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Glycosyltransferases: cell surface remodeling and regulation of receptor tyrosine kinases-induced signaling*

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INTRODUCTION

The biosynthesis and degradation of glycoconjugates are catalyzed by glycosyltransferases and glycosidases, respectively, and the genes which encode glycosyltransferases and related proteins are referred to as 'glyco-genes'. The expression of glycosyltransferases, the substrate specificity of the enzymes and their subcellular localization represent key determinants in the biosynthesis of sugar chain [1–6]. In recent years several glycosyltransferases have been identified, cloned and characterized. However, the biological significance of these genes and their precise physiological function still remain elusive. One of the strategies used in the elucidation of the physiological function of glycosyltransferases is to manipulate the expression levels of glyco-genes in mammalian cells. Even though a specific glycosyltransferase gene is over-expressed or knocked out in mammalian cells, due to the abundance of glycoconjugates, the resulting phenotypic changes are rather complex [4] because those changes are due to a direct effect of glycosyltransferase gene or an indirect one. It is essential to identify the likely target glycoconjugates under those conditions. It is noteworthy that the recent manipulation of glyco-genes in animal models in our and several other laboratories have opened new insights in the area of glycobiology or glycotechnology in terms of phenotypic changes of cells and tissues.

Our interest has focused on the *N*-glycan biosynthesis of glycoproteins and, in particular, branching enzymes such as N-acetylglucosaminyltransferase III (GnT-III), GnT-IV, GnT-V and α1-6 fucosyltransferase (α1-6FucT). These enzymes regulate the further processing of the *N*-glycan structures which play a pivotal role in tumor development, metastasis and invasion. Some of our earlier papers reported on the purification of *N*-acetyl-glucosaminyltransferases III, V and α1-6FucT from various mammalian cells or tissues and the cloning of their genes [7–11]. Figure 1 depicts enzymatic products of these glycosyltransferases. This paper will present an overview of the above three glycosyltransferase genes and will then focus on the physiological significance of GnT-III and its enzymatic product designated as bisecting GlcNAc which represents one of the signaling molecules in glycoproteins.

Glycosyltransferase gene expression is differentially regulated in a tissue- and organ-specific manner

Most of the glycosyltransferase genes consist of multiple promoters and are expressed in an organ- and tissue-specific manner. As shown in Fig. 2, this property probably results in a multiplicity of sugar chains in various organ, tissue and cells. Therefore, the expression of glycosyltransferase gene is complicated and varies in a tissue specific manner. For example, the human GnT-V gene is divided into 17 exons, spanning 155 kb [12] and analysis of the 5′ untranslated region of mRNAs from various cells showed multiple sequences depending on the cell types. Further analysis of the 5′ upstream region of exon 1 of

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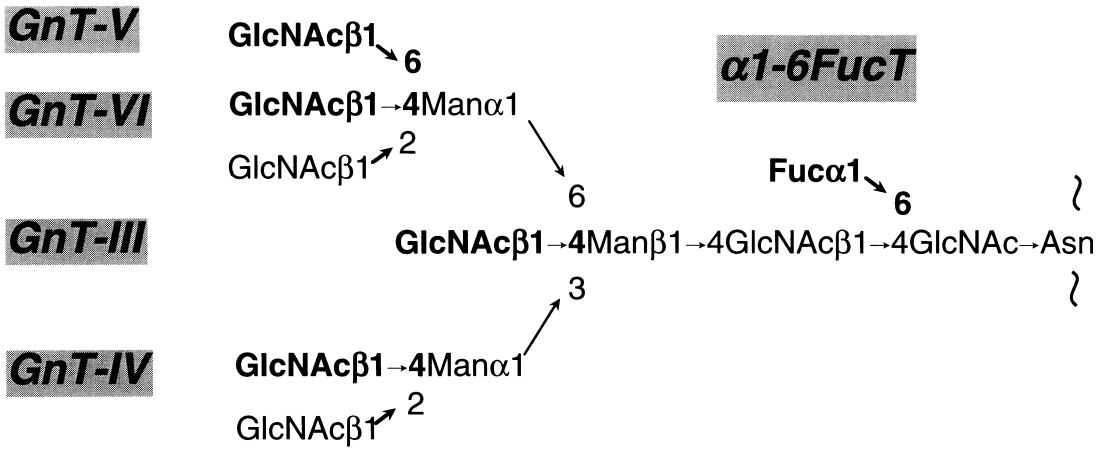

Fig. 1 Structure of enzymatic products of GnT-III, IV, V, VI and α1-6FucT.

