Glycoconjugate Journal 20, 71–78, 2004

MINI REVIEW

Glycosylation defining cancer cell motility and invasiveness

Masaya Ono* and Senitiroh Hakomori

Pacific Northwest Research Institute, 720 Broadway, Seattle, WA 98122-4302, USA and Departments of Pathobiology and Microbiology, University of Washington, Seattle, WA, USA

Published in 2004.

Keywords: **focal de-differentiation site, sialyl-Le^x, tetraspanin, N-glycosylation, CD82, apoptosis, metastasis, CD9/alpha3 integrin/GM3 complex**

Introduction

The degree of tumor malignancy is controlled by many factors, *e.g.,* (i) motility and invasiveness of tumor cells through pericellular matrix and basement membrane; (ii) adhesion of tumor cells to target cells, particularly to microvascular endothelial cells; (iii) matrix-destroying protease activity; (iv) angiogenesis in tumor cell surroundings; (v) apoptotic susceptibility of tumor cells, etc. The mechanism of each event is functionally maintained by a combination of defined molecules involved in the processes, each of which is affected directly or indirectly by N- or O-glycosylation of functional proteins or surrounding gangliosides.

During the past three decades, many of our studies have involved structural characterization of glycosylation changes associated with oncogenic transformation, and identification of tumor-associated carbohydrate antigens [1,2]. A subsequent series of studies has addressed the functional significance of aberrant glycosylation in tumor progression and metastasis.

(i) Carbohydrate epitopes in tumors *per se* mediate tumor cell adhesion through carbohydrate-to-carbohydrate interaction, *e.g.,* [3; for reviews see 4–6], or through interaction of carbohydrate with its binding protein (*e.g.,* selectin, siglec, galectin) [7–10]. Expression of sialyl-Le^x (SLe^x) and sialyl-Le^a (SLe^a), the E-selectin epitopes, in tumors is generally considered to promote tumor cell invasion and metastasis since such expression is correlated with shorter patient survival [11–13].

To whom correspondence should be addressed: Senitiroh Hakomori, Pacific Northwest Research Institute, 720 Broadway, Seattle, WA 98122-4302, USA. E-mail: hakomori@u.washington.edu
*Present address: Cancer Proteomics Project, National Cancer Center Research Institute, Tokyo 104-0045, Japan.

(ii) Carbohydrates in tumors, either N- or O-glycosylation of major membrane receptors, modulate their functions in controlling tumor cell adhesion, motility, and invasiveness. Lipid glycosylation, *i.e.,* gangliosides [14], surrounding integrins, and growth factor receptors, have recently been found to be effective modulators as well. Those receptors susceptible to glycosylation are integrins, cadherins, CD44, and growth factor receptors; for review see [2] and references therein.

This brief review is based on studies by one of the authors and his colleagues on a specific type of SLe^x expressed at the site of "focal de-differentiation" [15], and on glycosylation effect on integrin/tetraspanin complex in low-density membrane fraction, controlling tumor cell motility and invasiveness [16–18].

Sialyl-Le^x expressed at focal de-differentiation site of colorectal carcinoma promotes metastatic potential to liver

The functional role of SLe^x epitope in metastatic potential and invasiveness of colorectal carcinoma was studied using three groups of cases. Group A (24 cases) had simultaneous liver metastases; group B (24 cases) had only lymph node metastases without recurrence for 5 years; group C (20 cases) had recurrence of liver metastases. We focused on cancer cell morphology at the invasive front, with expression of SLe^x, defined by IgG3 mAb SNH4 [19], in the primary cancer.

