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Developmentally Regulated Expression of Cell Surface Carbohydrates During Mouse Embryogenesis

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Cell surface carbohydrates undergo marked alterations during mouse embryogenesis. In preimplantation embryos, many carbohydrate markers show stage-specific expression in diverse ways. In early postimplantation embryos, certain carbohydrate markers are localized in defined regions in the embryo. Important carriers of stage-specific carbohydrates are the lactoseries structure (Galβ1→4GlcNAc) and the globoseries structure (Galα1→4Gal). Notably, the glycoprotein-bound large carbohydrate of poly-N-acetyllactosamine-type ($[Gal\beta1 \rightarrow 4GlcNAc\beta1 \rightarrow 3]_n$) carries a number of markers preferentially expressed in early embryonic cells. These markers are of practical value in analyzing embryogenesis and cell differentiation. For example, in order to monitor in vitro differentiation of multipotential embryonal carcinoma cells, stage-specific embryonic antigen-1 (SSEA-1) and the *Lotus* agglutinin receptor have been used as markers of the undifferentiated cells, and the *Dolichos* agglutinin receptor has been used as a marker of extraembryonic endoderm cells. Developmental control of cell surface carbohydrates is attained by controlled expression of activities of key glycosyltransferases; for example, the activity of N-acetylglucosaminide α1→3 fucosyltransferase is lost during in vitro differentiation of embryonal carcinoma cells to parietal endoderm cells, in parallel to the disappearance of SSEA-1. Accumulating evidence suggests that poly-N-acetyllactosamine-type glycans that are abundant in early embryonic cells are involved in cell surface recognition of these cells.

Key words: glycoproteins, glycolipids, cell surface antigens, carbohydrate antigens, glycosyltransferases, mouse embryos, teratocarcinoma cells, poly-N-acetyllactosamines

Cell surface carbohydrates undergo remarkable alterations during differentiation and development [1–3]. Many of the developmentally regulated carbohydrate sequences are recognized by antibodies or lectins and serve as cell surface markers useful for identification and separation of cells in the process of differentiation. Furthermore, some stage-specific carbohydrate sequences may play key roles in intercellular recognition required to accomplish embryogenesis.

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The mouse is frequently used to study mammalian development, since it is easy to breed, has a short gestation period, and comprises many inbred strains, including mutant strains with developmental abnormalities. Although early embryos of the mouse cannot be obtained in large amounts, teratocarcinoma cells have been used as a convenient alternative to analyze the stage by biochemical methods and to elicit antibodies to certain subpopulations of early embryonic cells. Teratocarcinoma is a tumor composed of cells of all three germ layers plus the malignant stem cells called embryonal carcinoma (EC) cells, which resemble multipotential cells of early embryos [4,5].

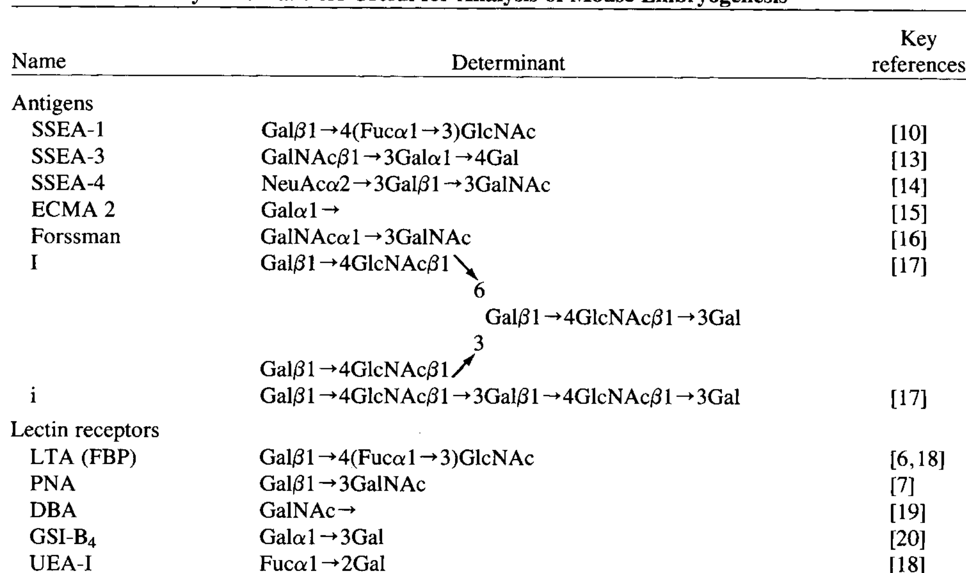
Breakthroughs in this area occurred in the late 1970s. Gachelin et al applied plant lectins to monitor in vitro differentiation of EC cells [6,7]. Muramatsu et al analyzed glycopeptides from EC cells and from early mouse embryos and found poly-N-acetyllactosamine-type glycans, which turned out to be carriers of many developmentally regulated carbohydrate markers present in early embryonic cells [2,8,9]. Applying the monoclonal-antibody technique, Solter and Knowles found an antigen, stage-specific embryonic antigen-1 (SSEA-1), which is preferentially expressed in EC cells and cells of early mouse embryos [10]. Subsequent studies disclosed the antigenic epitope to be so-called Le^x structure [11]. Now the phenomena of carbohydrate alterations are reasonably well described, and the knowledge is of practical value in analyzing embryogenesis. However, only a little is known still about the biological significance of the cell surface change and about the mechanism of developmental control. Much effort is being devoted to resolve the remaining problems.

