Carbohydrate Antigen Sialyl Lewis a – Its Pathophysiological Significance and Induction Mechanism in Cancer Progression

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Carbohydrate antigen sialyl Lewis a (CA19-9) is the most frequently applied serum tumor marker for diagnosis of cancers in the digestive organs. Recent progress disclosed the presence of a normal counterpart of the determinant, namely disialyl Lewis a, which is predominantly expressed in non-malignant epithelial cells of the digestive organs, while sialyl Lewis a is preferentially expressed in cancers. The disialyl Lewis a determinant carries one extra sialic residue attached through a 2→6 linkage to the GlcNAc moiety compared to cancer-associated sialyl Lewis a, which carries only one 2→3 linked sialic acid residue (monosialyl Lewis a). Disialyl Lewis a in normal epithelial cells serves as a ligand for immunosuppressive receptors such as sialic acid binding immunoglobulin (Ig)-like lectins (siglec-7) and -9 expressed on resident monocytes/macrophages and maintains immunological homeostasis of mucosal membranes in digestive organs. Epigenetic silencing of a gene for a 2→6 sialyltransferase in the early stages of carcinogenesis results in an impairment of 2→6 sialylation, leading to incomplete synthesis and accumulation of sialyl Lewis a, which lacks the 2→6 linked sialic acid residue, in cancer cells. Simultaneous determination of serum levels of sialyl- and disialyl Lewis a, and calculation of the monosialyl/disialyl Lewis a ratio provide information useful for excluding a false-positive serum diagnosis, and also for averting the undesired influence of the Lewis blood group of patients on serum antigen levels. During the course of cancer progression in locally advanced cancers, tumor hypoxia induces transcription of several glycogenes involved in sialyl Lewis a synthesis. Expression of the determinant, consequently, is further accelerated in more malignant hypoxia-resistant cancer cell clones, which become predominant clones in advanced stage cancers and frequently develop hematogenous metastasis. Sialyl Lewis a, as well as its positional isomer sialyl Lewis x, serves as a ligand for vascular cell adhesion molecule E-selectin and facilitates hematogenous metastasis through mediating adhesion of circulating cancer cells to vascular endothelium. Patients having both strong sialyl Lewis a expression on cancer cells and enhanced E-selectin expression on vascular beds are at a greater risk of developing distant hematogenous metastasis. *(Chang Gung Med J 2007;30:189-209)*

Key words: CA19-9, epigenetic gene silencing, hematogenous metastasis, tumor hypoxia, serum tumor marker, selectin

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The worldwide monoclonal antibody approach to cancer-associated antigens in the 1980's facilitated research on carbohydrate determinants, because many monoclonal antibodies that exhibited apparent cancer specificity in those studies were found to recognize carbohydrate determinants. Some of these carbohydrate determinants are clinically utilized in routine serum diagnosis of cancers. The sialyl Lewis a determinant (carbohydrate antigen 19-9) is a typical example of these tumor-associated carbohydrate determinants. In recent years the major issues in the research area of these determinants have been, first, to elucidate their functional roles in cancer progression and, second, to clarify the mechanism by which expression of these determinants is induced preferentially in cancers compared to non-malignant cells. This review will focus on the pathophysiological significance of sialyl Lewis a and the mechanisms for induction of its expression in cancer cells.

1. Carbohydrate antigen 19-9 as a serum tumor marker

N19-9, the monoclonal antibody recognizing carbohydrate antigen 19-9 (CA19-9), was first generated and found to have preferential reactivity to cancer tissues through the collaborative studies of researchers at the Wistar institute, including Dr. Tong H. Chang.[1-3] The antibody was initially raised against colon cancer cells, but was found to detect the serum glycoprotein antigens that appear more frequently in the sera of patients with pancreatic and biliary tract cancers.[3,4] The antibody was shown to recognize a carbohydrate determinant called sialyl Lewis a,[5] the structure of which is shown in Fig. 1A.

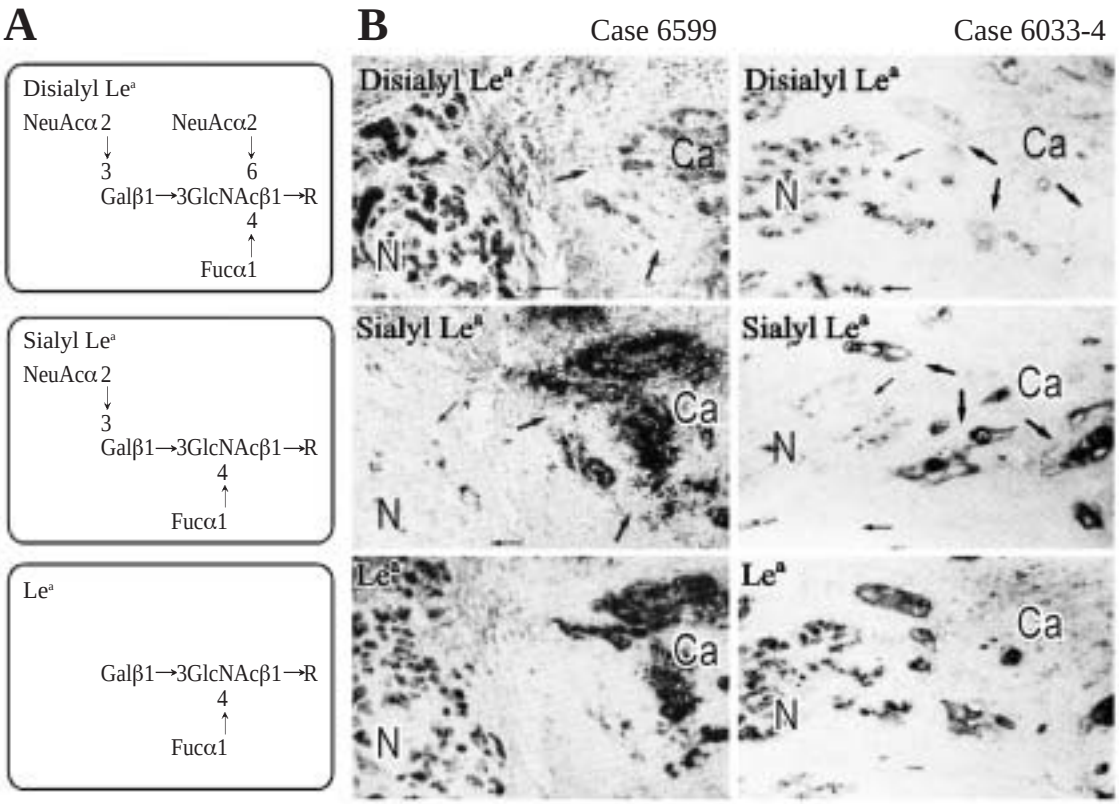

Fig. 1 Structures of three carbohydrate determinants, disialyl Lewis a, sialyl Lewis a and Lewis a (panel A) and their distribution in consecutive sections prepared from two specimens of pancreas cancer tissues (panel B). In panel A, note that the only difference between the three determinants is the linkage of sialic acid residues. In panel B, distributions of the three determinants are seen by immunohistochemical staining using specific monoclonal antibodies. Cancer cells are indicated by thick arrows and labeled "Ca", while non-malignant pancreatic epithelial cells are indicated by thin arrows and labeled "N." Modified from reference.[12] The α2→6 sialic acid residue in disialyl Lewis a was later found to be synthesized by a sialyltransferase ST6GalNAcVI, which shows a significant decrease in its mRNA level upon malignant transformation.

Several other antibodies raised against human cancers, including C50, Span-1, 2D3, and 1B4, were also shown to recognize essentially the same carbohydrate determinant.[6-9]

Serum diagnosis of cancers using sialyl Lewis a determinant has been covered by national medical insurance in Japan over the past two decades. Serum diagnosis with sialyl Lewis a determinant is widely accepted by clinicians as clinically beneficial. Among clinical serum tumor markers, CEA (carcinoembryonic antigen) and α-fetoprotein were most frequently utilized in Japan for years, but in 2004 and 2005, serum CA19-9 determinations were performed approximately 9,430,000 and 9,305,000 times, respectively (Fig. 2). This nearly equals the frequency of clinical tests for CEA, and is more than that for α-fetoprotein (Fig. 2). Serum diagnosis is utilized in the initial diagnosis of cancers, in detection of recurrence after surgery, and during monitoring of the therapeutic effects of radio- and chemotherapy.

Some monoclonal antibodies such as FH6, CSLEX-1 and NCC-ST-439, which detect carbohydrate antigens closely related to the sialyl Lewis x determinant, the positional isomer of sialyl Lewis a, were also found to be useful for serum diagnosis of cancers and are also covered by national medical insurance in Japan. While sialyl Lewis a is frequently detected in cancers of the pancreas, biliary tract, stomach, colon and rectum, sialyl Lewis x is preferentially expressed in cancers originating in the lung, ovary and mammary gland.[9-11]

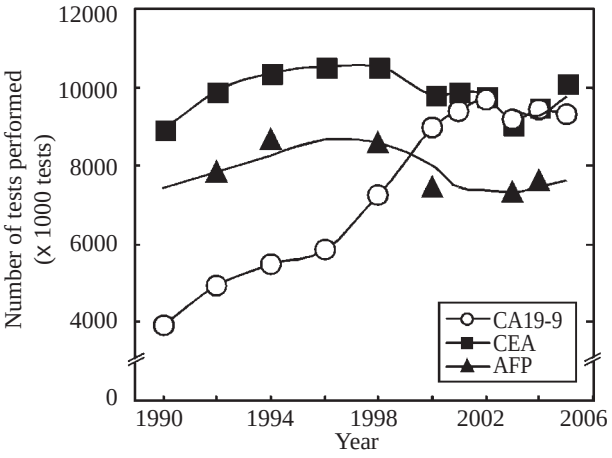

Fig. 2 Established standing of sialyl Lewis a among serum diagnostic tests for cancers in Japan. Comparison of the number of clinical tests for serum sialyl Lewis a determination performed all over Japan per year with that of serum CEA and α-fetoprotein determinations, which formerly ranked first and second, respectively, among serum diagnostic tests for cancers for much of the last few decades (adopted from the Yearbooks on Immunoassay Market 1993-2006, Fuji Keizai Co. Ltd., Tokyo, Japan).