It was frequently found at the invasive front in group A that polygonal (not columnar) cancer cells with a single or solitary trabecular form with indistinct polarity showed an infiltrative growth pattern. This type of morphology was termed "focal de-differentiation" and graded into four levels. Eleven out of 24 cases (46%), showing severe focal de-differentiation, were found in group A, one out of 24 cases (4%) in group B,

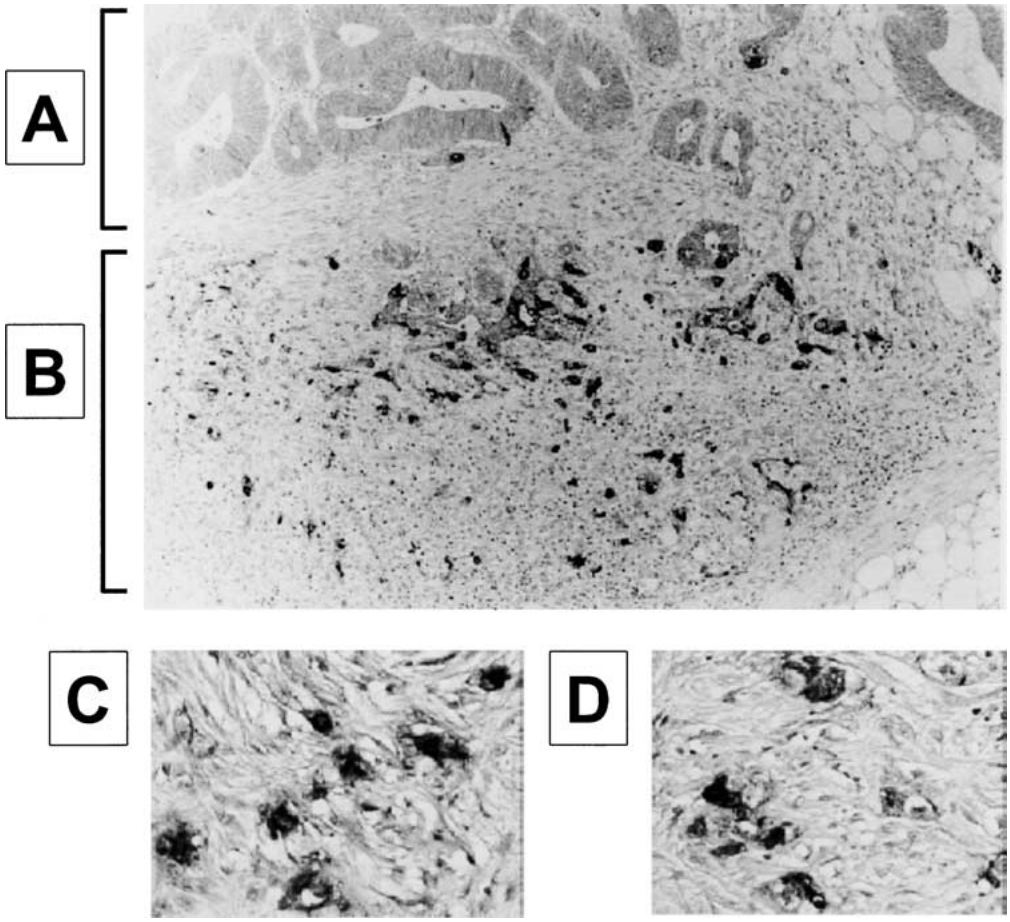

Figure 1. SLe^x expression at invasive front of colorectal cancer, in contrast to absence of SLe^x expression in differentiating tumor cells with glandular morphology. In non-invasive colorectal cancer, columnar cancer cells with glandular morphology are found, which are rarely stained by anti-SLe^x antibody (upper part; A). In contrast, the invasive front of colorectal cancer (lower part; B) has no columnar cancer cells with glandular morphology; rather, cancer cells are polygonal with single or solitary trabecular form and indistinct polarity (focal de-differentiation), and are often stained by anti-SLe^x antibody SNH4. Typical focal de-differentiation stained by anti-SLe^x mAb SNH4 at higher magnification (200×) is shown in Panels C and D. It should be noted that staining by SNH4 indicates modified cellular morphology prepared for invasiveness, regardless of E-selectin expressed at activated microvasculature.