OUTLINES OF THE CARBOHYDRATE ALTERATIONS

Both immunohistochemical methods and biochemical methods have been used to analyze developmental changes of cell surface carbohydrates during mouse embryogenesis. The former approach utilizing antibodies and lectins can be applied to a small amount of material, even to a single cell, and can identify regional differences in carbohydrate expression. However, results are sometimes influenced by experimental conditions such as fixation procedures, and the lower limit of detection is usually unclear. Furthermore, antibodies and lectins can detect only a partial structure of the whole carbohydrate chain. Moreover, rigidly speaking, structure of binding sites detected by these reagents can only be assumed, relying on their known specificities. On the other hand, biochemical analysis requires a substantial amount of material and cannot determine the distribution of the molecule in the specimen. The combination of the two different approaches, however, has been able to provide a rather comprehensive picture of the cell surface change.

Table I shows carbohydrate markers useful for the analysis of mouse embryogenesis. Examples of their appearance and disappearance are illustrated in Figure 1. These carbohydrate markers are mostly determined by structures around the nonreducing end of the carbohydrate chain. Two important structures carrying these determinants are the lactoseries structure (Galβ1$\rightarrow$4GlcNAc) and the globoseries structure (Galα1$\rightarrow$4Gal) [12]. Although the latter is apparently located only in glycolipids, the former is present both in glycoproteins and glycolipids. Notably, the glycoprotein-bound large carbohydrate of poly-N-acetyllactosamine-type ([Galβ1$\rightarrow$4GlcNAcβ1$\rightarrow$3]n) carries a number of markers. In addition, the ganglioseries

TABLE I. Carbohydrate Markers Useful for Analysis of Mouse Embryogenesis



Name	Determinant	Key references
Antigens		
SSEA-1	Galβ1→4(Fucα1→3)GlcNAc	[10]
SSEA-3	GalNAcβ1→3Galα1→4Gal	[13]
SSEA-4	NeuAcα2→3Galβ1→3GalNAc	[14]
ECMA 2	Galα1→	[15]
Forssman	GalNAcα1→3GalNAc	[16]
I	Galβ1→4GlcNAcβ1↘6 Galβ1→4GlcNAcβ1→3Gal; Galβ1→4GlcNAcβ1↗3	[17]
i	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Gal	[17]
Lectin receptors		
LTA (FBP)	Galβ1→4(Fucα1→3)GlcNAc	[6,18]
PNA	Galβ1→3GalNAc	[7]
DBA	GalNAc→	[19]
$GSI\text{-}B_4$	Galα1→3Gal	[20]
UEA-I	Fucα1→2Gal	[18]

structure (GalNAcβ1→4Gal) and the core structure of mucin-type oligosaccharides (Galβ1→3GalNAc) also serve as carriers of certain developmentally regulated sequences.

Figure 2 shows the outline of mouse embryogenesis and carbohydrate alterations during the course. At the beginning of embryogenesis, cells continue to divide without apparent signs of distinction from one another. At the 32-cell stage (blastocysts), the presence of two distinct cell groups becomes clear—namely, the externally located trophectoderm and the internally located inner cell mass. Blastocysts implant into uterine epithelium on day 4.5 of embryogenesis. In the preimplantation period, the most dramatic changes of the carbohydrate markers occur (Table II). The mode of expression is distinct for almost every marker. After implantation, the embryo proper develops from the inner cell mass. Between day 5 and day 7 of embryogenesis, the three germ layers are formed. In the early postimplantation embryos, many carbohydrate markers become segregated to restricted regions (Fig. 1). In late postimplantation embryogenesis, notably between days 11 and 15 of embryogenesis, when organogenesis is actively taking place, some of the markers are expressed in cells at the intermediate state of differentiation. For example, Forssman [21], SSEA-1 [22], ECMA-2 [23], and ECMA-3 [23] antigens are expressed in primordial germ cells during migration and in those newly migrated to the gonad; these antigens disappear from the cells in later stages.