2. Current problems in serum diagnosis with sialyl Lewis a awaiting solutions

2.1 Expression of sialyl Lewis a in non-malignant epithelial cells causing false-positive results in serum diagnosis

There are two major disadvantages in applying sialyl Lewis a for serum diagnosis of cancers. One is occasional elevation of its serum level in patients with benign disorders. Appearance of sialyl Lewis a in the serum is not specific to malignant disorders, and patients with benign disorders sometimes show elevated serum levels of sialyl Lewis a. This is because sialyl Lewis a is not cancer-specific in a strict sense. The determinant is expressed in a small number of normal cells, and its expression increases in non-malignant disorders such as inflammatory diseases. Thus, false-positive results in a given population of patients with benign disorders are unavoidable.

For instance, a small number of ductal epithelial cells express sialyl Lewis a in the normal pancreas. Sialyl Lewis a-positive ductal epithelial cells are usually strictly localized at the distal end of the pancreatic ducts near the centroacinar cells.[12] Upon inflammation, an increased number of pancreatic epithelial cells are induced to express sialyl Lewis a.[12,13] Therefore, eventually a significant amount of sialyl Lewis a can be released from the non-malignant pancreas, which causes false-positive results in serum diagnosis in patients with benign inflammatory disorders. Embryonic pancreatic primordial cells also significantly express the determinant.[14]

Because of the production of sialyl Lewis a by non-malignant epithelial cells, secreted mucins in pancreatic juice and bile, as well as saliva, contain a significant amount of the determinant. Occlusion of efferent ducts in these organs leads to the accumulation and backflow of the determinant into the general circulation, which also produces false-positive results in serum diagnosis. This type of false-positive result is sometimes encountered in patients with cystic diseases of the pancreas, salivary gland or kidney,

peritoneal pseudomyxoma,[15] and also in patients with chole- or urolithiasis.[16]

Patients with false-positive serum tests for sialyl Lewis a are sometimes hospitalized for long periods for differential diagnosis, and undergo pointless multiple clinical examinations including diagnostic imaging. To avoid this, we need some means to distinguish malignant from benign underlying disorders in patients with elevated serum sialyl Lewis a levels.

2.2 How to overcome false-positive results in serum diagnosis using sialyl Lewis a

Previously we have described that non-malignant epithelial cells of the digestive organs express a carbohydrate determinant closely-related to, and structurally more complex than, sialyl Lewis a. The structure of the determinant, 2→3, 2→6 disialyl Lewis a (or simply called disialyl Lewis a) is shown in Fig. 1A. Its structural similarity with sialyl Lewis a implies that both the disialyl- and sialyl Lewis a determinants are produced through a common synthetic pathway. Nevertheless, the disialyl Lewis a determinant is preferentially expressed on non-malignant epithelial cells in healthy individuals and in patients with non-malignant disorders. Immunohistochemical examination indicates a widespread distribution of disialyl Lewis a in normal epithelial cells of healthy individuals, and its increased expression in epithelial cells in patients with benign disorders of the pancreas,[12] biliary tract, stomach, and colon.[13] On the other hand, expression of the determinant is reduced in cancers originating in these organs, in clear contrast to that of sialyl Lewis a (Fig. 1B).

Because the disialyl Lewis a determinant, as well as sialyl Lewis a, is secreted into general circulation in the form of high-molecular mucin-like glycoproteins,[4] it is detectable in sera of patients with ELISA or other appropriate techniques which are also applied for serum diagnostic tests for sialyl Lewis a. The results of these assays indicate that patients with benign disorders tend to have a higher serum disialyl Lewis a level compared to the sialyl Lewis a level, while cancer patients have a higher serum sialyl Lewis a level relative to the disialyl Lewis a level in the same sample (Fig. 3A).[12,13] Consequently, the ratio of sialyl Lewis a/disialyl Lewis a in serum is high in cancer patients, and is low in patients with benign disorders (Fig. 3B). Calculation of the ratio of the two determinants was found to be informative in the differential serum diagnosis of benign and malignant disorders of the pancreas,[12] biliary tract, stomach and colon.[13] A high sialyl Lewis a/disialyl Lewis a ratio indicates that the serum antigens originated in malignant lesions, while a low ratio is indicative that the serum antigens are derived from non-malignant tissues.

2.3. Lewis blood group dependency of tissue expression and serum levels of sialyl Lewis a

Another problem in applying sialyl Lewis a for serum diagnosis is that its serum level, as well as its tissue expression, is highly dependent on the Lewis blood group status of the given patient (Fig. 4). This is because the glycosyltransferases involved in the synthesis of the determinant are closely related to the Lewis blood group system. Le^{a+b-} individuals (*Le/Le* or *Le/le* genotype) have higher serum sialyl Lewis a levels, while Le^{a-b-} individuals (*le/le*) give quite low or negative results (Fig. 4A, left panel), because an α(1,3/4) fucosyltransferase (*FUT3*), the *Le* gene in the Lewis blood group system, is involved in synthesis of the Fucα1→4 residue in the determinant. Le^{a-b+} individuals (*Se/Se* or *Se/se* genotype) have lower serum levels than Le^{a+b-} individuals (*se/se*) (Fig. 4A, left panel), because an α(1,2) fucosyltransferase (*FUT2*), the *Se* gene, competes for the galactose-terminal substrates with α(2,3) sialyltransferases, which are required for synthesis of the NeuAcα2→3 residue in the sialyl Lewis a determinant. Its serum level is affected by the heterozygosity in *FUT3* or *FUT2* genes,[17-19] and the use of multiple cut-off values depending on patient genotypes has been proposed. In applying such multiple cut-off values for serum diagnosis, detailed Lewis blood group typings and complicated PCR analyses are required,[20,21] although even the simplest conventional Lewis blood group typing is not routinely performed in most hospitals. We need some means to rid ourselves of the Lewis-blood group dependency of serum sialyl Lewis a levels to settle this problem.

Distribution of serum sialyl Lewis a levels in Le^{a-b+} individuals is widespread as shown in the left panel of Fig. 4A, because of the variance in α(1,2) fucosyltransferase activity depending on gene dosage and position of point mutation in the *Se* gene, or possible participation of other glycosyltransferases in the competition for galactose-terminal substrates.[22,23]

