Review

Challenge to the suppression of tumor growth by the β4-galactosyltransferase genes

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Abstract: It has been well established that structural changes in glycans attached to proteins and lipids are associated with malignant transformation of cells. We focused on galactose residues among the sugars since they are involved in the galectin-mediated biology, and many carbohydrate antigens are frequently expressed on this sugar. We found changes in the expression of the β4-galactosyltransferase (β4GalT) 2 and 5 genes in cancer cells: decreased expression of the β4GalT2 gene and increased expression of the β4GalT5 gene. The growth of mouse melanoma cells showing enhanced expression of the β4GalT2 gene or reduced expression of the β4GalT5 gene is inhibited remarkably in syngeneic mice. Tumor growth inhibition is probably caused by the induction of apoptosis, inhibition of angiogenesis, and/or reduced MAPK signals. Direct transduction of human β4GalT2 cDNA together with the adenovirus vector into human hepatocellular carcinoma cells grown in SCID mice results in marked growth retardation of the tumors. β4GalT gene-transfer appears to be a potential tool for cancer therapy.

Keywords: galactose, N-glycans, lactosylceramide, β4-galactosyltransferase gene, tumor growth, cancer gene therapy

1. Introduction

Earlier studies have demonstrated that sialidase treatment of cancer cells reduces their electrophoretic mobility, causes a substantial decrease in oncogenicity, and increases immunogenicity in animals.1,2) Overall changes in cell surface glycoconjugates are also evident in studies on the differences in lectin binding and lectin-induced agglutination of cancer cells with a focus on the facts showing that lectin-resistant cancer cells acquire reduced tumorigenic and/or metastatic potentials as compared with the parental malignant cells.3) These observations sparked extensive investigations of glycoconjugates of malignant cells, and soon many investigators demonstrated that both quantitative and qualitative changes take place in cell surface glycoproteins and glycosphingolipids (GSLs) upon malignant transformation of cells.

One of the most characteristic features of glycoproteins in malignantly transformed cells is an increase in large N-glycans on the cell surface. This was discovered initially by Robbins’s group4) and by Warren’s group5) who compared the gel filtration patterns of the glycopeptides obtained by extensive pronase-digestion of metabolically labeled normal and malignant cellular glycoproteins. At first, the increased molecular weight of the glycopeptides was considered to be due to a higher content of sialic acid in their glycans. However, subsequent studies revealed that the large glycopeptides are not solely due to increased sialylation but also to increased branching of the glycans. In fact, structural analyses of N-glycans isolated from BHK cells and from polyoma virus- or Rous sarcoma virus-transformed BHK cells

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Abbreviations: β4GalT: β4-galactosyltransferase; CAR: coxsackie adenovirus receptor; Cer: ceramide; ERK: extracellular signal-regulated kinase; Glc-Cer: glucosylceramide; GSLs: glycosphingolipids; Lac-Cer: lactosylceramide; MAPK: mitogen-activated protein kinase; MEF: mouse embryonic fibroblast; MTAg: middle T antigen; RCA-I: Ricinus communis agglutinin-I; VEGF: vascular endothelial growth factor; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling.

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showed that the increase in GlcNAc(1→6) branching in the Man(1→6)Man arm of trimannosyl cores is the structural basis for the Warren-Glick phenomenon described above. A similar phenomenon has also been observed in the O-glycans of cancer cells. Most GlcNAc residues in the outer branches of N- and O-glycans are subsequently galactosylated by O4-galactosyltransferases (O4GalTs), and some of them are recognized by galectins to induce a variety of biological events such as apoptosis. Many carbohydrate antigens including polysialic acid (Fig. 1a), HNK-carbohydrate, (Fig. 1b), poly-N-acetyllactosamine (Fig. 1c), ABO blood group antigens (Fig. 1d, e and f), and sialyl Lewis X group (Fig. 1h) are expressed on the Gal(1→4)GlcNAc group (reviewed in ref. 10).

In the case of the GSLs in malignantly transformed cells, the expression of gangliosides, sialic acid-containing GSLs, is enhanced. In particular, gangliosides GM3 and GD3 are characteristic of many aggressive tumors such as melanomas and neuroblastomas. These gangliosides are derived directly from Gal(1→4)Glc(1→1)Cer (lactosylceramide: Lac-Cer) synthesized by lactosylceramide synthases, O4GalT5 and O4GalT6. Furthermore, lacto-, neolacto-, globo-, and isoglobo-series GSLs are also synthesized from Lac-Cer (reviewed in ref. 18). Therefore, the Lac-Cer synthesized by O4GalT5 and O4GalT6 is important for inducing characteristic features in cancer cells.

Thus, the galactosylation of not only N- and O-glycans on glycoproteins but also on glucosylceramide (Glc-Cer) and lactotriaosylceramide (Lc3Cer) by O4GalTs is very important for the altered cell surface properties of cancer cells. In the present article, the biological significance of O4GalT5s in cancer cells is described, and possible clinical applications of the O4GalT genes in cancer therapy are discussed.