Although certain markers are expressed in multiple sites, which are seemingly unrelated to each other in cell lineage (Fig. 1), this does not mean that the markers in different tissues have the same chemical structure. As will be described later, backbone structures are occasionally different for the same marker isolated from different cells. Thus, carbohydrate alterations during differentiation is a highly complex phenomenon: a certain carbohydrate structure appears to be specific for a given stage of cell differentiation.

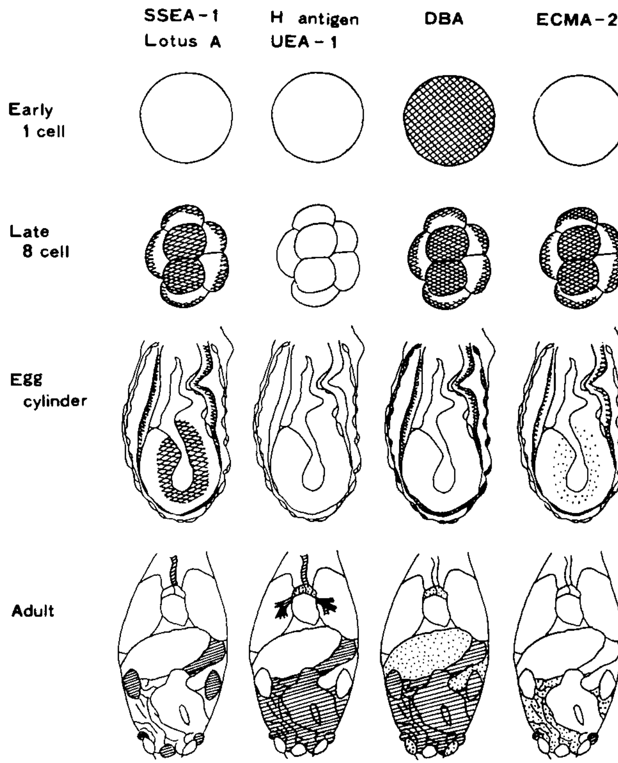


Fig. 1. Immunohistochemical demonstration of developmentally regulated carbohydrate expression in the mouse. [cross-hatched], majority of the cell expresses the marker; [hatched], a part of the cell in the area expresses the marker; [dotted], a few cells in the area express the marker. [Based on references 10, 15, 18, 19, 22, 23, 57, 58, 61, and 62].

POLY-N-ACETYLLACTOSAMINES

Muramatsu et al analyzed carbohydrate moieties of glycoproteins from EC cells and from preimplantation mouse embryos: the specimen was cultured in the presence of radioactive fucose, extensively digested with pronase, and analyzed by gel filtration on a column of Sephadex G-50 [8]. Glycopeptides eluted at the void volume of the column were found in large amounts; such large glycopeptides were scarcely found when many normal and cancerous cells were analyzed by similar methods. The large glycan had not been detected, although cell surface glycopeptides from the preimplantation embryo had been studied earlier [24].

The results of several experiments excluded the possibility that the large glycopeptides were glycosaminoglycans or mucin-type glycopeptides. Subsequent studies revealed that they indeed have large carbohydrate chains with a molecular weight of around 10,000 or more [25,26]. They have a repeating structure of (Galβ1$\rightarrow$4GlcNAcβ1$\rightarrow$3)n and are highly branched at the C-6 hydroxyl group of galactose [25,27]. The protein-carbohydrate linkage was shown to be the N-glycoside-type involving asparagine [26]. The large glycan is called embryoglycan.

Abundance of the fucosylated form of the large glycan is characteristic of the early embryonic phenotype. Thus, the fucose-labeled glycan progressively decreases

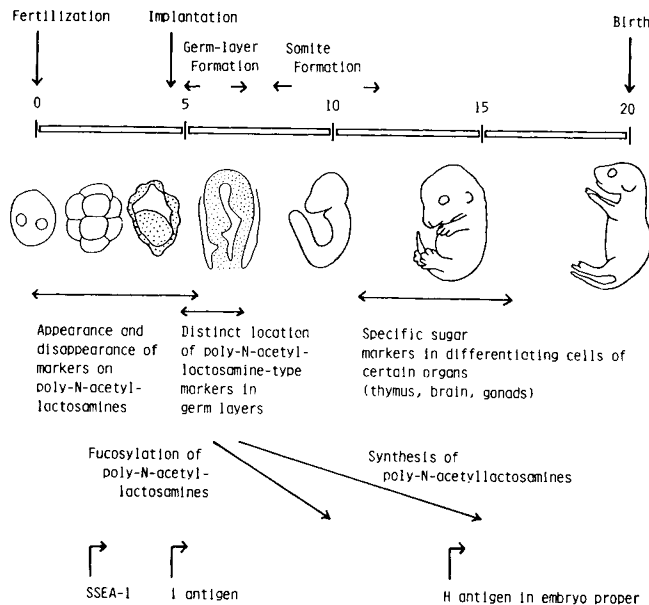


Fig. 2. Distinct events in carbohydrate alteration during mouse embryogenesis.