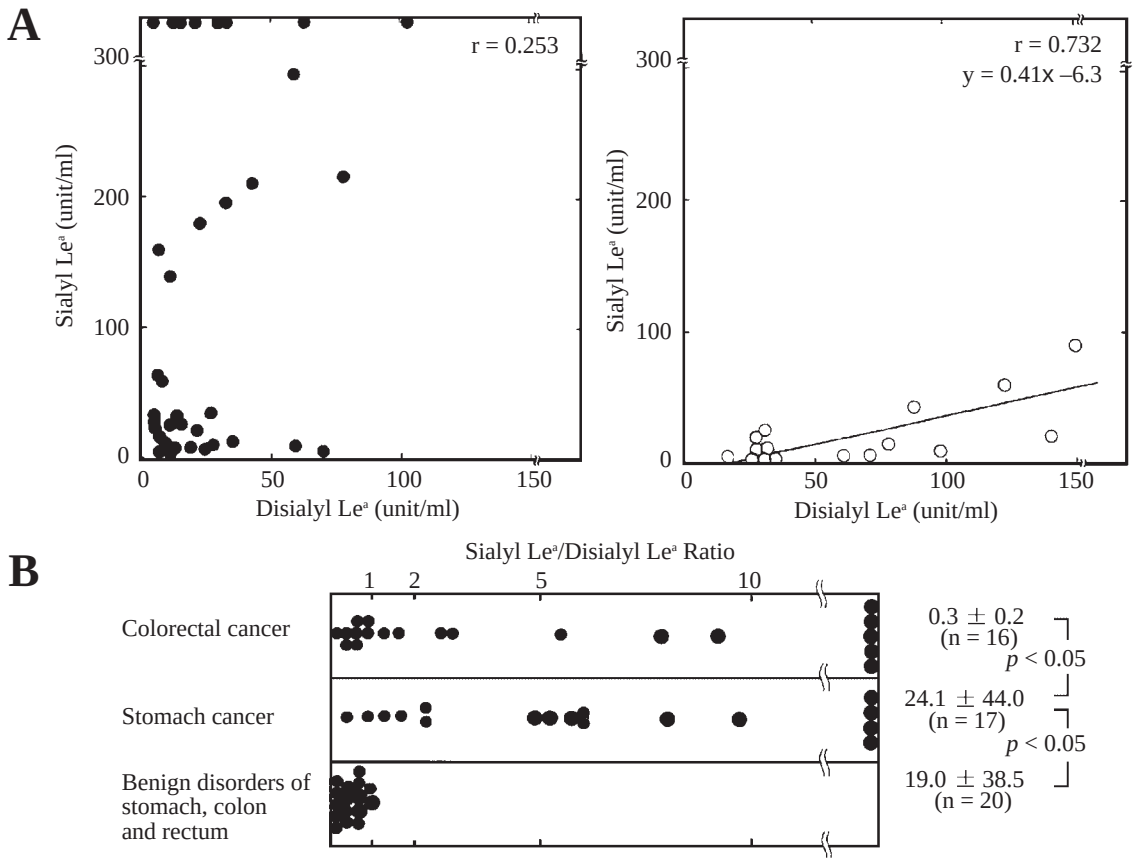

Fig. 3 Serum levels of disialyl- and sialyl Lewis a determinants in patients with malignant and benign disorders of the gastrointestinal tract. Panel A, two-dimensional distribution of serum levels of the two determinants indicating a good correlation is observed only in the sera from benign disorders, suggesting a fixed ratio in the content of the two determinants in non-malignant tissues. Panel B, sialyl Lewis a/disialyl Lewis a ratio in the sera of patients with malignant and benign disorders of the gastrointestinal tract. Note that although the ratio is high in malignant disorders, it is low and distributes within a narrow range in patients with benign disorders. Arbitrary units were applied for both determinants. Modified from reference.[13]

The presence of a unique Le^{a+b+} phenotype in Eastern Asia, which could be present in more than 20% of the total population,[20,24-27] would make the situation more complicated.

2.4 How to overcome Lewis-blood group dependent variation in serum diagnosis

Interestingly, the tissue expression and serum levels of disialyl Lewis a is also affected by the Lewis-blood group of patients. This is quite natural when we take into consideration that disialyl Lewis a is synthesized through essentially the same metabolic pathway as sialyl Lewis a (Fig. 4B). The serum levels of disialyl Lewis a are highest in Le^{a+b-} individuals, followed by Le^{a-b+} individuals, and quite low or undetectable in Le^{a-b-} individuals (Fig. 4A, middle panel), which is the same pattern as that of sialyl Lewis a. Moreover, as with sialyl Lewis a levels, disialyl Lewis a levels show a broad distribution in Le^{a-b+} individuals (Fig. 4A, middle panel), most probably due to the variance in $\alpha(1,2)$ fucosyltransferase activity in Lewis blood group subpopulations.

Accordingly, the ratio of sialyl Lewis a/disialyl Lewis a, which we proposed to provide information on cancer specificity in the previous section, is free from Lewis-blood group dependent variations (Fig. 4A, right panel). In serum diagnosis using sialyl Lewis a, it is highly recommended that the level of disialyl Lewis a be measured in parallel, and the ratio of sialyl/disialyl Lewis a be calculated for improved serum diagnosis to reduce false-positive results, and also to avert undesired Lewis-blood group dependent

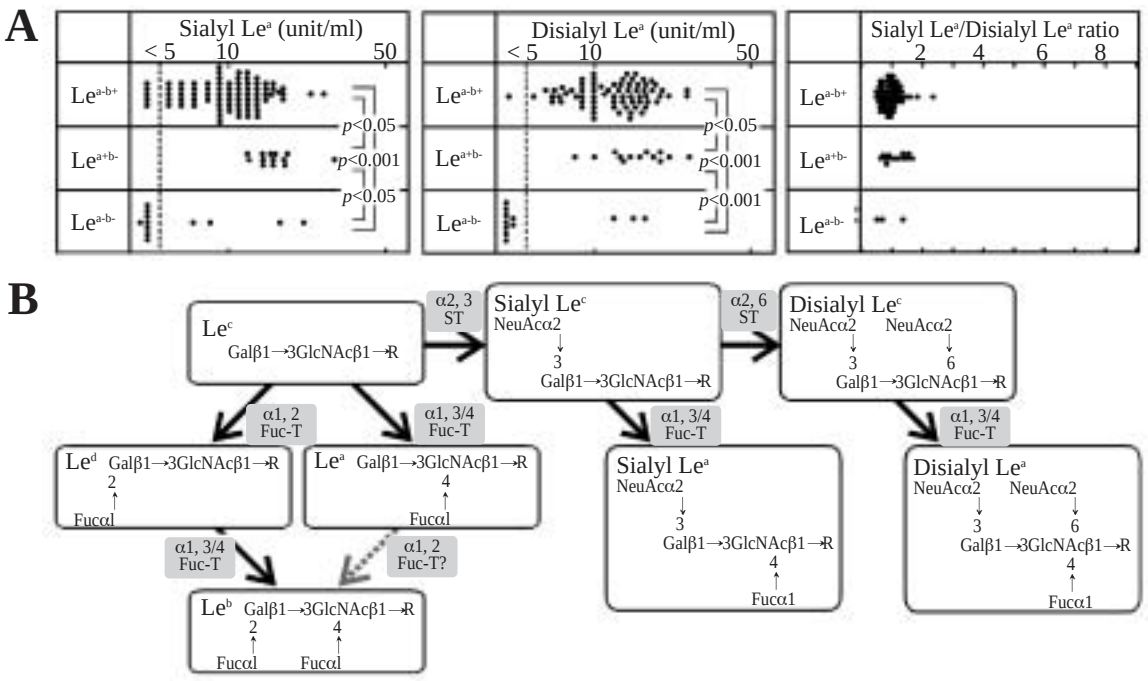

Fig. 4 Lewis blood group-dependent variation in serum levels of disialyl and sialyl Lewis a determinants in healthy individuals. Panel A, distribution of serum levels of sialyl- and disialyl Lewis a determinants and the ratio of the two determinants in Le^{a-b+}, Le^{a+b-} and Le^{a-b-} individuals. The ratio was calculated only when levels of both determinants were above the reliable detection limit of the assay methods. Note that the ratio distributes within a narrow range. Panel B, a scheme for synthetic pathways for Lewis blood group and related carbohydrate determinants indicating disialyl and sialyl Lewis a determinants are synthesized through a common pathway. Adopted from reference.[100]

variation.

What we can postulate at present is that perhaps every cancer-related carbohydrate determinant has its corresponding counterpart in normal cells. The combination of sialyl Lewis a in cancer cells versus disialyl Lewis a in non-malignant epithelial cells is a good example, and the pair of sialyl Lewis x in cancer cells versus the recently described sialyl 6-sulfo Lewis x in non-malignant epithelial cells is another good example.[28] Simultaneous determination of the set of determinants will yield useful information in serum diagnosis.

3. Mechanism for induction of sialyl Lewis a expression in early stage cancers

3.1 Glycosylation change responsible for preferential expression of sialyl Lewis a in cancer

Once again we need to carefully ponder the biological significance of the finding for preferential expression of disialyl Lewis a on non-malignant epithelial cells, which is in good contrast to the frequent expression of sialyl Lewis a in cancer cells. Notably, non-sialylated Lewis a determinant is expressed almost equally well in either non-malignant epithelial cells or in cancer cells (Fig. 1B).[12] From these findings we can predict that the fucosyltransferase which synthesizes the α1→4 fucose residue in the sialyl Lewis a determinant may not figure heavily in determining the cancer specificity of the determinant, because the fucose residue is shared by all three determinants exhibiting a pronounced contrast to each other in their cancer specificity. Taking the carbohydrate structures of these determinants into consideration (Fig. 1A), the relative cancer specificity of sialyl Lewis a can be suggested to heavily depend on the distinctiveness of the linkage of the sialic acid residue in the antigenic structure.

In line with this, there is no significant difference in α1,3/4 fucosyltransferase (*FUT3*) between cancer cells and non-malignant epithelial cells in digestive organs both in terms of enzymatic activity

and mRNA content.[29-31] No published evidence to date indicates a significant difference in α1,3/4 fucosyltransferase expression between malignant and non-malignant epithelial cells, as long as the epithelial cell samples are carefully prepared.

In contrast, significant alterations in sialyltransferases in cancers are frequently reported.[30,32,33] Judging from the structures of sialyl- and disialyl Lewis a in Figure 1A, the most relevant changes in sialyltransferases, which would account for the transition from disialyl Lewis a-dominant status in normal epithelial cells to sialyl-Lewis a-dominant status in cancer cells upon malignant transformation, must be the loss of the α(2,6) sialyltransferase responsible for 2-6 sialylation at the GlcNAc moiety. The sialyltransferase was recently identified to be ST6GalNAcVI,[34] and expression of the gene was shown to be drastically reduced in cancer cells compared to non-malignant epithelial cells (Fig. 5A),[35] which is quite consistent with the antigenic transition from disialyl Lewis a to sialyl-Lewis a upon carcinogenesis. An increase in α(2,3) sialyltransferase in cancers described in earlier reports was found to be mainly due to the increased transcription of ST3O (ST3Gal-I),[30] which may also subsidiarily enhance sialyl Lewis a expression.

3.2 Sialyl Lewis a and the classic concept of "incomplete synthesis"

Two principal mechanisms are known for the tumor-associated alteration of cell surface carbohydrate determinants; "incomplete synthesis" and "*neosynthesis,*" as first formulated by Hakomori and co-workers.[36] The synthesis of complex carbohydrate determinants well-developed on normal epithelial cells tends to be impaired upon malignant transformation, predisposing the cells to express less complex carbohydrate determinants, and this mechanism is referred to as "incomplete synthesis."