and six out of 20 cases in group C (30%). SLe^x staining was positive in twelve out of 24 cases (50%) in group A; in three out of 24 cases (13%) in group B; and in seven out of 20 cases (35%) in group C. With respect to staining of SLe^x at focal de-differentiation site (Figure 1), it was positive in 17 out of 24 cases (71%) in group A; in four out of 24 cases (17%) in group B; and in eleven out of 20 cases (55%) in group C. Focal de-differentiation and SLe^x staining in the primary cancer showed a significant difference between groups A and B. SLe^x staining at focal de-differentiation site showed a significant difference between groups A and B and groups B and C [15]. Characteristic properties of groups A, B, and C are shown in Table 1.

Another anti-SLe^x mAb with IgM isotype, KM93 [20], diffusely stained all tumor cells and did not show preferential staining of the focal de-differentiation site. It is possible that IgG3 has higher binding affinity than IgM, and that SNH4 binds to SLe^x preferentially at the focal de-differentiation site regardless of its carrier carbohydrate chain.

Thus, focal de-differentiation and expression of SLe^x antigen in the primary lesion are good markers for assessing the metastatic potential of colorectal cancer, or expression of SLe^x may cause de-differentiation. The rationale for this phenomenon is unclear at this time. It is possible that SNH4-positive focal de-differentiation site binds preferentially to selectin expressed at microvascular endothelial cells, or that the site is somehow a preferable assembly for tumor cells to infiltrate through surrounding connective tissue.

N-glycosylation and GM3 inhibit tumor cell motility through integrin/tetraspanin complex, and promote apoptosis

The membrane protein KAI-1 was originally identified in prostate tissue as a metastasis-suppressing gene product [21]. Its sequence is identical to that of CD82, and it is downregulated in metastatic deposits [22]. A similar metastasis-suppressing

Table 1. Incidence of focal de-differentiation, SLe^x expression, and liver metastasis in colorectal carcinoma

	Occurrence of focal de-diff'n (%)	*SLe^x expression (%)*	*SLe^x expression in focal de-diff'n site (%)*	*Metastasis of colorectal carcinoma (CRC) to liver*
Group A	11/24 (46%)	12/24 (50%)	17/24 (71%)	Complete resection of primary CRC tumor. Liver metastasis observed at time of resection.
Group B	1/24 (4%)	3/24 (13%)	4/24 (17%)	Complete resection of primary CRC tumor. No Liver metastasis observed at time of resection, but metastasis to lymph nodes was observed. No liver metastasis observed during subsequent 5-yr period.*
Group C	6/20 (30%)	7/20 (35%)	11/20 (55%)	Complete resection of primary CRC tumor. No liver metastasis observed at time of resection of primary CRC. However, recurrence of liver metastasis was observed subsequently (within 5 yr).*

*Clinicohistologically, Groups B and C underwent complete resection of primary CRC, and no liver metastasis was observed at the time of this operation. Group B did not show subsequent recurrence of liver metastasis; metastasis was observed only to lymph nodes. Group C did show recurrence of liver metastasis later (within 5 yr). This reflects higher SLe^x staining at focal de-differentiation site in Group C (55%, vs. 17% for Group B).

effect was assumed to occur for motility-regulatory protein (MRP) [23], which has the same sequence as CD9 and is downregulated in colorectal carcinoma [24]. Both CD82 and CD9 have four hydrophobic transmembrane domains and are members of the tetraspanin (Tsp) family. In many cases, they are highly N-glycosylated, and function as facilitators of various membrane receptors [25]. Many Tsp are known to be associated with various types of integrin receptors to form an integrin/ Tsp "web" [26–30].