TABLE II. Examples of Developmentally Regulated Expression of Carbohydrate Markers During Preimplantation Mouse Embryogenesis*

Marker	1 cell	2 cell	8 cell	16 cell	Blastocyst ICM[a]	Blastocyst TE[b]
SSEA-1, Lotus A (LTA)	–	–	–→+	++	±→+	++→+
H, UEA-1	–	–	–	–	–	+→–
ECMA-2	–→+	+	+	++	±	++→±
DBA	++	++	++	+	+→±	+→–

*[Based on references 10, 15, 18, 23, 57, and 58.]
[a]ICM, inner cell mass.
[b]TE, trophectoderm.

during in vitro differentiation of EC cells [8,9,28,29]. Determination of the amount of fucose by a chemical method confirmed that the decrease in the amount of fucose-labeled embryoglycan after differentiation is not the misinterpretation of an increased ratio of fucose incorporation into smaller glycopeptides [28]. The sharp decrease in the synthesis of the large glycan occurs between day 2 and day 4 of retinoic-acid-induced differentiation of aggregated multipotential EC cells [29]. In the differentiation system, cells commit to differentiate before day 2, and the direction of differentiation of the cell population is decided between day 2 and day 4. Therefore, the decreased synthesis of embryoglycan is not the cause of the induction of differentiation, while the possibility is not excluded that the glycan is somehow involved in selection of the direction of differentiation. In dissected mouse embryos, fucose incorporation into the large glycan becomes negligible on day 10 of embryogenesis [30]. That synthesis of the backbone structure of the poly-N-acetyllactosamine decreases more slowly is revealed by the incorporation of radioactive glucosamine into

the glycan [31,32]; synthetic activity of the high molecular weight glycan becomes scarcely detectable on day 15 [32].

Embryoglycan has been shown to carry many cell surface markers preferentially expressed in early embryonic cells; they include antigens such as SSEA-1 [33,34], ECMA-2 [35], ECMA-3 [35], TC [36], NL-9 [37], TEC-02 [38], and receptors for lectins such as LTA [9,26], PNA [9], DBA [39], and GS-I [40]. A simple way to demonstrate the presence of antigenic determinants on the glycan is a modified Farr's assay: the antibody to be examined is mixed with the radiolabeled glycan, and ammonium sulfate is then added to precipitate the resulting immune complex [41]. The experiment is a convenient way to demonstrate the carbohydrate nature of an antigen. To be used in such an experiment, the glycan preparation is preferably prepared by combining chloroform-methanol extraction, alkaline $NaBH_4$ treatment, and extensive pronase digestion to ensure the removal of lipids and undigested proteins [29].

In 1978, when an initial report on embryoglycan was published, a glycoprotein-bound large glycan of similar nature was also observed in human erythrocyte membrane: the glycan, termed "erythroglycan," was proved to carry determinants of ABH blood group antigens [42,43]. Subsequently, asparagine-linked carbohydrates with repeated arrangements of (Galβ1→4GlcNAcβ1→3)n were found in many sources [44–47]. These glycans are collectively called poly-N-acetyllactosamine(s) or (poly)lactosaminoglycan(s). Structures of poly-N-acetyllactosamines are diverse and differ according to the sources from which they are isolated. The one from embryonal carcinoma cells and the one from human erythrocytes have more complex structures than those from other sources; still, the one from EC cells is different from the one from erythrocytes with respect to the markers they carry. So far, the glycan characterized in EC cells appears to be specific for cells of early embryonic phenotype. Poly-N-acetyllactosamines may be regarded as a unique class of glycans, whose structural variety reflects a given stage of cell differentiation.

SSEA-1 AND OTHER FUCOSYL MARKERS

To analyze early stages of embryogenesis, Solter and Knowles prepared a monoclonal antibody against EC cells [10]; the antigen recognized by the antibody was named SSEA-1. SSEA-1 is intensely expressed in EC cells, but disappears from most of cells differentiated from EC cells in vitro. Thus, the antigen has been conveniently used as a marker to identify EC cells in their differentiating cell populations [48–50]. During mouse embryogenesis, SSEA-1 first appears in late eight-cell embryos [10] (Table II). It is expressed in embryonic ectoderm, visceral endoderm, and trophoblasts in early postimplantation embryos (Fig. 1) and progressively disappears from most of the embryos during embryogenesis. In adult mice, the antigen is detected in highly restricted sites such as the epididymis, renal tubules [22], and epithelium of the bladder [18].