For instance, it has been known that non-malignant colonic epithelial cells strongly express the blood group A- or B-determinants, Sd^a- and 3'-sulfated Lewis a determinants.[37-41] Upon malignant transformation, synthesis of these determinants is somehow impaired, and this leaves the galactose-terminated precursor substrates for synthesis of the sialyl Lewis a or sialyl Lewis x determinants. Disialyl Lewis a has a carbohydrate structure more complex than sialyl Lewis a, and antigenic transition of disia-

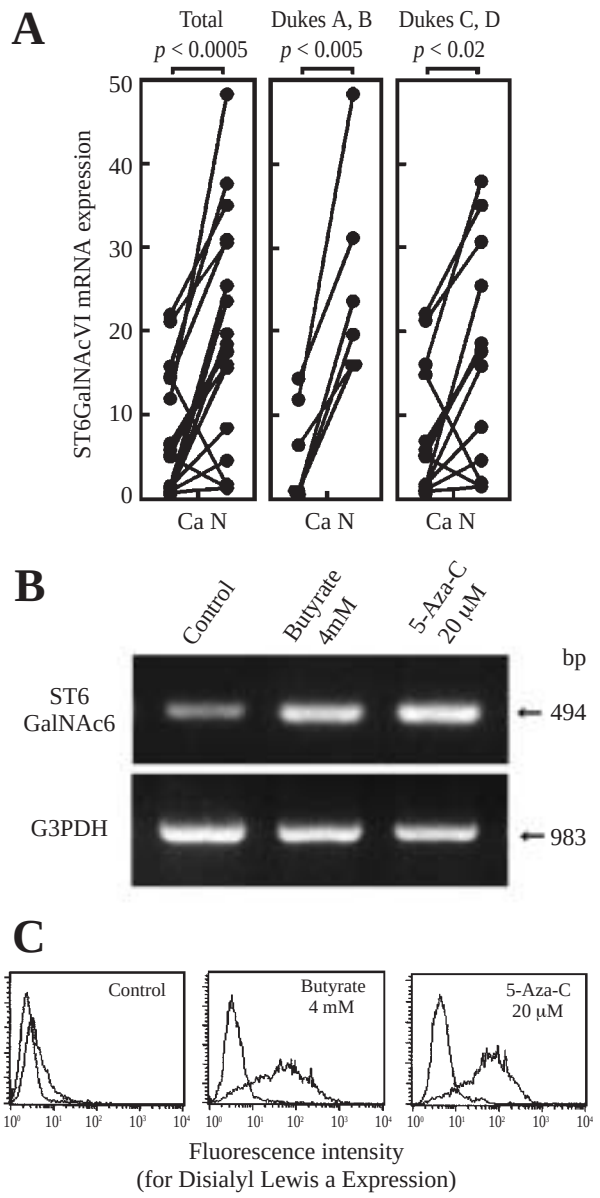

Fig. 5 Epigenetic silencing of the gene for α2→6 sialyltransferase responsible for disialyl Lewis a synthesis in colon cancer cells. Panel A, expression of mRNA for α2→6 sialyltransferase (ST6GalNAcVI) responsible for disialyl Lewis a synthesis in cancer cells (Ca) and non-malignant epithelial cells (N) in patients with colon cancer. Note that suppression of transcription is apparent in early stages such as Dukes' stages A and B. Panels B and C, induction of mRNA for α2→6 sialyltransferase (ST6GalNAcVI) responsible for disialyl Lewis a synthesis (panel B) and its product disialyl Lewis a (panel C) in cultured human colon cancer cells by a histone deacetylase, butyrate, or a DNA methylation inhibitor, 5-aza-cytidine (5-aza-C). Adapted from reference.[35]

lyl Lewis a to sialyl Lewis a due to reduction of ST6GAlNAcVI gene transcription can be regarded as a typical example of "incomplete synthesis." The decrease of GlcNAc 2,6-sialylation will directly result in the accumulation of sialyl Lewis a and a concomitant reduction in disialyl Lewis a expression.

Incomplete synthesis of disialyl Lewis a may have a key role in inducing sialyl Lewis a expression in cancers. Without the incomplete synthesis of disialyl Lewis a, what increases will be disialyl Lewis a instead of sialyl Lewis a when synthesis of other carbohydrate determinants, such as blood group A-, B-, Sd^a- or 3'-sulfated Lewis a determinants, is impaired, because the major product of the synthetic pathway will remain disialyl Lewis a rather than sialyl Lewis a.

3.3 Epigenetic silencing of glycogene expression as the principal mechanism for "incomplete synthesis"

It is notable that the suppression of ST6GAlNAcVI gene transcription is evident already in early cancers such as Dukes stage A or B (Fig. 5A), which is compatible with the well-known induction of sialyl Lewis a expression in early stage cancers. To determine the mechanism underlying the transcriptional suppression of the gene in cancers, we chose cultured colon cancer cell lines which strongly express sialyl Lewis a but no appreciable disialyl Lewis a. We then cultured the cells in the presence of a histone deacetylase inhibitor or an inhibitor of DNA methylation.[35] This resulted in significant induction of mRNA for ST6GAlNAcVI and surface expression of disialyl Lewis a in the treated cells (Fig. 5B and Fig. 5C), suggesting that ST6GAlNAcVI gene transcription is suppressed in cancer cells through epigenetic silencing mechanisms such as histone deacetylation and/or DNA methylation.

Recent expansion in knowledge of the relationship between chromatin organization and gene transcription has highlighted the importance of epigenetic mechanisms, such as DNA methylation and histone deacetylation, in the initiation and progression of cancers.[42] In fact, epigenetic gene silencing is now recognized as a predominant mechanism for tumor-suppressor gene inactivation in cancers and can affect gene function without genetic changes like somatic mutation, deletion or loss of heterozygosity. In fact, epigenetic silencing is observed to occur not only in tumor-suppressor genes, but in many other genes transcribed in normal cells. Increasing evidence has recently revealed that it is epigenetic gene silencing which lies behind the incomplete synthesis of normal carbohydrate determinants in cancers. For instance, DNA methylation is proposed to be a main mechanism for the decrease of ABO(H)[43,44] or Sd^a determinants,[41] and histone deacetylation for loss of disialyl Lewis a[35] in cancers.

3.4 Biological function of disialyl Lewis a in normal mucosal membranes

The sialyl Lewis a determinant on cancer cells is known to serve as a ligand for endothelial E-selectin, and mediates hematogenous metastasis of cancers.[45-47] On the other hand, the biological functions of its normal counterpart, disialyl Lewis a, remain unknown. Recently, we found that disialyl Lewis a determinant serves as a ligand for siglec-7 and -9, the members of a family of sialic acid recognizing molecules expressed mainly on leukocytes (Fig. 6A and Fig. 6B).[35]

This prompted us to study whether any leukocytes expressing siglec-7 or -9 are present on the normal mucosal membranes of the digestive organs which are aligned with the epithelial cells expressing their ligand, disialyl Lewis a. A significant number of mononuclear leukocytes were found in the lamina propria of the intestinal mucosal membranes, some of which showed adhesion to disialyl Lewis a-positive epithelial cells (Fig. 6C and Fig. 6D). Siglec-7 is known to be carried mainly by NK cells,[48] and siglec-9 by NK cells, mono- and granulocytes[49,50] among peripheral blood leukocytes. In the mucosal membranes, however, the number of NK cells was almost negligible, and the siglec-7/-9-positive cells were found to be mostly of monocyte/macrophage lineage.

Siglec-7 and -9 are known to have immunoreceptor tyrosine-based inhibition motifs (ITIM) in their cytoplasmic domains. When the tyrosine residues in the motifs are phosphorylated upon some cellular stimulation, they can function as docking sites for the phosphatases, Src homology 2 (SH2)-containing tyrosine phosphatase-1 (SHP-1) and/or Src homology 2 (SH2)-containing tyrosine phosphatase-2 (SHP-2), which suppress signal transduction. Thus, siglec-7 and -9 act as inhibitory receptors,