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Fig. 1. A variety of carbohydrate antigens expressed on the Gal(1→4)GlcNAc groups of N- and O-glycans and a neolacto-series of GSLs. a, polysialic acid; b, HNK-1 carbohydrate; c, poly-N-acetyllactosamine; d, blood group O-antigen; e, blood group A-antigen; f, blood group B-antigen; g, Lewis X; h, sialyl Lewis X; and i, Lewis Y.
2. Acceptor-specificities of 4GalTs

4GalT exists as a family comprising seven members (Fig. 2) (reviewed in ref. 19) with different acceptor-specificities, tissue distribution, chronological expression and/or biological functions although some aspects require further investigation. 4GalT1 and 4GalT2 show broad substrate specificities toward N-glycans and possess high activities for the poly-N-acetyllactosamine synthesis. Furthermore, 4GalT2 can effectively galactosylate the GlcNAcβ1→2Man chain on α-dystroglycan and the GlcNAcβ1→3Fuc chain on Notch receptors. Quite interestingly, the expression level of HNK-1 carbohydrate decreases dramatically in 4GalT2-deficient mouse brain. This indicates that HNK-1 carbohydrate is expressed predominantly on the Galβ1→4GlcNAc group synthesized by 4GalT2. In the case of 4GalT1-deficient mice, HNK-1 carbohydrate and polysialic acid appear to be expressed normally in the brain glycoproteins. These results indicate that polysialic acid is expressed on the Galβ1→4GlcNAc group synthesized by 4GalT(s) other than 4GalT1 and 4GalT2. In addition, it was shown that the GlcNAc residues in 4GalT1-deficient mouse liver and plasma glycoproteins are galactosylated in a 1,3-linkage instead of a 1,4-linkage, although there are some ungalactosylated N-glycans in the mutant mouse glycoproteins. 4GalT3 is involved in the synthesis of the first N-acetyllactosamine unit of poly-N-acetyllactosaminoglycans and of neolacto-series GSLs, and 4GalT4 in the synthesis of neolacto-series glycolipids. Recent studies have revealed that 4GalT3 is also responsible for the poly-N-acetyllactosamine synthesis on N-glycans. In addition, 4GalT4 can also act on the GlcNAc-6-O-sulfate residues, and may be involved in the syntheses of keratan sulfate-related glycans and be a precursor of 6-sulfosialyl-Lewis X group. In the case of 4GalT5, it can galactosylate not only N- and O-glycans but also Glc-Cer in vitro. Quite interestingly, 4GalT5 shows a higher specific activity toward Glc-Cer than 4GalT6, which was initially isolated as a lactosylceramide synthase from rat brain. To establish the substrate specificity of 4GalT5 in cells, we analyzed N- and O-glycans, and GSLs in fibroblast cells isolated from 4GalT5−/−-mouse embryos, and found marked decreases in the amounts of Luc-Cer and GM3, a major ganglioside, in the cells, but no changes in N- and O-glycosylation of the proteins. A similar result has also been reported. Therefore, we conclude that 4GalT5 is a lactosylceramide synthase, and is not involved in the galactosylation of N- and O-glycans in vivo. The difference in the substrate specificity of 4GalT5 between the in vitro and in vivo assays could be due to a difference in the accessibility of the transferase toward substrates. In the case of 4GalT6-deficient mice, they were born and grew up normally, and showed no significant changes in Lac-Cer synthase activity and GSL composition in the brain as compared with those of wild-type mice. 4GalT7 acts in the synthesis of the glycosaminoglycan-protein linkage region of proteoglycans.

Following the action of 4GalTs as described above, many other glycosyltransferases compete with one another to add sugars to the galactosylated products to express a wide range of biologically active carbohydrate determinants, some of which are shown in Fig. 1, and to express a series of GSLs and chondroitin sulfate and heparan sulfate chains. Therefore, the 4-galactosylation of glycans is again quite important for many biological events including the development of cancers. Although there have been numerous studies on the changes that occur in glycan structures in cancers, there are a limited number of reports on the functions of the galactose-containing glycans.

Fig. 2. Human 4GalT family proteins. For each transferase, the closed box indicates a putative transmembrane domain (TM), and other boxes in blue, green and red indicate amino acid sequences conserved among 4GalTs 1, 2, 3, 4, 5 and 6 but not 4GalT7. The positions of Cys residues (C) are also indicated. The total number of amino acids is shown at the right.
3. Transformation-associated changes in the expression of β4GalT genes