Feizi et al found that the antigenic determinant is Galβ1→4(Fucα1→3)GlcNAc as the result of hapten inhibition experiments [11]. This antigenic epitope has been known as Le^x or X. SSEA-1 is usually carried by the lactoseries structure. Notably in early embryonic cells, the poly-N-acetyllactosamine (embryoglyan) linked to high-molecular-weight glycoproteins is the major carrier of the antigen. This conclusion was reached by analysis of the antigen immunologically isolated from EC cells [33]

and by endo-β-galactosidase digestion of the antigen [34,51]. Endo-β-galactosidase from *Escherichia freundii* cleaves Galβ1→4GlcNAc linkage whose galactosyl residue is substituted by GlcNAcβ1→3 [52]; susceptibility to the enzyme implies that the substrate has a (Galβ1→4GlcNAcβ1→3)n unit. Endo-β-galactosidase does not cleave the linkage near the branching point of the poly-N-acetyllactosamine chain [53]. Thus, sensitivity of the enzyme indicates that SSEA-1 determinant is present in the linear domain of the poly-N-acetyllactosaminyl chain [51], although the entire glycan carrying the determinant structure is branched as judged from the result of chemical analysis [25,26].

Le^y (or Y) antigen [Fucα1→2Galβ1→4(Fucα1→3)GlcNAc] is also detected in some EC cells and early embryos of the mouse [51,54,55]. Mode of expression of Le^y is somewhat different from SSEA-1. Thus, EC cells are classified into Le^y-positive (F9, PCC4) and Le^y-negative (PCC3, PCC7-S-1009) [54]. Expression of the antigen in preimplantation embryos requires the uterine environment [55]. In early postimplantation embryos, the antigen is detected in embryonic ectoderm, visceral endoderm, and trophoblasts just as in the case of SSEA-1 [55].

The *Lotus tetragonolobus* agglutinin (LTA or FBP) reacts with EC cells and early mouse embryos in a manner identical with that of anti-SSEA-1 antibody [6,9,18]. Distribution of the lectin receptors in adult tissues is also restricted and in a fashion similar to that of SSEA-1 [18]. Receptors for LTA were isolated from EC cells and identified as glycoproteins with a molecular weight of around 100,000 [26]. Carbohydrate moieties constitutes about 28% of the receptors, and 37% of them are embryoglycan, which retains the binding activity to LTA. The fucosyl residue in the large glycan mostly has the structure of Galβ1→4(Fucα1→3)GlcNAc. Therefore, epitopes of SSEA-1 and LTA receptors appear to be identical, although hapten inhibition studies indicated that the lectin binding site had the structure of Fucα1→2Galβ1→4GlcNAc [56].

H antigen is defined by a Fucα1→2Gal linkage. It forms the basis of A and B blood group antigenic epitopes. *Ulex europaeus* agglutinin-I (UEA-I) also recognizes H antigenic structure. In contrast to SSEA-1, H antigen is scarcely detected in early mouse embryos [18,51]. An extensive survey using UEA-I revealed the temporal expression of the receptors in trophectoderm cells (18) (Table II). In embryo proper, the receptors are observed only after day 14 of embryogenesis [18]. In adult mice, UEA-I receptors are mainly expressed in epithelial tissues [18].

DBA RECEPTORS

Dolichos biflorus agglutinin (DBA) is a lectin recognizing terminal N-acetylgalactosamine residues. DBA receptors are expressed even in unfertilized ova, and their expression becomes decreased during preimplantation embryogenesis [57,58] (Table II). In early postimplantation embryos, the receptors are present in extraembryonic endoderm cells [19]. They are also expressed in embryonic thymocytes on day 13 of embryogenesis: during embryogenesis, the receptors progressively disappear from the surface of these cells [59]. A line of leukemia cells (GRSL) also expresses the receptors, probably owing to the immature phenotype of these cells [60]. In the adult mice, the receptors are detected in several sites, largely on tissue of endoderm origin [61,62].

DBA receptors are expressed in so-called quasinullipotent EC cells (ie, F9 and N4-1 cells) [39,63]: these cells have weak differentiation capabilities and mainly differentiate to extraembryonic endoderm cells upon retinoic-acid-induced differentiation. On the other hand, so called pluripotent EC cells (PCC3, PCC4, HM-1) do not express DBA receptors: these cells have strong differentiation capabilities and form cells of three germ layers upon induction of differentiation. Therefore, the presence or absence of DBA receptors may be used to predict the differentiation capability of EC cells. Furthermore, DBA is a useful reagent to detect extraembryonic endoderm cells upon in vitro differentiation of pluripotent EC cells [29].

Expression of DBA receptors in the adult organ is under genetic control. In most mice, DBA receptors are expressed in epithelial cells of the small intestine [62], but not in the endometrium of the uterus [61], while in DDK mice, epithelial cells of the small intestine lack the receptors and the endometrium expresses them [64]. The genetic difference occurring in the epithelium of the small intestine has been utilized to follow the cell lineage of epithelial cell differentiation by making chimera of the two mice genetically different in the expression [65].