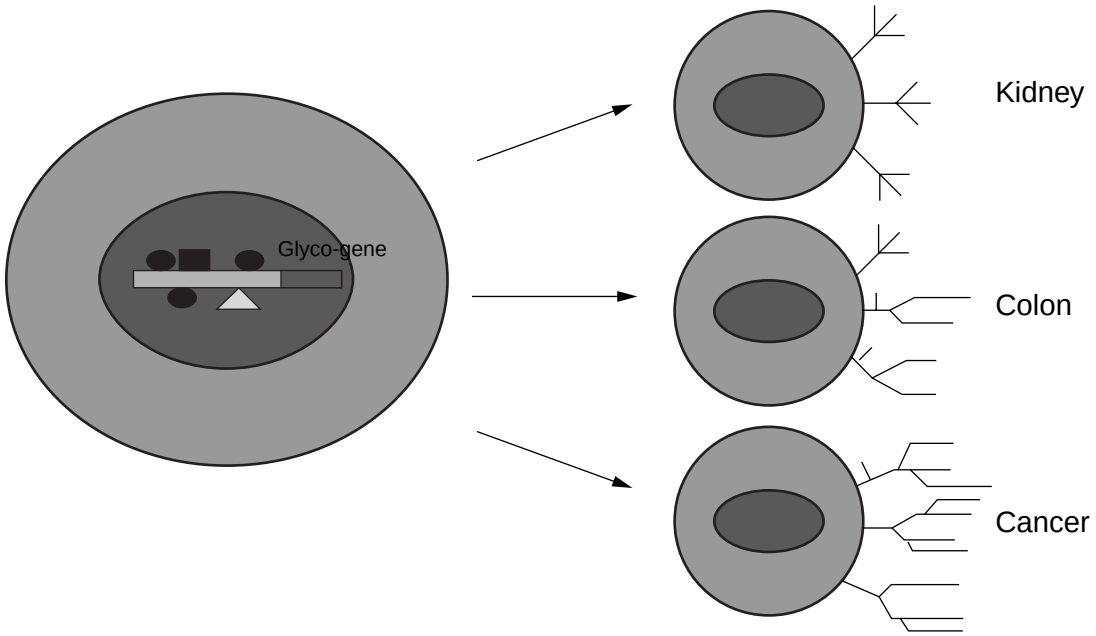

Fig. 2 Multiplicity of sugar chains in various organ, tissue and cells.

GnT-V gene revealed the presence of consensus motifs for transcription factors such as a TATA box, AP-1, AP-2 and for HNF-1, HP1. In addition, liver restricted transcription factor sites were also found in intron 1. The gene employs a multiple promoter system for transcription, and, as a result, gene expression may be regulated in a tissue-specific fashion. Moreover, multiple GnT-V transcripts have been reported in various cell lines and tissues [13]. In rodent cells, 7.5 and 3.5 kb mRNAs were found, while human cells expressed transcripts which were 4.5 and 9.5 kb in size for the GnT-V gene. We have shown that in murine melanoma cells, GnT-V mRNA expression is regulated by TGF-β1 due to the increased stability of its mRNAs [14]. However, in several cell types a discrepancy between the levels of mRNA expression and enzymatic activity has been reported [15], suggesting that a post translational modification of GnT-V is required for enzyme activation.