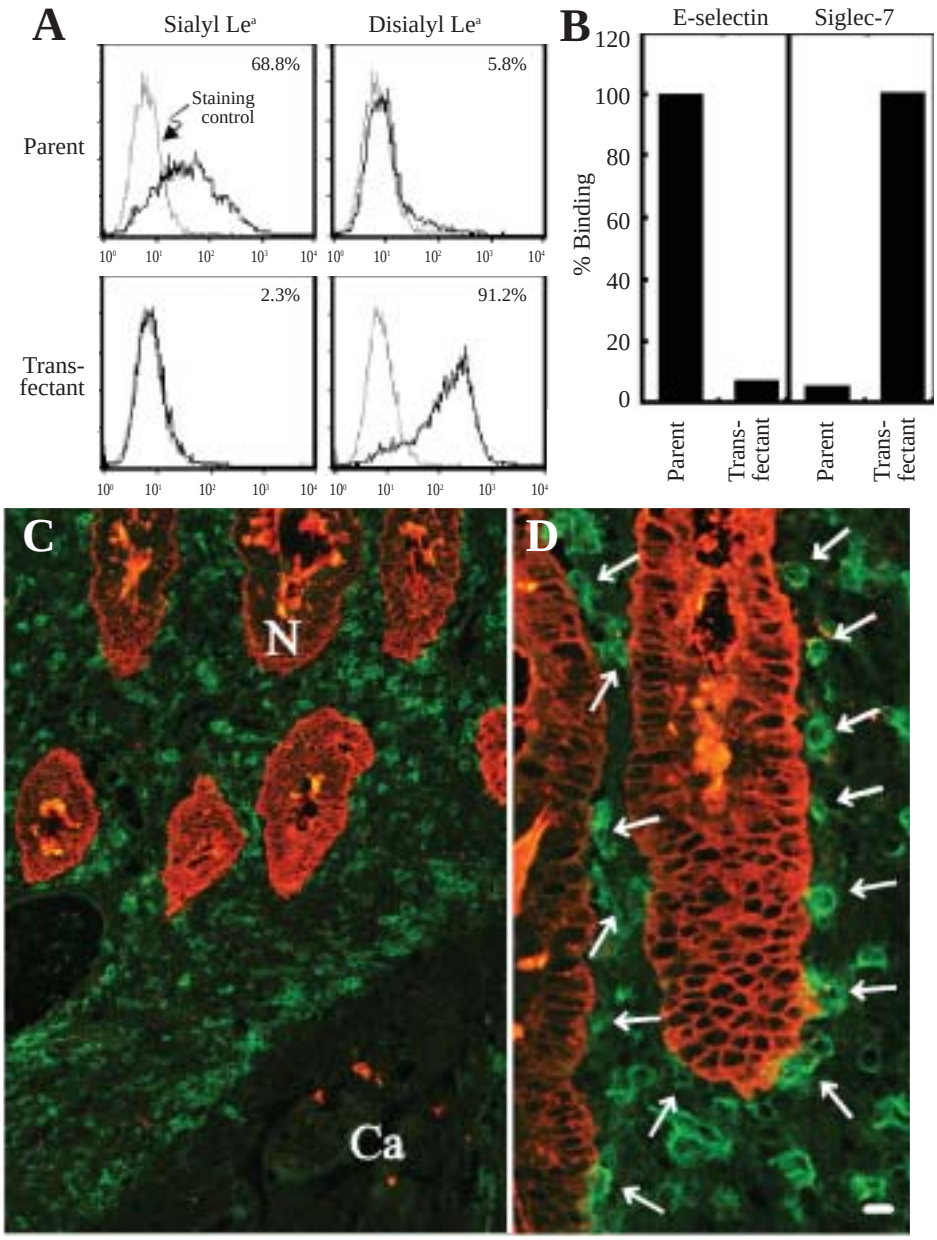

Fig. 6 Biological function of disialyl Lewis a expressed on normal epithelial cells as a ligand for an inhibitory immune receptor, siglec-7. Panel A, expression of disialyl- and sialyl Lewis a in a parental cultured human colon cancer cell line and its clone transfected with the gene for α2→6 sialyltransferase (ST6GalNAcVI). The parental cells (SW1083) express only sialyl Lewis a but no disialyl Lewis a, while the transfectant cells strongly express disialyl Lewis a. Note that expression of sialyl Lewis a is markedly reduced in the transfectant cells because of substrate competition, and the carbohydrate profile of the transfectant cells resembles that of non-malignant epithelial cells. Panel B, binding of recombinant E-selectin and siglec-7 to the transfectant- and parental cells, indicating only the parental cancer cells expressing sialyl Lewis a bind to E-selectin, whereas only the transfectant cells expressing disialyl Lewis a bind to siglec-7. Panel C, distribution of non-malignant epithelial cells (labeled as "N") expressing disialyl Lewis a (stained in red using anti-disialyl Lewis a antibody), and monocyte/macrophages expressing siglec-7 (stained in green using anti-siglec-7 antibody) in colonic mucosal membranes. Note that cancer cells (labeled as "Ca") do not express disialyl Lewis a. Panel D, a photograph with a higher magnification indicating adhesion of siglec-7-positive monocyte/macrophages to disialyl Lewis a-positive colonic epithelial cells. Bar, 10 μm. Adapted from reference.[35]

and prevent excessive activation of monocyte/macrophages in mucosal membranes, maintaining immunological homeostasis. Loss of disialyl Lewis a on normal epithelial cells by epigenetic suppression of ST6GalNAcVI gene expression in the early stages of carcinogenesis will result in abruption of the immunological homeostasis in mucosal membranes. In this context, it is noteworthy that carcinogenesis or even initial polypogenesis in the digestive organs involves inflammatory activation of mucosal monocyte/macrophages expressing excess cyclo-oxygenase-2 (COX2).[51] Full elucidation of the functional consequences conferred by the interaction of siglec-7/9-positive leukocytes and disialyl Lewis a-positive epithelial cells as well as the functional consequences brought about by the loss of this interaction in the early stages of carcinogenesis, awaits further investigation.

4. Enhancement of sialyl Lewis a/x in locally-advanced stage cancers

4.1 Tumor hypoxia and malignant progression of cancers

Progression of cancer is a long process which sometimes spans a few years. Long after the initiation of sialyl Lewis a expression through epigenetic gene silencing in the early stages, cancer cells in the locally-advanced stages gradually develop genetic abnormalities, and more malignant cancer cells with higher infiltrative and metastatic activities evolve according to the principle of survival of the fittest. This process is called cancer progression, and expression of sialyl Lewis a determinant is further accelerated during the process of cancer progression.

A major factor for the selection of more malignant cells during cancer progression is their resistance to tumor hypoxia.[52,53] Because of the uncontrolled proliferation of cancer cells, delivery of oxygen is reduced in peripheral areas of solid tumors, and some of the cancer cells are subjected to a hypoxic environment (Fig. 7A). The hypoxic environment leads to the evolution of cancer cell clones that acquire, through the accumulation of genetic changes, constitutive and irreversible expression of genes required to adapt to hypoxia. Acquisition of a high level of a transcription factor called hypoxia inducible factor (HIF) plays the major role in the process.[52,53]

One of the characteristics of hypoxia-resistant cancer cells is a particular deviation in intracellular carbohydrate metabolism, a metabolic shift from oxidative to elevated anaerobic glycolysis (the Warburg effect), which is correlated with the increased gene expression of some glycolytic enzymes and glucose transporters including *GLUT1*.[46] This enables the cells to produce enough ATP under extreme hypoxic conditions. Another consequence is facilitated production of humoral factors such as vascular endothelial growth factor (*VEGF*), which supports tumor angiogenesis. Both make cancer cells capable of surviving under hypoxic conditions in locally advanced tumor nests. Natural selection of hypoxia-resistant cancer cells in locally-advanced tumor nests results in the clonal expansion of cancer cells with higher invasive and metastatic activities.

4.2 Transcriptional induction of glycogenes by tumor hypoxia

We recently found a significant induction of sialyl Lewis a and sialyl Lewis x expression by hypoxia (Fig. 7B), with a concomitant increase in E-selectin binding activity.[54] The glycogenes induced by hypoxia included a glucose transporter *GLUT1*, a UDP-galactose transporter (*UGT1*), a sialic acid transporter (*SIALIN*), a sialyltransferase (*ST3O*) and some other genes closely related to carbohydrate metabolism as ascertained by DNA-microarray and/or RT-PCR techniques.[54,55] These genes are involved, directly or indirectly, in the synthesis of the sialyl Lewis a/x determinants, and their increased transcription leads to enhanced expression of the determinants. Transcription of these genes is significantly elevated in cancer cells prepared from surgical specimens of colon cancers, compared to non-malignant colonic epithelial cells taken from the same patients, especially those in advanced stages such as Dukes' C and D stages (Fig. 7C).[54,56]

These results indicate that augmentation of sialyl Lewis a/x expression on cancer cells is closely implicated in the process of cancer progression, and more malignant cancer cells tend to have a more enhanced expression of these carbohydrate determinants. This "*neo*synthesis" mechanism contributes to a further increase of sialyl Lewis a/x expression in advanced cancer cells, which had already been predisposed to express these determinants by epigenetic gene silencing in the early stages of carcinogenesis.