As described earlier, the activity of the N-acetylglucosaminyltransferase V, which is responsible for the formation of 2,6-branched N-glycans, has been shown to increase several fold upon malignant transformation of cells.37),38) and the expression levels of highly branched N-glycans have been shown to correlate with the metastatic potentials of cancer cells.39) However, there are only a few reports describing β4GalT activity upon cell transformation.40) We determined β4GalT activities towards GlcNAcβ-S-pNP and GlcNAc in NIH3T3 cells and their polyoma middle T antigen gene-mediated transformed cells (MTAg cells), and found only a slight increase in β4GalT activities in MTAg cells as compared with NIH3T3 cells.41) This indicates that β4GalT activity does not change significantly when cells undergo malignant transformation, or that β4GalT activity cannot be determined correctly using the above acceptor substrates. Unfortunately, nothing is known about the acceptor-substrates by which the individual β4GalT1, β4GalT2, β4GalT3, β4GalT4, β4GalT5 and β4GalT6 activities differentiate themselves in vitro. Instead of the enzymatic activities, we determined the expression levels of the β4GalT genes in NIH3T3 cells and MTAg cells by Northern blot analysis, and found that the expression level of the β4GalT2 gene decreases to one-fifth to one-tenth while that of the β4GalT5 gene increases two- to three-fold with no significant changes in the other β4GalT genes in MTAg cells as compared with NIH3T3 cells (Fig. 3).41) These changes were also observed in eight human cancer cell lines42) where the gene-expression levels were determined by Northern blot analysis and expressed as relative to the expression levels of the N-acetylglucosaminyltransferase V gene, a marker gene whose association with malignancy has been described.37),38) since no normal counter cells were available for the cell lines used. The results so far indicate that the decreased expression of the β4GalT2 gene and the increased expression of the β4GalT5 gene occur in several types of cancer cells and tumors.

In addition, changes in the gene expression levels of other β4GalTs have been reported in cancer cells. The increased expression of the β4GalT1 gene leads to the formation of the sialyl-Lewis X determinant that has been shown to correlate with the metastatic potentials of human lung cancer cells and U937 cells.43),44) The β4GalT1 and β4GalT5 genes have also been shown to be involved in the development of the multidrug resistance of human leukemic cells by activating hedgehog signaling and enhancing the expression of transporters, p-glycoprotein, and multidrug resistance-associated protein 1.45) Indeed, overexpression of the β4GalT1 gene or the β4GalT5 gene results in the acquirement of multidrug resistance in HL60 cells, and silencing of the β4GalT1 gene or the β4GalT5 gene produces cells sensitive to therapeutic drugs.40) Over-expression of the β4GalT2 gene has been shown to induce p53-mediated apoptosis in HeLa cells.46) The expression of the β4GalT3 gene enhances the migration and invasion of human neuroblastoma cells,28) whereas the expression of the same gene suppresses metastasis in human
colorectal cancer. Further, high expression of the \( \beta 4 \)GalT4 gene is associated with colorectal cancer metastasis and poor prognosis. Increased expression of the \( \beta 4 \)GalT5 gene is also associated with the malignant properties of several tumors and cancer cells.\(^{48}\) In the cases of the \( \beta 4 \)GalT6 and \( \beta 4 \)GalT7 genes, no knowledge is available concerning their relevance to cancers. Although the expression of several \( \beta 4 \)GalT genes changes markedly in cancers, only a few reports describe the mechanisms for the transcriptional regulation of these genes.\(^{49}-^{51}\) Overall, the reports indicate that \( \beta 4 \)GalT1, \( \beta 4 \)GalT2, \( \beta 4 \)GalT3, \( \beta 4 \)GalT4 and/or \( \beta 4 \)GalT5 are somehow involved in the acquirement of the malignant properties of cancer cells and tumors.

4. Modified expression of \( \beta 4 \)GalT gene in cancer cells

If the altered galactosylation of N-glycans or Glc-Cer in cancer cells is critical to their malignant properties, the effect can be neutralized by reverting the expression levels of the respective \( \beta 4 \)GalT genes to those of normal cells through gene transduction. As we reported that the expression level of the \( \beta 4 \)GalT2 gene decreases upon malignant transformation of cells,\(^{41}\) we wondered why this is the case. In order to elucidate the biological significance of this decrease, we simply transfected mouse \( \beta 4 \)GalT2-sense cDNA into B16-F10 mouse melanoma cells, and isolated several clones showing enhanced expression of the \( \beta 4 \)GalT2 gene. One of the clones, clone D4, which had a 2.5-fold higher expression level than clone C2 (mock cells), showed increased binding of Ricinus communis agglutinin-I (RCA-I), which interacts with glycans terminated with the Gal\( \beta 1 \rightarrow 4 \)GlcNAc/Glc group, to several membrane glycoproteins as compared with the control, indicating that the transfected cDNA is translated into the protein and stimulates the galactosylation of N-glycans. No significant difference in growth rate was observed between the two clones in culture (Fig. 4a), but a partial cellular interaction appeared to occur in clone D4 cells when they were at sub-confluence (Fig. 4b).\(^{53}\) This indicates that the cell surface properties are changed slightly upon the enhancement in \( \beta 4 \)GalT2 gene expression. Since galactose residues on N-glycans of several plasma membrane proteins have been suggested to be involved in the contact-dependent inhibition of growth of human embryonic fibroblast cells,\(^{54}\) higher galactosylation of N-glycans by the greatly enhanced expression of the \( \beta 4 \)GalT2 gene over that of the clone D4 may lead to the recovery of this growth inhibition mechanism.