In contrast, the open reading frame of human GnT-III gene lies on a single exon and the 5′ noncoding regions of the mRNA consists of three types in a hepatoma cell line. At least two of the active promoter regions of GnT-III gene have been identified in a hepatoblastoma cell line. Collectively, these

observations indicate that like GnT-V, the gene expression of GnT-III is also controlled by a multiple-promoter system. More recently, we have cloned the α1-6 FucT gene and the analysis of the 5′ region is now under way.

GnT-V expression is regulated by ets-1, a transcriptional factor which is highly implicated in tumor metastasis and invasion

GnT-V catalyzes the attachment of a β1-6GlcNAc residue, producing branched N-glycans. The high degree of branching of N-glycans appears to be related to the malignant potential of tumor cells. In particular, β1-6 branching of N-glycans is directly linked to tumor metastasis. The expression of the GnT-V gene is induced by viral and oncogene transfections and during hepatocarcinogenesis. In addition, GnT-V activity is also augmented by phorbol esters and transforming growth factor β1 [14,16]. These observations suggest the existence of complex regulatory mechanisms for GnT-V enzymatic activity.

As described earlier, the promoter region of the GnT-V gene has been characterized and recent studies from our laboratory demonstrated a role of the ets-1 transcription factor in the regulation of GnT-V gene expression [17]. Ets-1 is a nuclear phosphoprotein which binds to purine-rich DNA sequences and functions as a transcription factor. The presence of ets binding sequences in the promoters of c-myc and cdc-2 has also been reported. The c-myc and cdc-2 oncoproteins are implicated in the control of normal cell growth, and their deregulation is associated with neoplasia. These studies suggest that the ets family of proteins are associated with transformation properties [18]. In addition, ets-1 also transactivates the expression of an extracellular matrix metalloproteinase, membrane-type metalloproteinases, stromelysin-1, collagenase and stromelysin-3. The expression and regulation of these proteins are associated with invasion and metastasis of tumor cells.

Our studies indicate that the ets-1 protein binds to the ets binding sites of two *cis*-acting elements and *trans*-activates the promoter activity of the human GnT-V gene in human bile duct carcinoma cells. Over-expression of the ets-1 gene in cancer cells resulted in the concomitant increase of GnT-V gene expression, while the transfection of a dominant negative form of ets-1 abolished the expression of GnT-V gene expression. Very recently, we also found a correlation between the expression levels of GnT-V and ets-1 genes in most of the human hepatoma tissues tested, as well as in a number of tumor cell lines [*J. Biol. Chem.*, in press].

Enzymatic competition between GnT-III and GnT-V are important in terms of tumor metastasis

As described above, GnT-V activity is highly correlated with the metastatic potentials of several tumors. In fact some tumor cells contain increased GnT-V activity. We purified GnT-V from the conditioned medium of a human lung cancer cell line and confirmed that a bisected biantennary sugar chain does not serve as a substrate, whereas both biantennary and triantennary sugar chains can be utilized by GnT-V. Molecular modeling of the core mannose in the presence and absence of bisecting GlcNAc was carried out on a INDGO workstation (Silicon Graphics) using INSIGHT II/DISCOVER (Biosystem Technologies) software, based on nuclear magnetic resonance data [1]. The results showed that the biantennary structure of a core mannose was twisted in the presence of bisecting GlcNAc. These conformational changes rendered the substrate inaccessible to GnT-V and thus prevented the formation of the β1-6 structure, raising the possibility that β1-6 branching formation might be suppressed by the introduction of the GnT-III gene. Using this rationale, we attempted to analyze the role of GnT-V and its product β1-6 branches in metastasis. Yoshimura *et al*. established a highly metastatic subclone of B16 melanoma cells (B16-hm) and introduced the GnT-III gene into these cells [19]. In GnT-III transfectants, the glycoproteins showed a reduced affinity to leukoagglutinating phytohaemagglutinin (L-PHA), whereas their binding to erythro-agglutinating phytohaemagglutinin (E-PHA) was increased. E-PHA strongly reacts with bisected biantennary structures, while L-PHA is highly reactive to the β1-6 branches of tri- or tetra-antennary sugar chains. Two proteins, 95 kDa and 80 kDa in size, showed a particularly intense L-PHA binding. This indicated that the level of β1-6 structure was reduced, due to the competition between intrinsic GnT-V and ectopically expressed GnT-III for the substrate. The transfected cells displayed decreased invasiveness into matrigel, and inhibited cell attachment to collagen and laminin matrix.

