Absence of oligosaccharides C and D in Fig. 5 indicated that the newly expressed GnT IV can transfer N-acetylglucosamine to biantennary complex-type sugar chains but not to monoantennary sugar chains. This substrate specificity is the same as that of GnT IV in normal tissues which can produce the triantennary sugar chains, because oligosaccharides C and D are not detected in the glycoproteins produced by normal cells. In choriocarcinoma, the GnT IV might be modified to have wider specificity towards acceptor because oligosaccharides C and D are formed.

Most of the patients with hydatidiform mole regress spontaneously. However, about 10% will develop persistent gestational trophoblastic diseases such as invasive mole and will need treatment with therapeutic agents. In order to avoid indiscriminate prophylactic chemotherapy, and to determine choriocarcinoma at its early stage, the structural

changes found in the sugar moieties of invasive mole and choriocarcinoma hCGs may be effectively used to develop a new diagnostic method.

CONCLUDING REMARKS

As described in this review, changes of the sugar moieties of glycoproteins during oncogenesis are diverse. The changes can be in the form of both deletions and elongations. This is because the sugar chains are not direct gene products but are secondary products through the concerted action of glycosyltransferases coded by genes. Therefore, disorder in the controlled expression of genes in the tumor cells may result in various changes of the sugar chain structures.

As well documented in the story of choriocarcinoma hCG, abnormal sugar chains produced by cancer cells can include those which have never been detected in the glycoproteins produced by normal cells. As discussed earlier, these sugar chains might be produced because tumor glycosyltransferases acquired wider substrate specificity towards acceptor sugar chains than the enzymes of normal cells. That tumor glycosyltransferases might have wider or looser substrate specificities has also been suggested by comparative study of glycolipids in normal and tumor cells. Therefore, elucidation of the biochemical basis of such modification of glycosyltransferases in tumor cells is one of the important areas to be resolved in the future.

As is exemplified by the study of plasma membrane glycoproteins of fibroblasts and their malignant transformants, tumor-related alteration of the sugar chains might well be correlated with the metastatic behavior of the malignant cells. As many cell lines with different metastatic character have already been established, this area of study may develop quickly.

Studies of γ-GT and hCG indicate that elucidation of the whole sugar structures of glycoproteins is essential for the effective use of the altered sugars for clinical application, because strategies to develop new diagnostic methods can only be made by picking up fragmental changes distributed in may tumor oligosaccharides. Since the changes of sugar chains are qualitative, they will afford much better diagnostic methods than simple immunochemical methods which have been widely used for cancer diagnosis and prognosis.

Acknowledgments

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