4.1 Enhanced expression of the \( \beta 4 \)GalT2 gene in B16-F10 cells and its biology. The expression level of the \( \beta 4 \)GalT2 gene decreases markedly upon malignant transformation of cells,\(^{41}\) and we wondered why this is the case. In order to elucidate the biological significance of this decrease, we simply transfected mouse \( \beta 4 \)GalT2-sense cDNA into B16-F10 mouse melanoma cells, and isolated several clones showing enhanced expression of the \( \beta 4 \)GalT2 gene. One of the clones, clone D4, which had a 2.5-fold higher expression level than clone C2 (mock cells), showed increased binding of Ricinus communis agglutinin-I (RCA-I), which interacts with glycans terminated with the Gal\( \beta 1 \rightarrow 4 \)GlcNAc/Glc group, to several membrane glycoproteins as compared with the control, indicating that the transfected cDNA is translated into the protein and stimulates the galactosylation of N-glycans. No significant difference in growth rate was observed between the two clones in culture (Fig. 4a), but a partial cellular interaction appeared to occur in clone D4 cells when they were at sub-confluence (Fig. 4b).\(^{53}\) This indicates that the cell surface properties are changed slightly upon the enhancement in \( \beta 4 \)GalT2 gene expression. Since galactose residues on N-glycans of several plasma membrane proteins have been suggested to be involved in the contact-dependent inhibition of growth of human embryonic fibroblast cells,\(^{54}\) higher galactosylation of N-glycans by the greatly enhanced expression of the \( \beta 4 \)GalT2 gene over that of the clone D4 may lead to the recovery of this growth inhibition mechanism.
so called “contact inhibition” for B16-F10 cells and probably for other cancer cells in culture. Next, we transplanted clone C2 and clone D4 cells subcutaneously into C57BL/6 mice, monitored their tumor growth up to two weeks, and found marked growth inhibition of the tumors formed with clone D4 as compared with those formed with clone C2 (Table 1). Lectin blot analysis of membrane proteins from these tumors showed that a remarkable increase in RCA-I-binding of several proteins in clone D4-derived tumors as compared with clone C2-derived tumors, indicating that the modified galactosylation of N-glycans in membrane proteins contributes to growth inhibition/retardation of melanoma cells. Although clone D4-derived tumors show growth retardation, there is no significant growth inhibition in clone D4 cells in culture. This could be due to differences in their growth conditions such as the presence of growth factors abundant in culture media but less in vivo.

We pursued possible mechanisms of this anti-tumor effect induced by the enhanced expression of the β4GalT2 gene by immunohistochemical analysis. Apoptosis is often observed in tumors treated with anti-cancer reagents, and this is often mediated by a p53 transcription factor. Quite interestingly, it has been shown that β4GalT2 gene expression is enhanced with this same transcription factor. In fact, the expression level of the β4GalT2 transcript increases in adriamycin-induced apoptotic HeLa cells, and that reducing the β4GalT2 transcript level with siRNA inhibits the apoptosis, indicating that a product(s) galactosylated by β4GalT2 is involved in the induction of apoptosis in HeLa cells. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay of clone D4-derived tumors showed apoptotic cells to be present in greater numbers than those in clone C2-derived tumors (TUNEL in Fig. 5 and Table 2). Some of galectins are up-regulated in several types of tumors and are involved in their progression, aggressiveness, and metastatic potentials. Furthermore, galectins have been

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**Table 1. Tumorigenic potentials of clone C2 and clone D4 cells**

| Cells   | Tumors formed | Tumor size (mm) (n) | p-value* |
|---------|---------------|---------------------|----------|
| clone C2 | 10/10 (100%)  | 13.1 ± 0.76 (10)    | <0.01    |
| clone D4 | 8/10 (80%)    | 5.1 ± 1.13 (8)      | <0.01    |

*p-value refers to a comparison between the average sizes of tumors formed with clone C2 cells and those with clone D4 cells.

Values given are means ± SE’s (n).

(Modified from ref. 53 with permission from Nature Publishing Group.)

This and following studies were carried out in strict accordance with the recommendations in the Guidelines for the Care and Use of Laboratory Animals of the Japanese Society for Biochemistry. The protocols were approved by the Committees on the Ethics of Animal Experiments of Nagaoka University of Technology and of Chiba University, respectively. All surgery was performed under ether anesthesia, and all efforts were made to minimize suffering.

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**Table 2. Immunohistochemical analyses of B16-F10 mouse melanomas**

| Stainings | Tumors formed with | Number of positive cells a |
|-----------|---------------------|----------------------------|
| TUNEL     | clone C2            | 3.7 ± 1.20                 |
|           | clone D4            | 8.3 ± 0.88*                |
| CD31      | clone C2            | 95.0 ± 9.66                |
|           | clone D4            | 60.0 ± 1.53*               |
| Ki-67     | clone C2            | 98.0 ± 18.5                |
|           | clone D4            | 83.7 ± 9.95                |

Numbers of positively stained cells per arbitrary areas were counted for each tumor specimen (n = 3).

Values given are means ± SE’s (n = 3).

*p < 0.05; TUNEL-staining, clone D4-derived melanomas relative to clone C2-derived melanomas.

* p < 0.05; CD31-staining, clone D4-derived melanomas relative to clone C2-derived melanomas.