Lung colonization is decreased in GnT-III transfectants during experimental metastasis: the role of glycosylated-E-cadherin in suppressing lung metastasis

When GnT-III transfectants of B16-hm cells were injected into syngeneic mice, the number of metastatic nodules were drastically reduced, compared to parental cells or negative transfectants. These results indicate that GnT-III expression decreased the metastatic potential of B16-hm cells *in vivo* as the result of substrate competition between GnT-III and GnT-V.

To elucidate the mechanism by which lung metastasis is suppressed, we considered the possibility that adhesion molecules are involved and examined the expression of E-cadherin. In the parental B16-hm cells and negative transfectants, E-cadherin was weakly expressed at the cell-cell contacts [20]. Positive transfectants, however, showed intense fluorescence with condensation at the cell-cell contacts, indicating an elevated expression of E-cadherin at cell-cell contacts of GnT-III transfectants. E-cadherin is also highly glycosylated in GnT-III transfected cells. The prolonged turnover rate of E-cadherin in GnT-III transfectants suggested that glycosylation of E-cadherin by GnT-III resulted in an increased level and accumulation of E-cadherin molecules at cell-cell contacts.

GnT-III and its enzymatic product, bisecting GlcNAc have multifaced properties

Cell specific targets for GnT-III modification and their differential functions are summarized in Table 1. In the normal rat liver GnT-III activity is almost undetectable, but during liver regeneration and hepatocarcinogenesis its activity is markedly increased [21,22]. To determine the biological significance of GnT-III in hepatocytes, transgenic mice which specifically express GnT-III in the liver were established [23]. The hepatocytes from transgenic mice showed a swollen oval-like morphology with accumulation of lipid droplets. In addition, circulating levels of triglycerides, β- and pre-β-lipoprotein fractions and apolipoprotein B100 were significantly reduced in the serum of transgenic animals, compared to syngeneic controls. However, apolipoprotein B which contained an increased level of bisecting GlcNAc accumulated in the transgenic hepatocytes. Decreased levels of triglycerides in transgenic mice plasma may also be related to the impairment of microsomal triglyceride transport protein which has two putative N-glycosylation sites. Collectively, these data demonstrate that aberrant glycosylation, as a direct result of the formation of bisecting GlcNAc, disrupts certain functions of apolipoprotein B such as its transport or lipoprotein complex formation, leading to the generation of fatty liver. Very recently, Stanley and colleagues have reported that GnT-III knock out mice are resistant to diethylnitrosamine-induced tumorigenesis [24]. These findings are significant and demonstrate the critical role of bisecting GlcNAc in tumor potentiation and progression. We have also shown that in leukemic cells, GnT-III activity is highly induced during blast crisis [25].

Table 1 Cell specific targets for GnT-III modification and their differential functions

Cel types	Target molecules	Biological functions
hepatocytes	Apolipoprotein B 100	Lipoprotein sorting
hepatoma M31	Cell surface proteins lysosomeal proteins	Sorting
melanoma B16-hm	E-cadherin	Suppression of metastasis
melanoma B16-hm	CD44	Enhancement of metastasis
B16-F10	Tyrosinase	Melanogenesis
U373MG	EGF-R	Regulation of Signal Transduction Pathways
PC12	NGF-R	
K562 cells	NK receptors?	Resistance to NK cells
HB611	HBV related nuclear factor?	Regulation of gene expression
porcine endotherial cells	Glycoproteins	Xenotransplantation antigen