Our previous studies indicate that fibronectin-dependent adhesion through integrin $\alpha5\beta1$ is controlled by its N-glycosylation [31] and by surrounding GM3 ganglioside [32]. $\alpha5\beta1$ function is maintained by close association of $\alpha5$ with $\beta1$ through their N-glycosylation, since these two subunits are separated by de-N-glycosylation [31]. Fibronectin-dependent cell adhesion is mediated by $\alpha5\beta1$, and its function is maintained maximally when $\alpha5\beta1$ is present in membrane liposome with optimal concentration of GM3 [31]. These observations indicate that both N-glycosylation and a certain type of ganglioside (GM3 in the case of $\alpha5\beta1$) are essential to maintain optimal function of integrin receptor.

Tsp are usually N-glycosylated and may be susceptible to gangliosides. In order to study effects of N-glycosylation and gangliosides on Tsp-dependent cellular functions, we initially used Chinese Hamster Ovary cell mutant ldlD14 deficient in UDP-Glc 4-epimerase, transfected with CD9 or CD82 cDNA. These cells are incapable of synthesizing structures with complete N-glycosylation, O-glycosylation, LacCer, or essentially all glycosphingolipids (GSLs) and gangliosides derived from LacCer, unless Gal or GalNAc is added to culture medium [33,34]. Using these transfectants, we investigated the effect of glycosylation on CD9 and CD82 function, and obtained the following results.

(i) *Effect of CD82 and CD9 on haptotactic and phagokinetic motility, and on cell adhesion.* With CD82 or CD9 transfectants, N-linked glycosylation of CD82 was completed, showing higher molecular mass, and endogenous GM3 synthesis was observed when cells were cultured in chemically-defined, insulin/transferrin/selenium (ITS) medium with addition of Gal (+Gal condition) (Figure 2). In haptotactic motility assay using Boyden chamber with Matrigel-coated membrane, or in phagokinetic assay with gold sol-coated plate, under −Gal condition, motility was significantly enhanced in both CD82-or CD9-expressing cells in comparison to mock-transfected cells. However, under +Gal condition, motility of both CD82- or CD9-expressing cells was much lower, close to the level of mock-transfected cells; *i.e.,* the motility-inhibitory effect of CD82 or CD9 was manifested only when glycosylation was completed or when endogenous GM3 synthesis occurred [16]. In contrast to the effect of Matrigel, fibronectin-dependent adhesion of CD82-transfected cells under +Gal condition was not enhanced while fibronectin-dependent motility was enhanced in comparison to −Gal condition [17].

CD82- or CD9-expressing cells grown under +Gal condition showed massive cell death after a latent period, characteristic of apoptosis [16]. Thus, the malignancy-suppressing effect of CD82 or CD9 is based on cell motility inhibition as well as apoptosis promotion, both of which are induced by GM3 synthesis and complete N-glycosylation. Further detailed study is necessary to determine the signaling mechanism that underlies these phenomena.

(ii) *Glycosylation effect on Tsp/integrin interaction.* To clarify the effect of glycosylation on interaction of CD82 with integrins, we applied the co-immunoprecipitation approach using CD82-transfected ldlD14 cells (ldlD/CD82), under three conditions: (a) complete vs. incomplete glycosylation state; (b) native state, vs. removal of sialic acid with sialidase; (c) mutation of CD82 at N-linked consensus gene of various sites.

In ldlD/CD82, the molecular weights of CD82 and integrin $\alpha5$ were changed with or without Gal due to