The determinant of DBA receptors appears to be highly antigenic: TC antigen recognized by rabbit antibody [36] and OR 19 antigen recognized by monoclonal rat antibody [66,67] are likely to share the determinant with the receptors. It is also interesting to examine its possible correlation with TEC-02 antigen, which is defined by GalNAcβ1$\rightarrow$4Gal linkage [38].

Carbohydrate moieties of DBA receptors isolated from different sources were compared and found to be substantially different [68]. The majority of them from F9 EC cells are embryoglycan, while those from the small intestine are much smaller. Those from extraembryonic endoderm cells of postimplantation embryos and of teratocarcinoma cells are of medium sizes. This result is an example showing the complex pattern of glycosylation changes during differentiation.

Since DBA receptors are intensely expressed in extraembryonic endoderm cells, affinity chromatography on DBA agarose can be utilized to isolate glycoproteins located on the endoderm cells. Using this approach, Ozawa et al identified a high-molecular-weight glycoprotein named "brushin," which is expressed only in primitive endoderm, visceral endoderm, and renal tubular brush border [69]. Judging from its localization, brushin appears to be involved in the absorption process. Furthermore, a renal antigen causing Hymann nephritis [70] is most probably identical with brushin. FT-1 is a glycoprotein antigen initially found in DBA receptors of GRSL leukemia cells [71]. It has also been found in visceral endoderm but not in primitive endoderm; a combination of brushin and FT-1 proved to be efficient in monitoring in vitro differentiation of EC cells to visceral endoderm cells [63]. The antibody to DBA receptors was also used to clone genes coding for core proteins of the receptor glycoproteins. Gp 70 found by the approach is an interesting glycoprotein. It was detected in EC cells, teratocarcinoma cells, and also in some other cells. Sequence analysis indicated that Gp 70 is a typical transmembrane glycoprotein and carries nine potential Asn glycosylation sites [72].

α-GALACTOSYL MARKERS

Griffonia simplicifolia (*Bandeirae simplicifolia*) agglutinin I-B_4 (GS-I-B_4) is a lectin recognizing α-galactosyl residue. Galα1$\rightarrow$3Gal is the most potent haptenic

inhibitor of the lectin [73]. GS-I, which is a mixture of isolectin including GS-I-B_4, binds to extraembryonic endoderm and trophoblasts of early postimplantation mouse embryos [20]. The preimplantation embryos are unreactive. In adult mice, GS-I-B_4 binding sites are mainly distributed in the basement membrane and capillaries of certain organs [74].

GS-I receptors isolated from F9 EC cells and teratocarcinoma OTT6050 are high-molecular-weight glycoproteins, and their poly-N-acetyllactosamines carry the binding sites [40]. Using endo-β-galactosidase C, a new endo-β-galactosidase isolated from the culture fluid of *Clostridium perfringens* [75], the structure of the binding site in the teratocarcinoma glycan was determined to be Galα1→3Gal [76].

A similar structure may be expressed on human germ cell tumors and recognized by the host's immune system. Thus, Kawata et al found that in 60% of the cases examined, sera from patients with germ cell tumors react with embryoglycan from F9 EC cells upon a modified Farr's assay [41]. No positive reaction is observed when sera from normal human subjects are used. α-Galactosidase digestion of embryoglycan abolishes the antigenic activity [77]. Among the glycans, those retained by GS-I agarose have higher antigenic activity [78]. The poly-N-acetyllactosamine-type glycan from teratocarcinoma OTT6050 also has antigenic activity, and it is completely destroyed by endo-β-galactosidase C digestion [76].

ECMA 2 is also determined by an α-galactosyl residue, but it is distinct from GS-I binding sites. The antigen is defined by a rat monoclonal antibody raised against EC cells [15]. In preimplantation embryos, the antigen becomes expressed in late one-cell embryos [23] (Table II). The antigen disappears during early postimplantation embryogenesis [23] and reappears in primordial germ cells. In the adult, the antigen is detected in severely restricted sites—namely, the oviduct, cauda epididymis, and in a small number of unidentified cells of the small intestine [23]. Thus, the antigen is closely related to early embryogenesis and reproduction. EMCA 2 antigen isolated from EC cells is a mixture of high-molecular-weight glycoproteins, and the antigenic site is again located in embryoglycan [35]. α-Galactosidase digestion of embryoglycan abolishes the antigenic activity [35]. However, it is not destroyed by digestion with endo-β-galactosidase C. When sections of early postimplantation embryos are digested with α-galactosidase, reactivities of anti-SSEA-1 antibody and anti-Le^y antibody increase; the result suggests the occurrence of novel determinants such as Galα1→XGalβ1→4(Fucα1→3)GlcNAc and Galα1→X(Fucα1→2)Galβ1→4(Fucα1→3)GlcNAc [51]. ECMA 2 determinant may be related to such structures.