Growth factor signaling and GnT-III transfectants

GnT-III is highly expressed in rat kidney and brain tissues. To examine the intracellular role of GnT-III and its product in neural cells, the GnT-III gene was over-expressed in a rat pheochromocytoma cell line, PC-12 [26]. The GnT-III transfectants showed a significant increase in E-PHA binding on lectin blot analysis, indicating that some glycoproteins contained elevated levels of bisecting GlcNAc structures. The parental control cells differentiated into sympathetic neurons upon treatment with nerve growth factor (NGF), but the GnT-III transfectants showed neither morphological response nor changes in cell growth rate. In addition, upon NGF treatment, tyrosine phosphorylation of the Trk/NGF receptor could not be detected in GnT-III-transfected PC-12 cells, although the degree of NGF binding to control and GnT-III transfectants remained unaltered. The above study also showed that the modification of Trk/NGF receptor as the result of the over-expression of GnT-III in PC-12 blocked the ligand-induced receptor dimerization. Taken together, these results indicate that the over-expression of the GnT-III gene in PC-12 cells results in some aberrant glycosylation of the Trk receptor which, in turn, affects NGF-induced signaling in PC-12 cells.

The role of N-linked sugars in the modulation of epidermal growth factor receptor (EGF-R) function has been reported by several authors via the use of glycosylation inhibitors such as tunicamycin. Rebbaa *et al*. for the first time reported that the binding of E-PHA lectin from *Phaseolus vulgaris* to bisecting structures on the EGF receptor in U373 glioma cells blocks EGF binding and receptor autophosphorylation [27]. In addition, it has also been reported that over-expression of the GnT-III gene in the glioma cell line inhibits EGF-R functions.

EGF-R is a glycoprotein and, as discussed above, its oligosaccharide residues play an important role in ligand binding, receptor dimerization and the phosphorylation of its tyrosine. In addition, we have recently found that glycosylation of EGF-R by GnT-III modulates its mitogenic effect and suppresses the metastatic potential of murine melanoma B16-F1 cells *in vivo*. However, in contrast to U373 glioma cells, ligand-induced tyrosine kinase activity of EGF-R was not altered in B16 melanoma cells. As detected by anti-phosphotyrosine antibodies, the EGF-R-induced phosphorylation of intracellular proteins is similar in both parental and GnT-III transfected B16-F1 melanoma cells (Fig. 3, upper panel). However, further analysis of EGF-R specific substrates showed a significant hypophosphorylation of oncoprotein c-cbl (Fig. 3, bottom panel). Oncoprotein c-cbl is a novel 120 kDa adopter protein which is implicated in the regulation of PI 3-Kinase and the MAPK/ERK pathways of signal transduction [28]. We have also shown that EGF-induced MEK1 and 2 phosphorylation is inhibited in GnT-III transfected B16-F1 cells. Since MEKs are upstream kinases which phosphorylate and activate ERKs, we also observed the down regulation of EGF-induced ERK activation in GnT-III transfected B16 melanoma cells. The MAP kinases are central transducers of transcytoplasmic signaling from hormones, growth factors, cytokines and environmental stresses to the nucleus. MAP kinase isoforms ERK1 and 2 have been implicated in regulation of cellular proliferation and differentiation via the phosphorylation of transcription factors and cytoskeletal proteins. It is, therefore, possible that down-regulation of EGF-induced phosphorylation of ERK/MAP kinases in GnT-III-transfected B16-F1 cells is involved in the suppression of metastasis.

GnT-III regulates melanogenesis in B16-F10 melanoma cells

The vast majority of studies to date have focused on the role of *N*-glycans on glycoproteins which are destined for secretion or cell surface expression. However, little information is available on the behavior of intracellular glycoproteins. We recently examined the function of an intracellular enzyme when it is modified by the activity of GnT-III. This study involved an examination of the catalytic properties of tyrosinase in B16-F10 murine melanoma cells which over-express GnT-III. Tyrosinase (monophenol, β 3,4-dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1), is a rate limiting enzyme which plays a pivotal role in melanogenesis. It is a single chain glycoprotein catalyzing the hydroxylation of tyrosine to β 3,4-dihydroxyphenylalanine (DOPA) and the further oxidation of DOPA to DOPA quinone [29]. We found that in B16-F10 melanoma cells which over-express GnT-III, the melanin content and tyrosine hydroxylase activity were markedly increased (Fig. 4). Previous studies in our laboratory have also shown that increased levels of cAMP by forskolin induces GnT-III activity [30]. Incubation of B16-F10 melanoma cells with forskolin induced GnT-III activity and melanogenesis in these cells. *In vivo*,