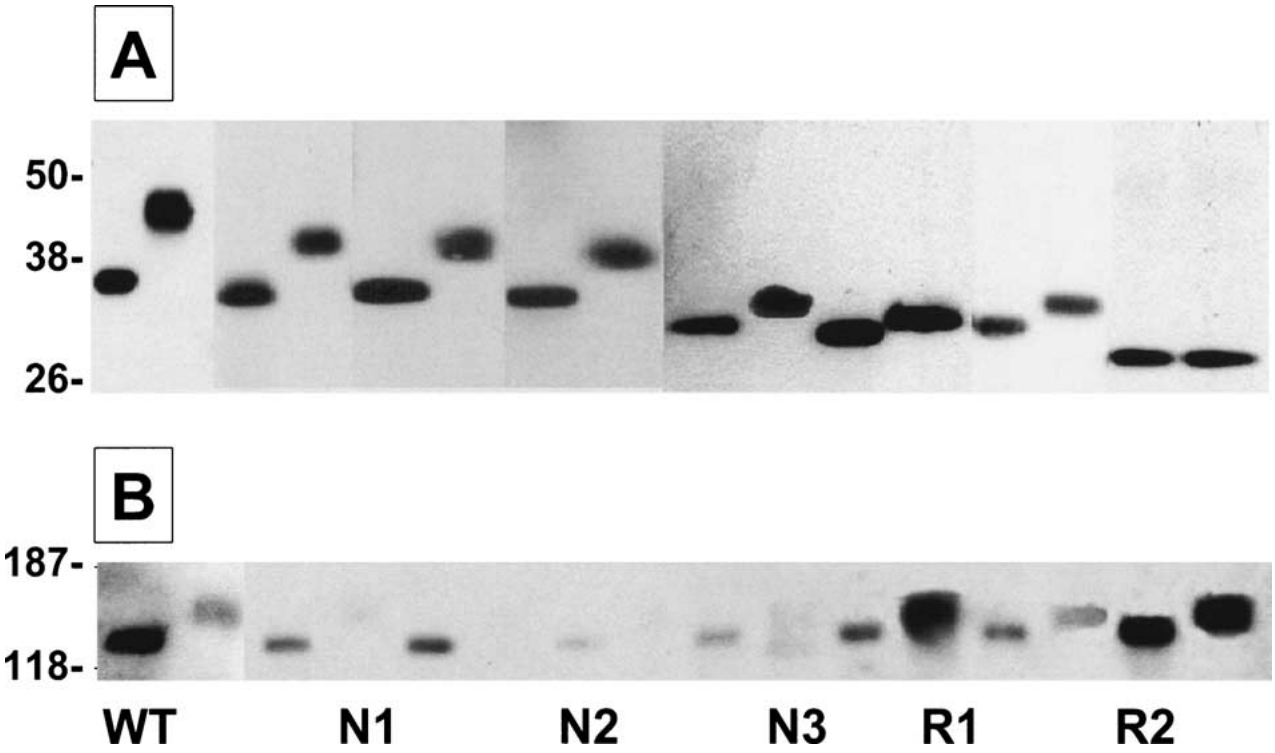

Figure 2. Co-immunoprecipitation of integrin $\alpha 5$ and CD82 mutant with N-linked glycosylation consensus gene. Asn 129, 157 and 198 of CD82, N-linked glycosylation consensus gene, were mutated to Gln. (A) CD82 molecular weight decreased according to increase of mutation site. (B) CD82 mutant co-immunoprecipitated with integrin $\alpha 5$ when both Asn129 and 198 were mutated. The mutation site of CD82 were N1: 129, N2: 157, N3: 198, R1: 157and 198, R2: 129 and 198, R3: 129 and 157, T: all of 129, 157 and 198. WT is wild type. In every wild and mutated cell lane, left side is cultured without Gal and right side with Gal.

elongation with completion of N-linked glycosylation. Co-immunoprecipitation of CD82 and $\alpha 5$ occurred to a much lesser extent in extracts from cells grown under +Gal compared to −Gal condition; *i.e.,* degree of CD82/$\alpha 5$ interaction was much lower when N-glycosylation was completed and ganglioside synthesis was present.

To investigate the effect of sialic acid located at the end of N-linked glycosylation, sialic acid was detached by sialidase. Under +Gal condition, cells were harvested and the lysate was mixed with sialidase and incubated. The molecular weights of CD82 and $\alpha 5$ were decreased by sialidase treatment. After sialidase treatment, $\alpha 5$ was co-immunoprecipitated with CD82 even under +Gal condition. The sialic acid at N-linked glycosylation is important for coupling of CD82 and $\alpha 5$.