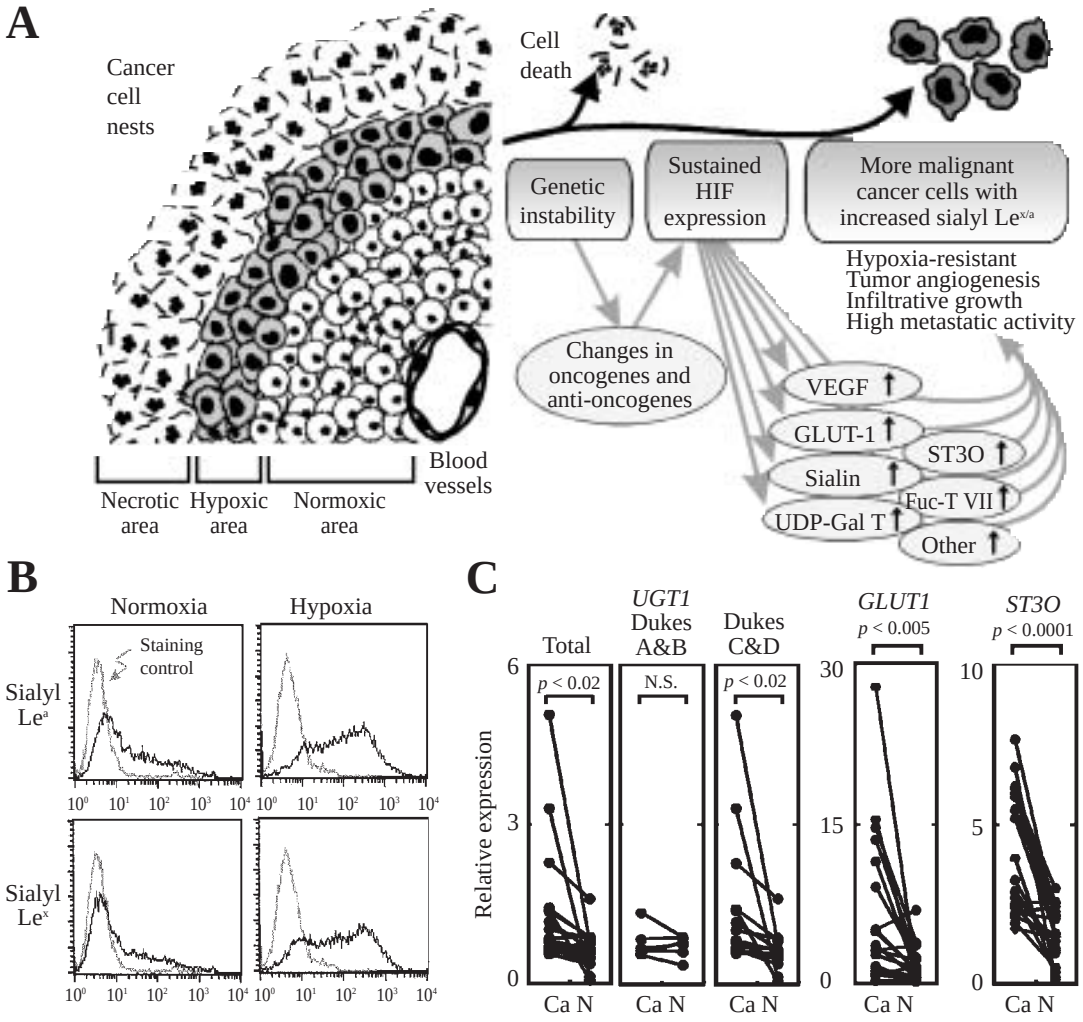

Fig. 7 Expression of sialyl Lewis a/x is further enhanced during hypoxia-induced cancer progression in locally advanced cancers. Panel A, schematic illustration of hypoxia-induced cancer progression and sialyl Lewis a/x accumulation in locally advanced cancer nests. Panel B, induction of sialyl Lewis a/x expression in human colon cancer cells SW480 cultured under hypoxic conditions.[54] Panel C, real-time RT-PCR analysis of mRNA levels of hypoxia-inducible glycogenes, *UGT1, GLUT1,* and *ST3O* in human colon cancer tissues (Ca) and non-malignant mucosa (N) prepared from surgical specimens. The results on *UGT1* were stratified according to Dukes' stage classification, indicating preferential elevation in advanced cancers.[54,56]

The reason why expression of these genes is induced by hypoxia in the context of general carbohydrate metabolism remains unclear. Increased expression of *GLUT1* provides more glucose molecules required for anaerobic glycolysis. As the monosaccharide specificity of *GLUT1* is not exclusive, this would lead to an increased cellular uptake of other monosaccharides such as galactose, which is to be incorporated into cell surface glycoconjugates. Otherwise galactose is eventually toxic for cells when metabolized through other pathways such as an aldose reductase. Galactose is transformed to UDP-galactose and transported into the Golgi apparatus by *UGT1*, to be incorporated into glycolipids and glycoproteins. A galactokinase gene (*GALK1*) is also upregulated in cancer cells.[46] Increased galactose-terminated glycoconjugates should then be covered by sialyltransferases such as *ST3O* using sialic acids transported by *SIALIN*, lest cells with exposed galactose terminals invite an attack by reticuloendothelial cells. In this sense, hypoxia-mediated induction of a set of genes including *UGT1*, *SIALIN* and *ST3O* may

play a role in detoxication of excessive intracellular galactose.

4.3 Growth advantage for cancer cells exhibiting elevated expression of sialyl Lewis a in tumor hypoxia

The advantage of expressing sialyl Lewis a or sialyl Lewis x for hypoxic cancer cells is that the determinants facilitate vascularisation of tumors by mediating cancer cell adhesion to endothelial cells. We assessed the significance of this cell adhesion in tumor vascularisation by employing a model using a cultured endothelial cell line, which expressed selectins and could adhere to human cancer cells expressing sialyl Lewis a/x.[57] The cultured endothelial cell line retained an ability to form tube-like structures when cultured on Matrigel (Fig. 8A), and co-culture of the endothelial cells with human cancer

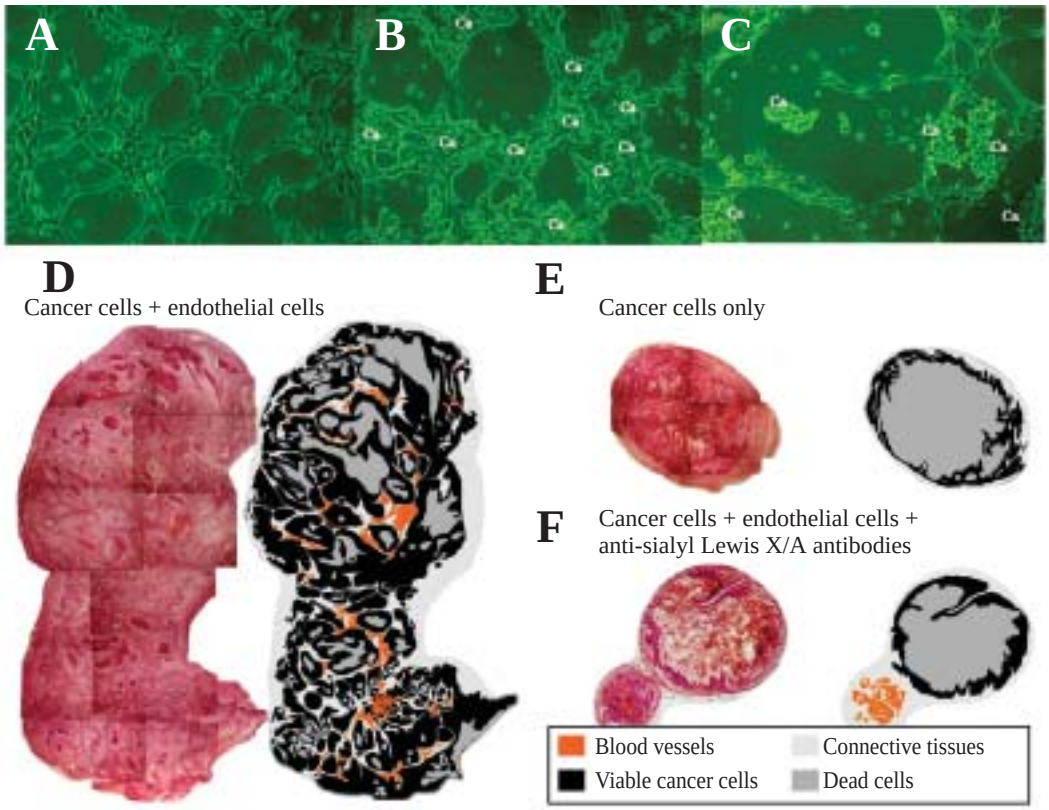

Fig. 8 Cell adhesion mediated by selectin and sialyl Lewis a/x is involved in tumor vascularisation. Panels A~C, *in vitro* experiments indicating contribution of cell adhesion mediated by selectin and sialyl Lewis a/x in tumor vascularisation. Panel A, phase-contrast morphology of cultured endothelial F-2 cells exhibiting tube formation induced by culture on Matrigel. Panel B, phase-contrast morphology of human cancer cells A431 co-cultured with the endothelial cells F-2. A mixture of A431 and F-2 cells at a ratio of 2:5 was plated on Matrigel and cultured for 8 h. The human cancer cells fit in the framework of tube-like structures formed by the endothelial cells. Panel C, phase-contrast morphology of human cancer cells A431 co-cultured with the cultured endothelial F-2 cells in the presence of anti-sialyl Lewis a/x neutralizing antibodies. Human cancer cells are outside the framework and form clumps independent of the tube-like structures of endothelial cells. Ca, cancer cells. Panels D~F, *in vivo* experiments indicating contribution of cell adhesion mediated by selectin and sialyl Lewis a/x in tumor vascularisation. Gross appearances of in vivo tumors formed in nude rats by transplantation of human cancer cells with (panel D) or without (panel E) endothelial cell supplementation, and those after treatment with anti-sialyl Lewis a/x antibodies (panel F) are shown. Panel D, a typical tumor formed 21 days after co-injection of A431 cells (5×10^6 cells) and F-2 cells (5×10^5 cells). Scheme showing the distribution of tissue components is attached. Panel E, a typical control tumor formed 21 days after the injection of A431 cells (5×10^6 cells) without F-2 cells. Panel F, a typical tumor produced by the same protocol as in panel D and treated with a mixture of anti-sialyl Lewis a and anti-sialyl Lewis x antibodies, indicating a marked reduction in the size of the main tumor, which is accompanied by a small vascular clump independent from the main tumor. Modified from reference.[57]

cells resulted in formation of vascular structures surrounding cancer cell nests *in vitro* (Fig. 8B). Addition of antibodies against sialyl Lewis a/x led to the formation of cancer cell clumps independent of the tube-like structures in cell culture dishes (Fig. 8C).