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suggested to be involved in angiogenesis. The binding of galectin-1 secreted from tumors upon hypoxia to N-glycans on vascular endothelial growth factor (VEGF)-receptors in endothelial cells can promote vascularization to tumors. Therefore, the galactosylation of N-glycans induced by β4GalT2 in the tumors may alter the binding of galectins so that angiogenesis is not induced. In fact, clone D4-derived tumors show decreased numbers of CD31-positive vessels as compared with clone C2-derived tumors (CD31 in Fig. 5 and Table 2). This indicates that angiogenesis is also inhibited by the enhanced expression of the β4GalT2 gene. However, the numbers of Ki-67-positive cells, which indicate proliferating cells, remain the same in both tumors regardless of the β4GalT2 gene expression level (Ki-67 in Fig. 5 and Table 2). These results suggest that the growth retardation of clone D4-derived tumors by the enhanced expression of the β4GalT2 gene is partly attributable to augmented apoptosis and inhibited angiogenesis.

As β4GalT2 has been shown to be involved in mouse neuronal development and medaka (Oryzias latipes) gastrulation, the β4-galactosylation of N-glycans by β4GalT2 must be important for animal embryonic development and growth. In fact, β4GalT2-deficient mice show impaired learning/memory and, simultaneously, a significant decrease in the amount of the HNK-1 carbohydrate antigen expressed on the Galβ1→4GlcNAc group of N-glycans in the brain, which is involved in neuronal development (reviewed in ref. 61). These results indicate that the galactosylation of N-glycans by β4GalT2 is essential for brain function. Quite interestingly, this antigen is expressed normally in the β4GalT1-deficient mice, indicating that the galactosylation of N-glycans by β4GalT2 is different from that by β4GalT1. In accordance with this, the β4GalT1 and β4GalT2 show slightly different acceptor specificities towards branched N-glycans, and our preliminary studies show that β4GalT2 has a preference to galactosylate the GlcNAcβ1→2Manα1→3Man branch as compared with β4GalT1 (unpublished data). This acceptor specificity of β4GalT2 may determine whether or not N-glycans bind or not to the respective galectins.

It has recently been shown that β4GalT1 and β4GalT5 are involved in hedgehog signaling and multidrug resistance. Since the enhanced expression of the β4GalT2 gene induces apoptosis and inhibits angiogenesis significantly in melanomas, and since hedgehog signaling regulates angiogenesis, apoptosis, and cell cycle progression, hedgehog signaling may be involved in the growth retardation of β4GalT2 gene-transduced tumors. An examination of this signaling in the β4GalT2 gene-transduced tumors will lead to further understanding of the molecular mechanism of growth retardation.

4.2 Reduced expression of the β4GalT5 gene in B16-F10 cells and its biology. To date, two lactosylceramide synthases are known to be present in mammalian tissues, β4GalT5 and β4GalT6. β4GalT5 is expressed constitutively while β4GalT6 is expressed in a tissue-specific manner and possibly in a species-specific manner (unpublished data). A variety of GSLs are synthesized in the Golgi apparatus by successive glycosylation of Lac-Cer. Changes in the composition of GSLs have been reported in a variety of tumors and cancer cells in which there is an enhanced expression of gangliosides as described earlier.

Our previous studies and those of others suggest that the expression level of the β4GalT5 gene but not that of the β4GalT6 gene increases upon malignant transformation of cells and increased progression of astrocytomas. In the case of human colorectal cancer-derived epithelial cells, the expression level of the β4GalT5 transcript has been shown to increase by 4.5-fold as compared to its normal counterpart without any change in the expression level of the β4GalT6 transcript. These studies indicate that β4GalT5 is solely involved in the changes in GSL composition that occur upon malignant transformation of cells.

Again, we wondered why the expression of the β4GalT5 gene increases in cancer cells. To elucidate the biological significance of this increased expression, we introduced the mouse β4GalT5 anti-sense cDNA into B16-F10 cells, and isolated several clones showing reduced expression levels of the β4GalT5 gene. One of these clones, clone E5, with a β4GalT5 gene expression level reduced to 35% that of clone C1 (mock cells) showed decreased amounts of Lac-Cer and its derivative GM3, a major ganglioside, as compared with clone C1 (unpublished data) indicating that the transfected anti-sense cDNA causes a decrease in the amount of protein translated, which results in the decreased amounts of GSLs. It appears that the growth rate of clone C1 cells is faster than that of clone E5 cells at high cell densities in culture, which reflects differences in the number of days needed to reach the stationary phase and to start the death phase (Fig. 6a). In this case, a clear partial cellular...
interaction was observed in clone E5 cells at sub-confluence (Fig. 6b). Since the biosynthesis and the amounts of GSLs have been shown to change between the sparse and confluent (contact inhibited) stages of human skin fibroblast cells in culture, the decreased growth rate and partial recovery of the cellular interaction of clone E5 cells might be induced by the lowered expression of Lac-Cer that results from the reduced expression of the \(O4\text{GalT5}\) gene.