To clarify the N-linked glycosylation site of CD82 responsible for coupling with $\alpha 5$, CD82 was mutated at three N-glycosylation sites (Asn129, Asn157, Asn198 to Gln129, Gln157, Gln198 respectively). By combination of three mutated sites of CD82, seven mutants were obtained. The CD82 mutants were transfected to ldlD14 cells, and transfectants were cultured with or without Gal. The molecular weight of CD82 was decreased according to the increase of Asn mutation site. The molecular weight of CD82 mutated all Asn sites to Gln was no different with or without Gal, because of no elongation of the N-linked glycosylation. When CD82 was mutated at both Asn129 and 198, coimmunoprecipitation of CD82 and $\alpha 5$ occurred under +Gal condition [17].

Interaction of CD82 and $\alpha 5$ is controlled by CD82 glycosylation. The CD82 glycosylation sites of Asn129 and Asn198 are particularly important (Figure 3). Glycosylation profoundly affects interaction of $\alpha 5$ with CD82, leading to significant change of cell motility.

Functional interaction of CD9 with GM3 ganglioside. A cooperative inhibitory effect of GM3, together with CD9, on haptotactic cell motility was demonstrated by the observations described below.

Haptotactic motility of colorectal carcinoma cell lines SW480, SW620, and HRT18, which express a high level of CD9, was inhibited by exogenous GM3, but not by GM1. In contrast, motility of gastric cancer cell line MKN74, which expresses a low level of CD9, was not affected by exogenous GM3. However, motility of MKN74 became susceptible to exogenous GM3 when CD9 level of the cells was enhanced by its gene transfection. The motility was not susceptible to GM1 regardless of CD9 level.

These findings suggest that haptotactic tumor cell motility is cooperatively inhibited by coexpression of CD9 and GM3. This