When the cancer cells and endothelial cells at a ratio of 10:1 were co-transplanted subcutaneously into the back of nude rats *in vivo*, the size of the tumors formed far exceeded that of control tumors formed without supplementation of endothelial cells, which were poorly vascularized (Fig. 8D and Fig. 8E).[57] With endothelial cell supplementation, the tumors were extensively vascularized throughout by blood vessel-like meshwork structures woven from the infused endothelial cells (Fig. 8D), the lumens of which contained the host blood cells. The administration of anti-Lewis a/x antibodies resulted in a marked reduction in the size of the tumors, which were not vascularized and were accompanied by independent tiny remnant clumps composed of endothelial cells (Fig. 8F).[57] These results served to corroborate that cell adhesion mediated by selectins and sialyl Lewis a/x determinants is significantly involved in tumor vascularisation.

In the early 1990's, selectin-mediated cell adhesion was proposed to be implicated in angiogenesis in general by supporting homologous cell adhesion among activated endothelial cells,[58,59] which were supposed to express both selectins and their carbohydrate ligands. This concept was not fully supported by our experimental results, because angiogenesis *per se* did not seem to be impaired by anti-sialyl a/x antibodies, as indicated by the presence of remnant clumps of hemangioma-like vascular structures composed of infused endothelial cells in the presence of antibodies (Fig. 8F). This suggested that angiogenesis *per se* by infused endothelial cells occurred independently of the inhibition of selectin-mediated cell adhesion by the antibodies. We suggest that selectin-mediated cell adhesion is closely involved in tumor vascularisation, *i.e.*, it is required for cancer cells to fit in the framework of the vascular structures by supporting heterologous cell adhesion between cancer cells and endothelial cells.

Cancer cells seek to survive under hypoxic conditions by inducing expression of a set of genes required to adapt to or cope with hypoxia. Hypoxia-induced enhancement of sialyl Lewis a/x expression on cancers also seems to be a link in the chains of these unfolding events, given that sialyl Lewis a/x determinants promote tumor vascularisation[57] and confer on cells a growth advantage in hypoxic local tumor environments.

5. Sialyl Lewis a as a risk factor for hematogenous metastasis of cancers

5.1 Accumulation of clinical statistics indicating importance of sialyl Lewis a in hematogenous metastasis

The thus-selected hypoxia-resistant cancer cells become predominant clones in locally-advanced tumors in the late stages of cancers.[53,60] What happens next is distant hematogenous metastasis, which characterizes the terminal stage of the malady. The sialyl Lewis a/x determinants, expression of which is exacerbated during natural selection of aggressive cancer cell clones in advanced tumors, mediate adhesion of cancer cells in the bloodstream to the vessel wall, and facilitate hematogenous metastasis (Fig. 9A).

The pathophysiological significance of sialyl Lewis a determinant in cancer progression had remained unclear when its application for serum diagnosis was started in the 1980's. It was not until the early 1990's that sialyl Lewis a on the surface of cancer cells, as well as sialyl Lewis x, was found to serve as a ligand for the endothelial cell adhesion molecule, E-selectin.[61,62] Sialyl Lewis a was shown to figure heavily in the adhesion to the endothelium of cancer cells derived from the lower digestive organs such as the colon and rectum as well as those from the pancreas and biliary tract, while another carbohydrate ligand for E-selectin, sialyl Lewis x, was found to figure heavily in the adhesion of breast, ovarian and pulmonary cancer cells.[61,63] These cell-biological results immediately raised the possibility that the adhesion of cancer cells to the endometrium mediated by E-selectin and specific carbohydrate ligands might be involved in the hematogenous metastasis of cancers.[45,64]

The numerous clinical statistics made available to date show that the intensity of sialyl Lewis a/x expression on cancer cells significantly correlates with the prognosis of patients (reviewed in refs).[45,65,66] A statistically significant correlation between the postoperative patient prognosis and sialyl Lewis a in cancer tissues was reported for cancers

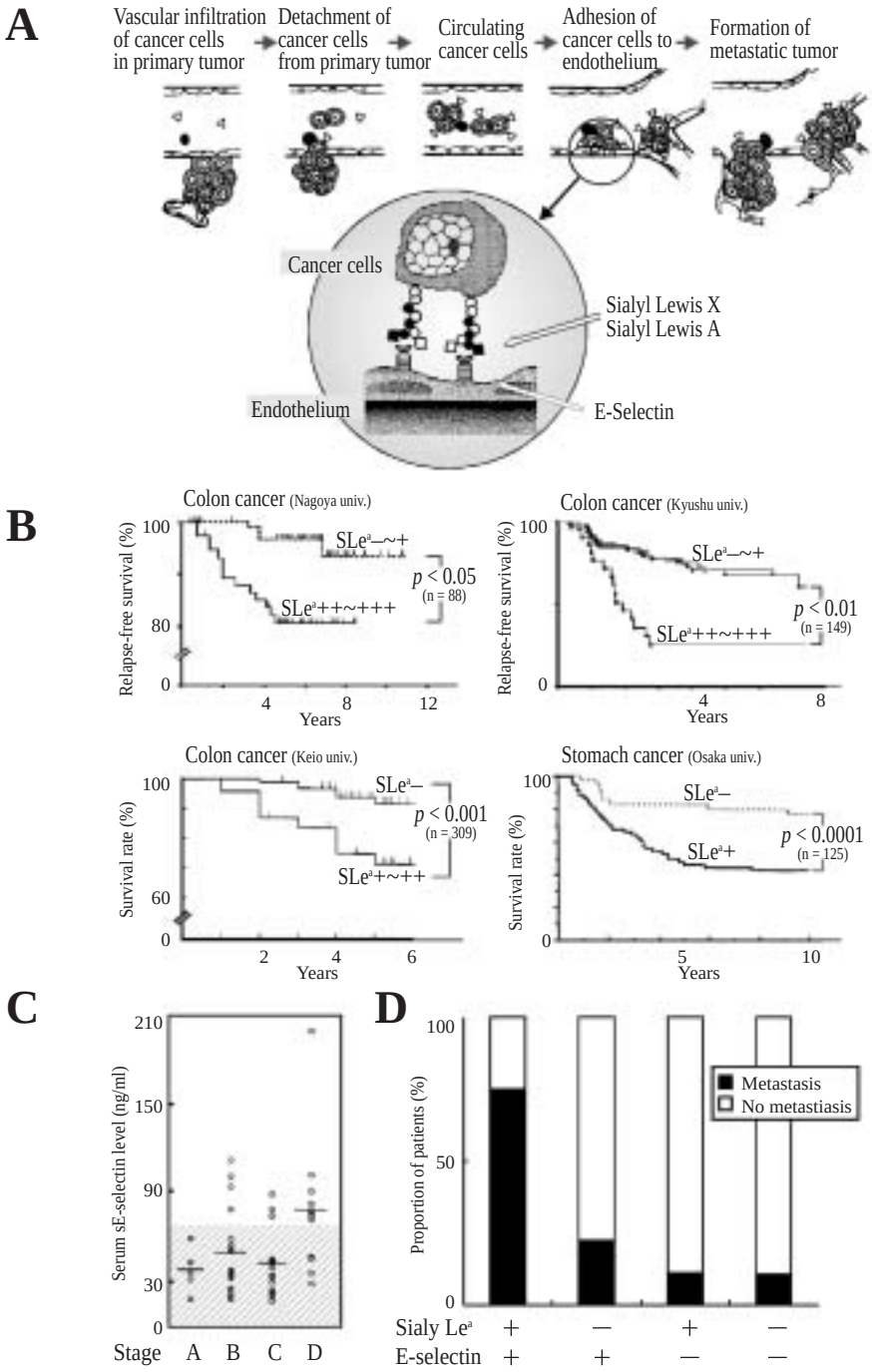

Fig. 9 Sialyl Lewis a accumulated in advanced cancers facilitates hematogenous metastasis. Panel A, schematic illustration for the role of sialyl Lewis a/x in adhesion of cancer cells to vascular endothelial cells during hematogenous metastasis of terminal stage cancers.[47] Panel B, clinical statistics indicating significant correlation between sialyl Lewis a expression in cancer cells and postoperative survival of patients with gastrointestinal cancers (SLe^a, sialyl lewis A; adapted from references).[67-70] Panel C, serum E-selectin levels in patients with colorectal cancer stratified according to Dukes' stage.[88] Panel D, proportions of patients with or without metastasis in groups having various combinations of serum sialyl Lewis a and E-selectin levels (+, elevated; –, non-elevated).[88]

of the colon and stomach (Fig. 9B),[67-70] while its correlation with sialyl Lewis x expression was reported for cancers of the lung, breast, prostate, stomach, colon and urinary bladder.[71-77] Correlations of sialyl Lewis a and -x expression with patient prognosis have been found mainly in patients with adenocarcinoma, but their significant expression is noted even in squamous cell carcinomas such as esophageal cancers, and the expression reportedly underscores a risk for hematogenous metastasis of squamous carcinoma.[78] The frequency of hematogenous metastasis is the most important factor in determining the prognosis of patients with advanced cancers. The patient prognosis may well be predicted by evaluating the degree of sialyl Lewis a/x determinants in cancer tissues obtained during surgical resection.