We next transplanted clone C1 and clone E5 cells subcutaneously into syngeneic mice, and monitored their tumor growth up to two weeks. We observed remarkable growth inhibition of the tumors formed with clone E5 as compared with those formed with clone C1 (Fig. 7a, Table 3). Analysis of neutral and acidic GSL fractions of the tumors revealed that the amounts of Lac-Cer and GM3, a major ganglioside in B16 melanoma cells, were significantly less in clone E5-derived tumors as compared with clone C1-derived tumors (Fig. 7b). Cer is known to play important roles in regulating apoptosis, cell senescence, and cell cycle arrest, and an accumulation of Cer was expected in tumors upon reducing the level of \(O4\text{galT5}\) gene expression. However, no such observation was made in clone E5-derived tumors, indicating that the growth retardation of the tumors is not due to the induction of apoptosis or senescence of the cells. As to the metastatic potential, the number of nodules formed with clone E5 cells was only about half of that of nodules formed with clone C1 cells (Table 4). These results indicate that not only the tumor growth activity, but also the metastatic activity of B16-F10 cells are suppressed by reducing the expression level of the \(O4\text{GalT5}\) gene.

Similar results have been reported for glioma cells where the expression of the \(O4\text{GalT5}\) gene is reduced by RNA interference. Since other clones whose \(O4\text{GalT5}\) gene expressions were more than that of clone E5 showed weaker inhibition of tumor growth activity than clone E5, we assumed that the growth activity of tumors is correlated with the expression level of the \(O4\text{GalT5}\) gene. To address this, we used mouse embryonic fibroblast (MEF) cells prepared from

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**Table 3. Tumorigenic potentials of B16-F10 mouse melanoma cells transfected with \(O4\text{GalT5}\) anti-sense cDNA**

| Cells   | tumors formed (%) | tumor size (mm) | \(p\) value* |
|---------|-------------------|-----------------|--------------|
| clone C1 | 100% (10)         | 13.3 ± 0.5 (10) | –            |
| clone E5 | 90% (10)          | 5.9 ± 0.7 (10)  | <0.01        |

*\(p\) values refer to a comparison between the average sizes of tumors formed with clone C1 and clone E5 cells.

Values given are averages ± S.E. (n).

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**Table 4. Experimental metastatic potentials of B16-F10 mouse melanoma cells transfected with \(O4\text{GalT5}\) anti-sense cDNA**

| Cells   | No. of nodules formed (No. of animals) | \(p\) value* |
|---------|---------------------------------------|--------------|
| clone C1 | 101 ± 2.7 (10) (100%)                 | –            |
| clone E5 | 49 ± 1.4 (10) (48.5%)                 | <0.01        |

*\(p\) values refer to a comparison between the average numbers of nodules in the lungs formed with clone C1 and clone E5 cells.

Values given are averages ± S.E. (n).

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O4galt5/−, O4galt5+/− and O4galt5+/+−-mouse embryos since these cells possess defined numbers of the O4GalT5 gene or defined amounts of Lac-Cer expressed in the cells as compared with clones showing arbitrary expression levels of the O4GalT5 gene, transformed them with the polyoma virus middle T antigen-oncogene, and finally examined their malignancies. The sizes and numbers of colonies formed with these transformed cells in soft agarose gels were larger in the order of O4galt5+/−, O4galt5+/− and O4galt5+/+−-derived MEF clones. The volumes of tumors formed in nude mice were also larger in same order (Fig. 8). These results indicate that the growth activity of the transformed MEF cells correlates with the gene dosage of O4GalT5, that is, the amount of Lac-Cer synthesized in the cells. Collectively, the malignancies of cancer cells and tumors may correlate with the expression levels of the O4GalT5 gene.

Several reports have shown that Lac-Cer and GM3 promote cell migration and proliferation by activating the MAPK pathway. These molecules are considered to localize in GSL-enriched domains where various transmembrane signals are modulated. In fact, Lac-Cer can stimulate the expression of integrin molecules and the phosphorylation of extracellular signal-regulated kinase (ERK) proteins in the mitogen-activated protein kinase (MAPK) pathway, and GM3 is involved in the activation of RPTPα, a tyrosine phosphatase. Therefore, the reduced amounts of Lac-Cer and GM3 at the cell surface in clone E5-derived tumors can alter the cell adhesion properties and transmembrane signals that lead to the suppression of the original tumorigenic and metastatic potentials. An examination of the activation levels of the signaling molecules involved in the Ras-MAPK pathway by Western blot analysis revealed a significant increase.

Fig. 7. Tumors and their GSL compositions. Tumors formed with clone C1 and clone E5 cells in mice two weeks after subcutaneous transplantation are shown in panel a. Panel b shows the GSL components of tumors formed with clone C1 and clone E5 cells. Neutral (left lanes) and acidic (right lanes) GSLs separated on TLC plates are shown. (Reproduced from ref. 67 with permission from Oxford University Press.)