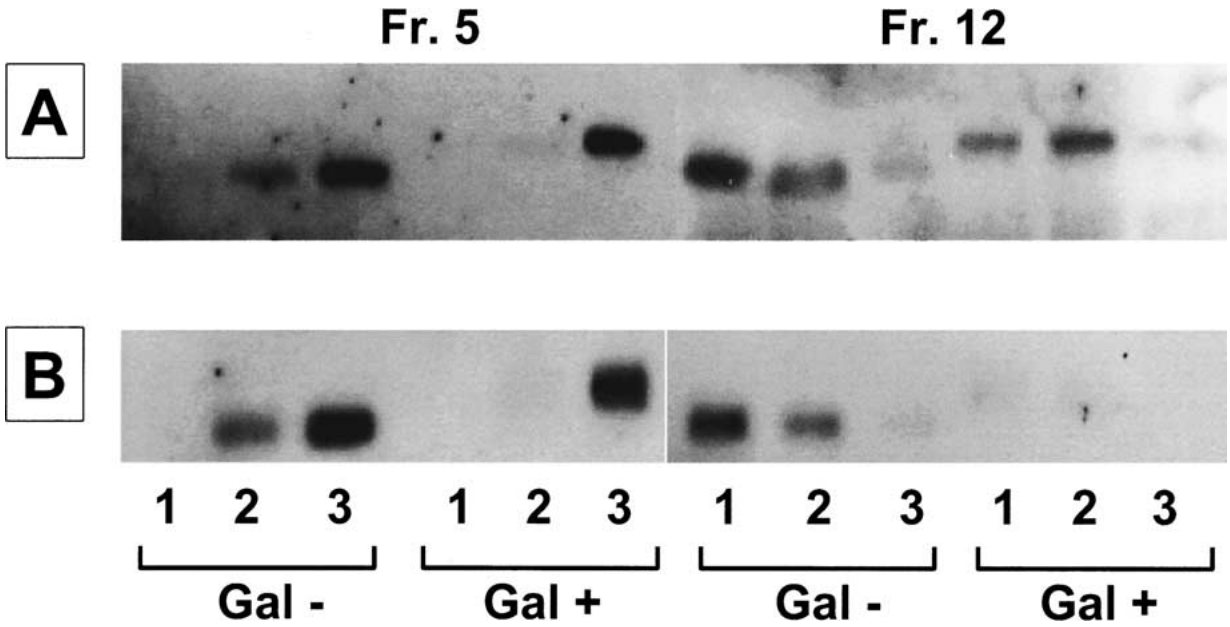

Figure 3. Association pattern of α3 (A) and α5 (B) integrin receptors in low-density, buoyant Fr. 5 and high-density Fr. 12 prepared from ldlD cells grown in the absence (Gal−) or presence (Gal+) of galactose. Lanes 1, 2, 3: Fr. 5 or 12 prepared in 1%, 0.5%, and 0.25% Triton X-100, respectively. Note that, for Fr. 5, α3 and α5 are absent or very low in lane 1 (1% Triton X-100 extract), but present at significant levels in lanes 2 and 3 (0.5%, and 0.25% Triton X-100) in the absence of Gal.

possibility was further demonstrated using cell line ldlD14, and its derivative ldlD/CD9 expressing high level of CD9 through its gene transfection. Haptotactic motility of parental ldlD cells is low, and shows no difference in the presence vs. absence of Gal. In contrast, motility of ldlD/CD9 cells is very high under −Gal condition whereby endogenous GM3 synthesis does not occur, and motility is greatly reduced under +Gal condition whereby endogenous GM3 synthesis occurs.

In order to further assess interaction of GM3 with CD9, photoactivatable ^{3}H-labeled GM3 [35] was employed as a probe. Addition of the probe to HRT18 cells, followed by UV irradiation, caused cross-linking of GM3 to CD9, as evidenced by ^{3}H-labeling of CD9, which was immunoprecipitated by anti-CD9 antibody [18]. These findings suggest that CD9 is a target molecule interacting with GM3, and that CD9 and GM3 cooperatively down-regulate tumor cell motility.

CD9/integrin α3/GM3 complex inhibits laminin-5-dependent cell motility

In further studies using ldlD/CD9 cells, we made the following observations: (i) CD9 has the novel feature of being soluble in chloroform/methanol, and classifiable as "proteolipid"; (ii) CD9 and integrin α3 were concentrated together in low-density glycolipid-enriched microdomain (GEM) of ldlD/CD9; and α3 expression ratio (value for cells grown under +Gal divided by value for −Gal condition) in GEM of ldlD/CD9 was higher than the ratio in control ldlD/moc, suggesting that CD9 recruits α3 in GEM under +Gal condition, whereby GM3 is present; (iii) chemical levels of α3 and CD9 in total extracts or membrane fractions from cells grown under +Gal vs. −Gal condition were nearly identical, whereas α3 expressed at the cell surface, probed by antibody binding in flow cytometry, was higher under −Gal than +Gal condition. These results suggest that GM3 synthesized under +Gal condition promotes interaction of α3 with CD9 which restricts α3 binding to its antibody. A concept of α3/CD9 interaction promoted by GM3 was further supported by (i) co-immunoprecipitation of CD9 and α3 under +Gal but not −Gal condition; (ii) enhanced co-immunoprecipitation of CD9 and α3 when GM3 was added exogenously to cells under −Gal condition; (iii) co-localization image of CD9 with α3, and of GM3 with CD9, in fluorescence laser scanning confocal microscopy. Based on promotion of α3/CD9 interaction by GM3, and status of laminin-5 as true ligand for α3, laminin-5/α3-dependent motility of ldlD/CD9 was found to be greatly enhanced under −Gal but strongly inhibited under +Gal condition. Such motility difference under +Gal vs. −Gal condition was not observed for ldlD/moc cells. The inhibitory effect observed in ldlD/CD9 under +Gal condition was reversed upon addition of anti-α3 antibody, and is therefore based on interaction between α3, CD9, and GM3 in GEM [36].