5.2 Induction of vascular E-selectin expression by cancer cells

E-selectin is a highly inducible protein; its expression is induced by a variety of stimuli including inflammatory cytokines such as TNF-α, IL-1α, and IL-1β, and the extent of its expression is highly variable in each patient. *In vitro* experiments testing the ability of culture supernatants of human cancer cells to induce E-selectin expression on cultured endothelial cells indicated that cancer cells can actually induce endothelial E-selectin expression through two alternative mechanisms.[79,80] One is the induction of E-selectin expression directly by a humoral factor produced by cancer cells, and this factor was found to be mostly IL-1α (Fig. 10). VEGF, which is produced by some cancer cells, is also known to induce vascular E-selectin expression.[81,82] The other is an indirect mechanism, in which a humoral factor, as yet unidentified, produced by cancer cells stimulates mononuclear leukocytes to produce IL-1β, and this in turn induces vascular E-selectin expression (Fig. 10). While the latter mechanism applies for many cancer cells, the former mechanism applies to relatively few. With either mechanism, vascular E-selectin expression is enhanced even in patients in the early stages of cancer having no overt metastasis, and this paves the way for actual metastasis. There are many other factors that lead to an increment of vascular E-selectin expression, such as surgery, chemotherapy, radiotherapy, and opportunistic infections, while glucocorticoids suppress its expression.[83,84]

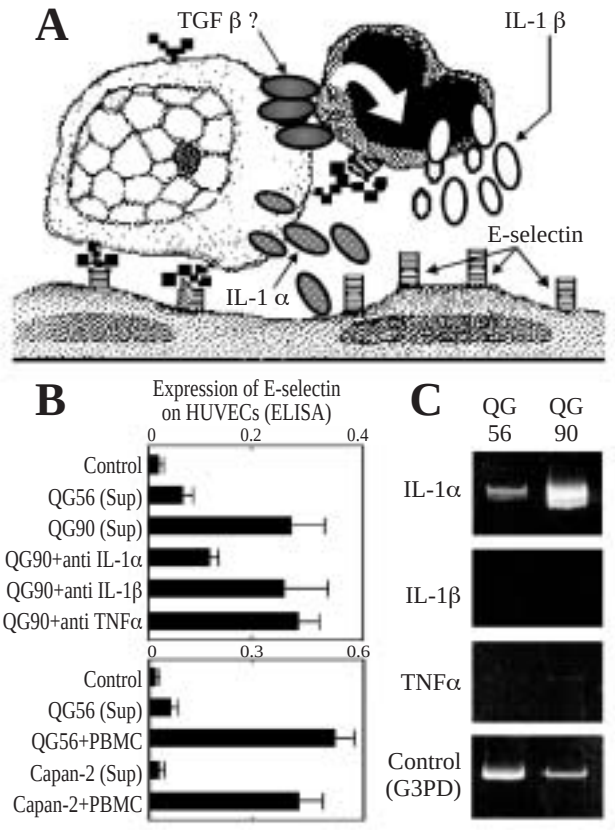

Fig. 10 Induction of vascular E-selectin expression by cancer cells. Panel A, schematic illustration of the role of cancer cells in the induction of vascular E-selectin expression. Panel B, *in vitro* experimental results showing induction of E-selectin expression on human umbilical vein endothelial cells (HUVECs) by cancer cells. QG90 cells secrete a humoral factor that directly induces E-selectin expression on HUVECs, while QG56 and Capan-2 cells secrete a factor which indirectly induces E-selectin expression through activation of PMBC. The factor produced by QG90 was identified as IL-1α by a neutralization experiment (panel B) and also RT-PCR (panel C). The humoral factor produced by PMBC in the indirect induction was identified as IL-β (data not shown). The factor produced by cancer cells in the indirect induction mechanism remains unidentified at this moment.

5.3 Vascular E-selectin expression as a second important risk factor for hematogenous metastasis

The second important factor in this context in predicting patient prognosis is the level of E-selectin expression on the vascular bed of patients. This can be evaluated by measuring soluble E-selectin in the sera of patients, because once expressed on endothelial cells, E-selectin is proteolytically released from the surface of endothelial cells and enters the general

circulation. Levels of soluble E-selectin in the sera reflect the extent of E-selectin expression on the vessel walls in patients. Our estimation of serum E-selectin yielded a normal value of 35 ng/ml in the sera of healthy individuals, indicating a certain basal level of vascular E-selectin expression. In patients, the level fluctuates depending upon various conditions.[85,86]

As cancer cells produce humoral factors that facilitate endothelial E-selectin expression, patients with advanced stage cancers generally have elevated serum E-selectin levels (Fig. 9C).[79,85-90] In immunohistochemical studies on colon cancer tissues, the small blood vessels surrounding cancer cell nests were found to frequently express E-selectin, and its expression was inversely correlated with the distance of blood vessels from the cancer nests.[85]

Elevated levels of serum E-selectin in patients with sialyl Lewis a/x-positive tumors predict a high risk for developing metastasis. Some patients have elevated levels even at relatively early stages; this subset of patients is at greater risk of developing hematogenous metastasis.

Interestingly, clinical statistics on patients with non-small cell lung cancer indicated a good correlation of E-selectin expression and outcome in patients with cancer cells expressing sialyl Lewis a/x determinants, while no significant correlation was observed in patients with sialyl Lewis a/x-negative tumor cells.[91] Simultaneous expression of sialyl Lewis a and vascular E-selectin is also associated with a higher risk of hematogenous metastasis in colon cancers (Fig. 9D).[88] These findings indicate that the level of vascular E-selectin expression can be regarded as a risk factor for metastasis when the sialyl Lewis a/x determinants are significantly expressed on cancer cells. Paired evaluation of sialyl Lewis a/x expression on cancer cells and vascular E-selectin expression would be useful in predicting risks for hematogenous metastasis and the postoperative prognosis of cancer patients. An anti-peptic ulcer agent, cimetidine, which is a classic histamine receptor antagonist, is known to suppress vascular E-selectin expression, and is reported to prevent hematogenous metastasis only when cancer cells express the sialyl Lewis a/x determinants.[92]

5.4 Number of circulating cancer cells as a third risk factor for hematogenous metastasis

Obviously cell adhesion mediated by selectins and sialyl Lewis a/x determinants is not the sole factor determining the frequency of hematogenous metastasis. Numerous other factors have been suggested to have significant influence by affecting the process of detachment of cancer cells from primary tumors into the bloodstream, such as cadherins, integrins and metalloproteinases.[93] The overall effect of these factors would eventually be reflected in the number of circulating cancer cells, which can be evaluated either by flow-cytometry using specific antibodies, or by RT-PCR using primers for epithelial cell-specific genes.[94-99] At present, the following three factors should be taken into consideration in predicting the risk for hematogenous metastasis in cancer patients: (i) degree of sialyl Lewis a/x expression on cancer cells; (ii) degree of E-selectin expression on vessel walls; and (iii) number of cancer cells circulating in the bloodstream.

Conclusions

Proteins and nucleic acids are generally believed to be indispensable functional molecules, but the role of carbohydrates as the third biologically functional molecule has long remained obscure. Biological functions and induction mechanisms for the sialyl Lewis a determinant in human cancers, although discovered as early as the beginning of the 1980s', have long remained unclear. The determination of the complete nucleotide sequence of the human genome has made it possible to systemically analyze genetic mechanisms for cell surface glycoconjugate expression, and to reconsider the genetic mechanism in the context of functional biology or pathophysiology. Elucidation of glycogenes involved in the hypoxia-mediated induction of sialyl Lewis a/x determinants in advanced cancers using DNA microarray techniques is a good example of such an approach. One of the most important goals of biological science in the post-genomic era is to clarify the mechanism of the induction of cell surface glycoconjugates and their functional roles. In this review we attempted to summarize the *status quo* of studies on the induction mechanisms and functional consequences of the sialyl Lewis a determinant in human cancers, as summarized in the Table 1. The knowledge obtained from these studies may well be useful in clinical application of the determinant, for differential diagnosis of benign and malignant disorders, and for pre-

Table 1. Summary of Induction Mechanisms and Functional Consequences of Sialyl Lewis a Expression during Progression of Human Cancers

Stage of cancer progression	Induction mechanism	Functional consequence	Possible clinical application
Early stage	Induction due to epigenetic glycogene silencing through histone deacetylation and DNA methylation (classic "*incomplete synthesis*")	Disturbance in immunological homeostasis of mucous membranes	Differential serum diagnosis through parallel determination of normal counterpart of determinant
Locally-advanced stage	Further enhanced expression due to hypoxia-induced glycogene transcription (formerly called "*neosynthesis*")	Tumor vascularisation	Prediction of risk of developing hematogenous metastasis
Terminal stage	Preferential expression in hypoxia-resistant cancer cells which become predominant clones in terminal stage cancers	Distant hematogenous metastasis	Monitoring of postoperative recurrence and distant metastasis

diction of a patient's risk of developing hematogenous metastasis.

Acknowledgements

This work was supported in part by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology (15590263 and on priority areas 14030092), Grants-in-Aid for the Second Term Comprehensive Ten-year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare, and a grant from the Core Research for Evolutional Science and Technology program of the Japan Science and Technology Agency, Japan.

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