GLOBOSERIES MARKERS

Forssman antigen is carried by globoseries glycolipids, notably by Forssman glycolipid, and the antigenic determinant is GalNAcα1→3GalNAc. Using a monoclonal antibody against Forssman antigen, Willison and Stern found that Forssman antigen is expressed in both trophectoderm and inner cell mass of blastocysts [79]. After implantation, the antigen persists in extraembryonic endoderm cells [21].

Two other antigenic epitopes—namely, SSEA-3 and SSEA-4—are also carried by globoseries glycolipids. SSEA-3 is defined by a monoclonal antibody against 4–8-cell mouse embryos [13], while SSEA-4 is defined by a monoclonal antibody raised against a human teratocarcinoma cell line [14]. Both antigens are expressed through-

out the preimplantation period except for trophoblasts of the late blastocysts [13,14]. In early postimplantation embryos, SSEA-3 serves as a marker of visceral endoderm [80]. Human teratocarcinoma 2102Ep cells express SSEA-3 and 4, but not SSEA-1, and upon differentiation the former two antigens decrease with a concomitant increase of SSEA-1 [14]. Therefore, the mode of cell surface change is different from that observed during differentiation of mouse EC cells. Kannagi et al isolated a series of globoseries glycolipids from human teratocarcinoma 2102Ep cells and determined their structure [81]. Then, they examined the reactivity of each glycolipid to SSEA-3 and SSEA-4 monoclonal antibodies and identified the antigenic epitopes of the two antigens (Table I).

In the case of some globoseries glycolipids—namely, Forssman glycolipid and ceramide dihexoside—the amount of glycolipids and the intensity of their reactivity to antibodies do not always correlate [82]. It is probably because the degree of exposure of antigenic epitopes on relatively short oligosaccharide chains is strongly influenced by other membrane components.

PNA RECEPTORS

Peanut agglutinin (PNA) is a lectin recognizing Galβ1→3GalNAc sequence. PNA receptors are expressed throughout the preimplantation embryogenesis (M. Sato, unpublished). In early postimplantation embryos, they are expressed in all germ layers [83]. In the somite, expression of PNA receptors is more intense in cells near neural tube (M. Sato, unpublished). Tissues expressing the receptors in the adult mice are rather restricted [61,62].

PNA receptors are expressed in EC cells, but not in cells differentiated from them [7], except for their presence on visceral endoderm cells (H. Hamada, unpublished). Therefore, the receptors are useful in monitoring the differentiation of EC cells. Furthermore, by mixed hemagglutination with rabbit erythrocytes in the presence of PNA, EC cells can be separated from differentiated cells devoid of PNA receptors [7].

In EC cells, PNA binding sites are also carried by embryoglycan [9]. In human gastric cancer cells, however, the binding site was revealed to be Galβ1→3GalNAc linked to the protein moiety [84].

I AND i

Patients with the autoimmune hemolytic disorder known as cold agglutinin syndrome produce high titers of antibodies recognizing antigens present in human erythrocytes. One of the antigens, I, is expressed in erythrocytes of most of the adults. The other antigen, i, is expressed in fetal erythrocytes, and on rare occasions in adult erythrocytes. The antigenic determinants of I corresponds to the branched domain of poly-N-acetyllactosamines and, i to the linear domain. I antigen is intensely expressed through the preimplantation embryogenesis and the early postimplantation embryogenesis [17,51]. On the other hand, i antigen first appears in parietal and visceral endoderm of the early postimplantation embryos, and then some other parts of the embryo come to express it [17,51]. In teratocarcinoma cells both I and i antigens are carried by high-molecular-weight glycoproteins [34].

Antigens related to Ii antigens—namely, C6 and A5—are detected by mouse IgM monoclonal antibodies [85]. Although both these antibodies are inhibited by N-acetyllactosamine, the mode of the antigen distribution is not identical with that of I or i antigen.