Discussion

This brief review is focused on a limited type of glycosylation that affects tumor cell motility and invasiveness. SLe^x expressed at the "focal de-differentiation" site can be considered in two different ways. One is in line with the general consideration that SLe^x promotes binding of tumor cells at an invasion focus to endothelial cells, through E-selectin. This is a generally-accepted

idea, as described in many papers since SLe^x expression was correlated with metastatic potential of human tumors grown in athymic mice, *e.g.* [37], as well as in clinicopathological studies [11,12]. Another yet-unexplored possibility is that SLe^x is carried by specific membrane receptors that control tumor cell motility, such as specific types of integrin (*e.g.,* $\alpha 6\beta 4$, $\alpha 3\beta 1$), or CD44. The presence of SLe^x in such receptors may enhance cell motility and invasiveness.

This review is also focused on a series of studies along another line, indicating that glycosylation oppositely affects, *i.e.,* inhibits, tumor cell motility/invasiveness. This is typically based on the inhibitory effect of GM3 on various types of human tumor cell lines, as well as ldlD cell line, having high level of CD9 and $\alpha 3$ expression in which CD9 and $\alpha 3$ form a stable complex in the presence of GM3. The complex may be further stabilized through N-glycosylation state of $\alpha 3$. Since laminin-5 is the principal ligand for $\alpha 3$, laminin-5-dependent motility of ldlD/CD9 cells was examined. The motility was strongly inhibited under +Gal condition, where complete N-glycosylation and endogenous GM3 synthesis occur. The inhibitory effect of GM3 may occur in the complex at GM3-enriched microdomain, where CD9 and $\alpha 3$ co-occur. The complex appears to be stable at low concentration (0.25–0.5%) of Triton X-100, but not at 1% concentration [38]. The complex appears to be more stable in 1% Brij (polyoxyethylene alkylether) in which CD9/$\alpha 3$/$\alpha 5$/GM3/cSrc were detectable, although no supporting data are available to show whether they form single or multiple complex.

The presence of low-density membrane microdomains involved in cell adhesion based on GSL-to-GSL interaction has been studied using B16 melanoma cells [3,39], in which GM3-dependent cell adhesion is coupled with activation of cSrc, RhoA, and FAK [40,41]. These studies led to the concept that GSL-dependent cell adhesion is coupled with signal transduction to alter cellular phenotype [42,43]. The concept of glycosylation microdomain involved in cell adhesion was further extended to mucin-type O-glycans such as MUC1 and PSGL-1 [44], and to another type of complex microdomain including N-linked glycan together with Tsp, *e.g.,* integrin/Tsp/ganglioside complex, as discussed in this review.

Thus, three types of microdomains showing glycosylation-dependent cell adhesion coupled with signal transduction have been proposed as "glycosynapses" [45]. Low-density membrane domains insoluble in detergent, termed "raft" or "caveolar membrane", have been the subject of many recent studies. However, these studies do not include any concept of glycosylation-dependent cell adhesion coupled with signal transduction. Cell adhesion/recognition initiate signal transduction to alter cellular phenotype, as typically observed in the compaction process of preimplantation embryo [46]. We therefore assume that each step of differentiation during ontogenesis requires a different type of cell adhesion coupled with signal transduction, although our knowledge in this area is highly limited. We also assume that the process of oncogenesis and tumor progression is analogous, through tumor cell microenvironment, *i.e.,* interfacing of tumor cell glycosynapse with glycosynapse of adjacent normal cells [47].

Acknowledgment

This study was supported by NIH/NCI grant CA80054 (to SH). We thank Dr. Stephen Anderson for assistance in preparation of the manuscript.

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