melanin pigment plays a key role against the carcinogenic effects of solar ultraviolet light in normal melanocytes, while increased melanin synthesis is a hallmark of the differentiation of melanoma cells. Thus, the modification of tyrosinase activity by GnT-III could be a novel approach in modulating the properties of malignant melanoma cells.

Other faces of GnT-III:

Bisecting GlcNAc on K562 cells suppresses NK(Natural Killer) cytotoxicity and promotes spleen colonization

NK cells, which comprise 10–15% of the lymphocytes in human peripheral blood, are morphologically large granular lymphocytes with CD3−, CD16+, CD56+ and are able to lyze target cells without prior sensitization or MHC restriction. The K562 cell line is commonly used as target cells in standard NK activity assays. Previous studies have suggested that the potential target structures for NK cells are not only proteins but also carbohydrate determinants including N-glycans. However, the nature of specific NK receptors, or receptor ligands on the target cells have remained obscure, since NK cells neither recognize nor kill all tumor targets equally effectively.

Ectopic expression of GnT-III in K562 cells results in an increase in bisecting GlcNAc and a decrease in external sialic acid and tri- and tetraantennary structures by blocking branching formation [31]. These aberrant changes of sugar chains are associated with a decreased susceptibility to NK cytotoxicity. Compared to controls, NK cell cytotoxicity was completely blocked against GnT-III transfectants. The binding of effector cells to positive transfectants was also decreased considerably. After subcutaneous injection into nude mice, the GnT-III transfectants produced spleen colonization, while none were

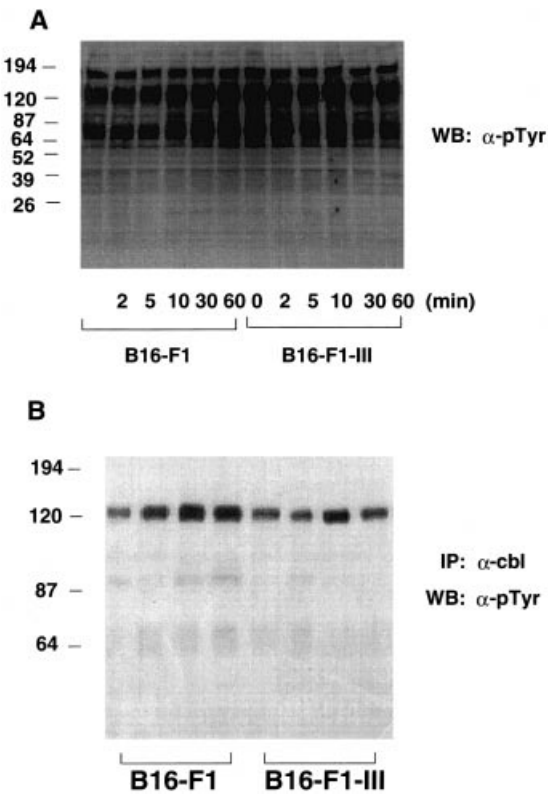

Fig. 3 EGF-dependent protein tyrosine phosphorylation in B16F1 parental and transfected cells. (A) Cells were treated with 50 ng/mL of EGF for the indicated times. The proteins (80 μg) from these cells were electrophoresed and subjected to immunoblot analysis with anti-phosphotyrosine antibody. (B) The proteins (2.0 mg) from cells were immunoprecipitated with anti-cbl antibody. The immunoprecipitated proteins were resolved by gel electrophoresis and subjected to immunoblot analysis with anti-phosphotyrosine antibody.

observed in the control cells. These results indicate that K562 cells which express GnT-III are resistant to NK cytotoxicity, probably as the result of binding to endogenous lectin which has not yet been identified. This binding then results in spleen colonization in nude mice.