REGULATION OF BIOSYNTHESIS

Carbohydrate structures are generally formed by enzymatic transfer of sugar residues from sugar nucleotides to the acceptor. Thus, the structure of glycoconjugates depends on the specificity of the transferase: the developmental alteration of the structure of cell surface carbohydrates is usually due to the developmental alteration of glycosyltransferase activities. This is clearly shown in the case of disappearance of SSEA-1 during differentiation of EC cells to parietal endoderm cells. Muramatsu and Muramatsu found that activity of a fucosyltransferase forming the determinant structure—namely, Galβ1→4(Fucα1→3)GlcNAc linkage sharply decreases in parallel to the disappearance of SSEA-1 [86]. Thus, the enzyme is one of the key enzyme-forming cell surface structures under developmental control. The enzyme has been highly purified from EC cells [87]. On the other hand, a sialyltransferase activity increases during differentiation of EC cells [88]. The enzyme may compete with other glycosyltransferases for an acceptor, resulting in inhibited synthesis of carbohydrates which are abundant in early embryonic cells. Developmental regulation of the synthesis of poly-N-acetyllactosaminyl chains is, however, still poorly understood. The linear chain may be formed by concerted action of a galactosyltransferase forming Galβ1→4GlcNAc linkage and an N-acetylglucosaminyltransferase forming GlcNAcβ1→3Gal linkage. A galactosyltransferase apparently involved in the synthesis of poly-N-acetyllactosamines has been purified from EC cells [89]. The enzyme efficiently uses agalacto-embryoglycan as an acceptor; N-acetylglucosamine and asialoagalactofetuin are also effective as acceptors. The molecular weights of the enzyme are 68,000 and 59,000. In the presence of α-lactalbumin, the enzyme can also transfer galactose to glucose. Thus, the enzyme has properties common to other galactosyltransferases so far described.

BIOLOGICAL FUNCTION OF STAGE-SPECIFIC CARBOHYDRATES

Cell-surface carbohydrates with strictly regulated expression during embryogenesis may be expected to play important roles in this process. So far most of the studies have been performed on poly-N-acetyllactosamines and epitopes carried by them.

Bird and Kimber added an oligosaccharide with the SSEA-1 determinant into an in vitro culture of preimplantation mouse embryos [90]. They found decreased cell adhesiveness resulting in inhibition of compaction. Compaction is observed from the late eight-cell-embryo stage onward and is a phenomenon of increased cell adhesiveness resulting in unclear cell boundaries. On the other hand, using simple oligosaccharides, Fenderson et al could not observe inhibition of compaction; instead they found that SSEA-1 determinants linked to a high-molecular-weight carrier inhibited compaction [91]. Feizi and co-workers digested preimplantation embryos with endo-β-galactosidase from *Escherichia freundii*. When the embryo had been treated with Ca^{++}-free medium, and the cell-to-cell adhesion decreased, the digested embryos were more difficult to reaggregate than untreated embryos [92]. These findings

suggest that fucosyl poly-N-acetyllactosamines play some role in cell adhesion in preimplantation embryos.

Studies using EC cells support the above suggestion. Shur fixed poly-N-acetyllactosamines to a plastic dish and plated EC cells on the surface. Adhesiveness of EC cells to the surface was significantly improved by the procedure [93]. Muramatsu and co-workers found that a monoclonal antibody reacting with fucosyl poly-N-acetyllactosamines inhibited cell-substratum adhesion of F9 EC cells [94]. Studies using mutant lines of EC cells, however, did not lead to a unifying conclusion. Kartha et al isolated three mutant EC cell lines defective in cell aggregation, all of which had reduced amounts of a PNA receptor protein of 94,000 mol wt [95]. Rosenstraus obtained a mutant EC cell line defective in SSEA-1. The mutant cell could form aggregates, differentiate into extraembryonic endoderm cells, and form tumors in the host just as the parental EC cell did [96]. In the mutant cell, other cell surface components may compensate for the absence of fucosyl poly-N-acetyllactosamines.

If these carbohydrate sequences play some roles in cell-surface recognition, a logical consequence is that some protein-recognizing cell surface carbohydrate is present on the surface of early embryonic cells. Shur postulated that a galactosyltransferase is present on the surface of EC cells [97] and in cells of early embryos [93] and plays a key role in cell surface recognition. This argument is mainly based on enzymological evidence of galactosyltransferase on the cell surface and on inhibition of certain cellular activities by α-lactalbumin, which is a specific modifier protein of a galactosyltransferase forming Galβ1$\rightarrow$4GlcNAc linkage.

Other candidates of the recognition molecules are lectins. Grabel et al found that fucoidan, a sulfated polysaccharide rich in fucose, inhibited aggregation of EC cells [98]. Furthermore, they found lectin activity in the cells, and the lectin is apparently specific for fucoidan-related molecules [98]. However, its detailed properties remain to be elucidated.

For studying the function of stage-specific carbohydrates, it is necessary to isolate cDNA clones of glycosyltransferases forming stage-specific carbohydrates, of endogeneous proteins recognizing the carbohydrate sequence, and of the core proteins carrying these carbohydrates. Since molecular biology has already been introduced in the field of glycoconjugate research, the above-mentioned tasks will be attained before long. When these genes are isolated, it will be possible to introduce the gene and its modified form into cultured cells capable of differentiation and into fertilized eggs. If some of the phenomena observed during differentiation are altered by such manipulation, we will be able to understand definitively the significance of carbohydrate alterations during differentiation.

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