Fig. 8. Tumorigenic potentials of oncogene-transformed MEF cells. Panel a: Numbers of colonies formed from transformed MEF cells in soft agarose gels. Panel b: Sizes of colonies formed from transformed MEF cells in soft agarose gels. Panel c: Volumes of tumors formed from transformed MEF cells in athymic mice three weeks after transplantation. Panel d: Representative tumors formed from transformed MEF cells. +/+ , +/− , and −/− indicate MEF cells isolated from O4galt5+/+−, O4galt5+/− , and O4galt5+/+−-mice, respectively, and transformed with the viral oncogene. *p < 0.05 compared with O4galt5+/+−-MEF cells. (Modified from ref. 67 with permission from Oxford University Press.)
in the amount of GDP-bound Ras together with a significant decrease of the amount of GTP-bound Ras, and significant decreases in the phosphorylation levels of c-Raf, MEK and ERK1/2 in clone E5-derived tumors as compared with those of clone C1-derived tumors without any differences in the amounts of the respective proteins expressed between these tumors (Fig. 9). These results indicate that a tumor growth signal mediated by the MAPK pathway is attenuated due to the decreased amounts of Lac-Cer and GM3 in clone E5-derived tumors.

Since Lac-Cer (as a second messenger) has been also suggested to be involved in angiogenesis where the binding of VEGF to its receptors stimulates Lac-Cer biosynthesis followed by the activation of PI3 kinase and since the ablation of the β4GalT5 gene results in reduced VEGF-mediated angiogenesis in human endothelial cells, it may also be possible that the reduced expression of Lac-Cer leads to an attenuation of angiogenesis of tumors. Since the blockage of angiogenesis is another promising tool for inhibiting tumor growth, it is worth investigating the dose effect of Lac-Cer on angiogenesis in tumors in detail.

MEF cells, whose gangliosides are completely depleted by the abrogation of the GM3 synthase/GM2 synthase genes, have been shown to impair tumor growth when they were transformed with an oncogene, indicating that certain types of gangliosides are important for expressing the malignant properties of cancer cells and tumors. However, even when gangliosides are completely removed from malignantly transformed cells, the cells still possess tumorigenic potentials. Our recent study has shown that MEF cells isolated from β4galT5−/− mice show the lowest susceptibility to malignant transformation, while those from β4galT5+/− and β4galT5+/-mice show intermediate and high susceptibilities to malignant transformation upon transfection with a viral oncogene based on their colony-forming capabilities in soft agarose gels and their tumor-forming capabilities. This indicates that Lac-Cer itself is quite important for the malignant transformation of cells apart from being the precursor molecule for gangliosides. Lac-Cer may serve as a ligand for galectins to form galectin-lattices on cell surfaces, and such lattices modulate or alter the functions of cell surface molecules such as cell adhesion molecules and growth factor receptors. Although such binding has not yet been found, there is one possible study describing an interaction between Lac-Cer and galectin-3. More directly, the amount of Lac-Cer may be critical to membrane dynamics such as in the formation of microdomains at the cell surface. Further studies are required to establish the intrinsic functions of Lac-Cer at the cell surface, although some Lac-Cer also localizes in cytoplasmic vesicles in cells. In any case, reducing the expression level of Lac-Cer in tumors using enzymatic or genetic tools or by the use of biosynthetic inhibitors may be useful for suppressing tumor growth and metastasis not only in B16-F10 cells, but also in many other types of tumors.
5. Challenge to the suppression of human tumor growth by β4GalT genes

It appears that the transduction of the β4GalT2 sense cDNA or the β4GalT5 anti-sense cDNA into B16-F10 cells is an effective means of suppressing the tumor growth in the animals. In general, it is currently much easier to enhance the level of the β4GalT2 gene than to reduce the level of the β4GalT5 gene in cells. Therefore, we addressed whether or not the growth of human tumors in animals can be suppressed by the transduction of the β4GalT2 sense cDNA with an eye toward eventual clinical application. For this purpose, human β4GalT2 cDNA was cloned into an adenovirus vector (Ad/β4GalT2), and HuH-7 human hepatocellular carcinoma cells were allowed to grow in SCID mice. Ad/β4GalT2 or Ad/mock was injected into the tumors directly, and subsequent tumor growth was monitored. Murine cells, but not human cells, are resistant to type 5 Ad-mediated transduction due to their poor expression of the coxsackie adenovirus receptor (CAR), a major cellular receptor. The reason we did not use human melanomas in this study is that melanoma cells generally express a very low level of the CAR, but HuH-7 cells express relatively high levels of the receptor. Marked growth retardation was observed for the tumors injected along with the Ad/β4GalT2 as compared with those injected along with the Ad/mock (Fig. 10a and 10b).53)

The tumors injected with Ad/β4GalT2 contained five times more β4GalT2 transcript than those injected with Ad/mock (Fig. 10c), 1.4 times higher β4GalT activity toward 1 mM GlcNAcβ1-4GalNAc-β-S-pNP than the control tumors (Fig. 10d), and membrane glycoproteins with a significant increase in RCA-I-binding as indicated by the arrows in Fig. 10e, indicating that the β4GalT2 gene injected into tumors is translated into an enzyme that affects galactosylation of the membrane glycoproteins, which eventually results in marked growth retardation of HuH-7 carcinomas.53)

The continuous growth observed in tumors (Fig. 10a) could be due to the presence of untranslated tumor cells. In this way, there are several factors that affect Ad-mediated gene transfer into human tumors such as the expression level of the virus receptors on tumor cells, the penetration of Ad into tumor tissues, homogenous distribution of the reagent among tumor tissues following injection, and, therefore, the development of enhanced vector delivering systems will be a key issue for the feasible clinical application of this reagent.