Downregulation of gene expression of hepatitis B associated antigens in GnT-III transfectants

Numerous viruses, including the AIDS virus, HBV and mouse Molony virus, have specific sugar chains in their structural proteins, although their functions *in vivo* are not fully understood. *In vivo* experiments suggest that sugar chains may be important for infection or viral secretion. The cell line HB611 established by transfecting the HBV genome into a human hepatoblastoma cell line Huh 6 produces a large amount of hepatitis B surface antigen, hepatitis B envelope antigen and HBV virion into the medium. We transfected the GnT-III gene into this cell line and found that the levels of these antigens in the medium were markedly lower in positive transfectants, compared to negative transfectants or the parental cells [32]. Northern blot analysis showed a marked suppression of HBV related mRNAs in the positive transfectants. When positive transfectants cells were treated with the oligosaccharide processing inhibitors such as tunicamycin and swainsonine, the expression of HBV-related mRNAs was dramatically increased. These data indicate that some glycoproteins whose oligosaccharide structures are altered by the over-expression of GnT-III suppress HBV-related gene expression.

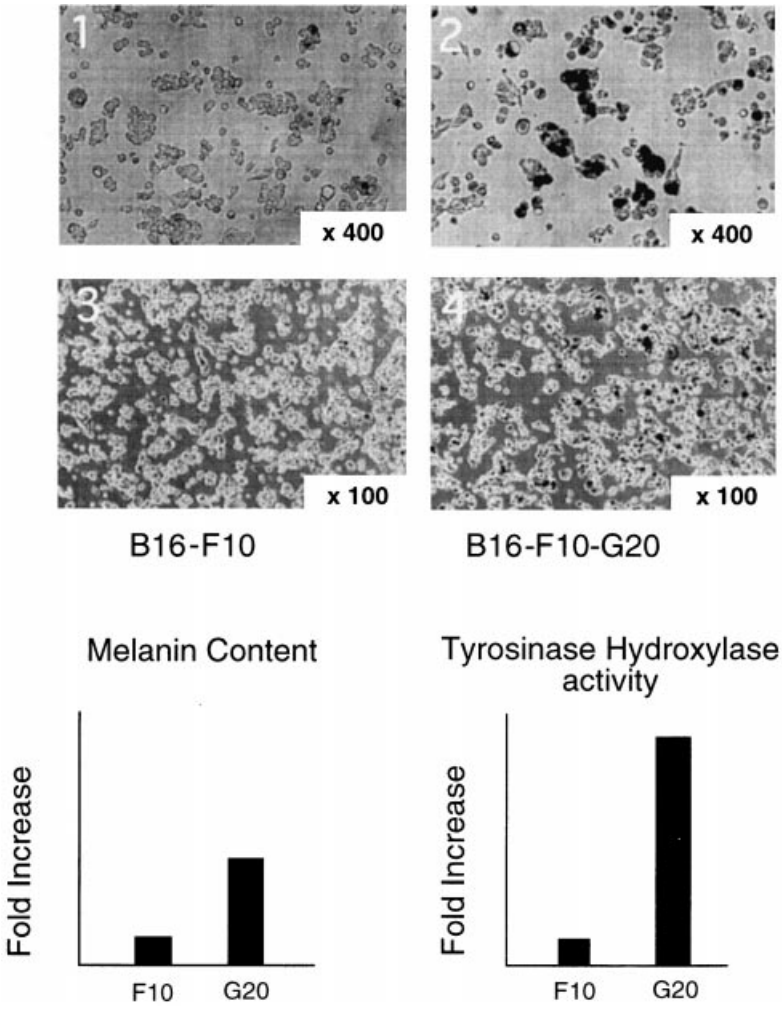

Fig. 4 Melanin content and tyrosinase hydroxylase activity in B16-F10 parental and transfected cells.