The HuH-7 hepatocellular carcinomas showing growth retardation by Ad/β4GalT2 transduction were examined by immunohistochemical analysis, but no significant differences were detected statistically for the induction of apoptosis and the inhibition of angiogenesis although the Ad/β4GalT2-transduced tumors showed increased numbers of apoptotic cells and minimally decreased numbers of CD31-positive vessels in comparison with the Ad/mock-transduced tumors.53) This is probably due to the reasons that the β4GalT2 gene was not transduced
to all cells consisting of the tumors by the method of intratumor injection and the materials analyzed contained the tumor cells not undergoing transduction of the \( \beta 4 \text{GalT2} \) gene. Therefore, it is considered that no statistical significance was obtained in that study.

Recently, we isolated several B16-F10 clones showing enhanced expression of the \( \beta 4 \text{GalT2} \) gene together with reduced expression of the \( \beta 4 \text{GalT5} \) gene, and found that the growth activity of the tumors formed from one of these clones in syngeneic mice is suppressed much more strongly than tumors formed from the single gene-manipulated clones. This indicates that remodeling of the galactosylation of N-glycans by \( \beta 4 \text{galT2} \) and of the composition of GSLs by \( \beta 4 \text{GalT5} \) is a strong tool for cancer gene therapy, although the development of an effective delivery system for the \( \beta 4 \text{GalT2} \) sense cDNA and the \( \beta 4 \text{GalT5} \) anti-sense cDNA or its siRNA remains another task.

As the up-regulation or down-regulation of \( \beta 4 \text{GalT} \) gene expression with their sense cDNAs, anti-sense cDNAs or siRNAs can reduce the malignancy of tumors, it is also important to elucidate the mechanisms of the transcriptional regulation of the individual \( \beta 4 \text{GalT} \) genes.\(^{49)}\)–\(^{51)}\) Once these are elucidated, controlling \( \beta 4 \text{GalT} \) gene expression with the appropriate transcription factor is another approach to suppressing tumor growth assuming consideration is given to the fact that the transcription factors also affect the expressions of other genes.

**Conclusion**

Reversing the alterations seen in the expressions of the \( \beta 4 \text{GalT2} \) and \( \beta 4 \text{GalT5} \) genes in mouse melanoma cells results in a marked suppression of tumor growth via the induction of apoptosis, inhibition of angiogenesis or attenuation of the MAPK signalings. Enhanced expression of the \( \beta 4 \text{GalT5} \) gene has been shown to be involved in the acquisition of multi-drug resistance. Therefore, manipulating the expression of the \( \beta 4 \text{GalT2} \) and/or \( \beta 4 \text{GalT5} \) genes in tumors by the gene transduction represents a promising tool for suppressing tumor growth. In fact, the administration of the \( \beta 4 \text{GalT2} \) cDNA with the aid of an Ad vector into human hepatocellular carcinoma cells grown in SCID mice results in the successful suppression of tumor growth.

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Profile

Kiyoshi Furukawa, born in 1949, received his BS degree from Faculty of Science, Shizuoka University in 1974, and his PhD degree (under the supervision of Prof. Hiroshi Terayama) from the Zoological Institute, Faculty of Science, the University of Tokyo in 1979. He did post-doctoral research in the laboratories of Prof. Veer P. Bhavanandan in Department of Biological Chemistry, College of Medicine/Milton S. Hershey Medical Center at the Pennsylvania State University, Hershey, PA (1979–1982) and Prof. Steven Roth in Department of Biology/Josef Leidy Laboratory at University of Pennsylvania, Philadelphia, PA (1982–1985). In 1986, he returned to Japan, and was initially a Research Associate in Institute of Medical Science at the University of Tokyo (in the laboratory of Prof. Akira Kobata) (1986–1994), and then a Head of the Department of Biosignal Research at Tokyo Metropolitan Institute of Gerontology (1994–2005). He was promoted to Professor in Department of Bioengineering at Nagaoka University of Technology, Niigata in 2005, and elected to Chair of the Department (2008–2014). His research has centered on the roles of O4-galactosylated glycans attached to proteins and lipids in mammalian embryonic development and growth, and in diseases. Currently, his interest extends to the roles of N-glycans, if any, in the regeneration of parts of the body lost using planarians and earthworms that possess high regenerative potentials. In the meantime, he was a Secretary in General in the XVth International Symposium on Glycoconjugates (President: Prof. Akira Kobata) in Tokyo, 1999, and a Chief Organizer of the 30th Annual Meeting of the Japanese Society of Carbohydrate Research in Nagaoka, 2011. Awarded Tokyo Metropolitan Governor Prize in Science, 2003, and Mizutani Foundation for Glycoscience Research Grant in 2006.