Major swine xenoantigen, Galα1-3 Gal epitope is down-regulated by GnT-III transfection in swine endothelial cells

Xenotransplantation is a potential solution to the world-wide shortage of organs for transplantation. However, the α-galactosyl epitope is the major antigen in swine to human xenotransplantation. GnT-III gene transfection into swine endothelial cells reduces their susceptibility to normal human serum in complement-mediated cell lysis and also suppresses the antigenicity to human natural antibodies [33]. Western blot and other analyses indicate that proteins smaller than 66 KDa have diminished reactivity to normal human serum which contain natural antibodies to the α-Gal epitope. In addition, the 1B 4 lectin which specifically binds to the α-Gal epitope also supports this conclusion. Collectively, these data suggest that GnT-III gene transfection into swine endothelial cells down-regulates the expression of the α-Gal epitope in swine endothelial cells. These findings point to the potential and promising role of the GnT-III enzyme in xenotransplantation.

α1-6FucT, a new member for fucosyltransferase family

α1-6 FucT is a newly discovered fucosyltransferase which catalyses the α1-6 addition of a fucose residue at the innermost GlcNAc residues in *N*-glycans. We have reported the successful purification and cDNA cloning of α1-6FucT both from porcine brain and from a human gastric cancer cell line [11,34]. The enzyme is a typical type-II transmembrane protein and the homology between the human and porcine enzyme is 92% and 96% at the amino acid level and nucleic acid level, respectively. No putative N-glycosylation sites were found in the primary sequence of α1-6 FucT. The enzymatic product, i.e. α1-6fucosylated glycoproteins are widely distributed over many tissues. α1-6 FucT is a novel glycosyltransferase and has no sequence homology with other members of the fucosyltransferase gene family such as α1-2, α-1-3 and α 1-4 fucosyltransferases. Of special interest in terms of carcinogenesis is the appearance of α1-6 fucosylated α-fetoprotein in serum of patients with hepatoma. This is a promising tumor marker for the diagnosis of primary hepatoma which will develop from the stage of liver cirrhosis. The up-regulation of α1-6 FucT has also been observed in rat hepatocarcinogenesis and in some of the human hepatoma tissues [35,36]. Recently, in our laboratory the α1-6 FucT transgene has been over-expressed in mice. Our preliminary data indicate a morphological alteration in the liver and kidney of these mice.

FUTURE PERSPECTIVES

Gene expression of glycosyltransferases are regulated in a tissue and organ specific manner and this may reflect the multiplicity and diversity of sugar chains in various organs. Therefore, the expression of glycosyltransferase(s) in a certain type of cell may modify the sugar chains of glycoconjugate. The most important remaining issue, however, is the identification of the target molecule and the characterization of the physiological implications related to these modifications. A broad range of target molecules for GnT-III modification in different cells and their physiological role have been described in this review (Table 1). For example, apoB100 is one of the target proteins which undergoes glycosylation in GnT-III transgenic mice. In highly metastatic B16 and B16-F10 melanoma cells, E-cadherin and tyrosinase, respectively, represent the potential targets for GnT-III modifications. In addition, receptor protein tyrosine kinases, such as Trk in PC 12 and EGF-R in U373 glioma cells have also been identified as targets which are modified by GnT-III. As judged by lectin blotting using E-PHA which preferentially recognizes bisecting GlcNAc, the number of glycoproteins which undergo aberrant glycosylation is very limited. This suggests that there are limited numbers of glycoproteins which undergo glycosylation when the glyco-genes are highly expressed.

It is clear that a thorough understanding of normal cell growth control is of paramount importance to understanding glycosyltransferase-mediated cellular proliferation and differentiation processes, since the functions of glyco-genes can only be understood in the context of mechanisms regulating these processes in a normal cell. Indeed, bisecting GlcNAc has multifaced properties and may utilize multiple signaling pathways to execute its ultimate effect in a cell. The down-regulation of MAP kinases in a GnT-III over-expressing cell implies that glyco-genes may also modify certain transcriptional factors, thus modulating

gene expression in various cells. Therefore, an elucidation of the role of sugar chain(s) in growth signaling may open new insights in the area of glycobiology and glycotechnology.

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