Jpn. J. Cancer Res. **93**, 1129–1137, October 2002

Expression of Glycolipids Bearing Lewis Phenotypes in Tissues and Cultured Cells of Human Gynecological Cancers

Kyoko Takehara,[1] Kaneyuki Kubushiro,[1] Kazushige Kiguchi,[2] Isamu Ishiwata,[3] Katsumi Tsukazaki,[1] Shiro Nozawa[1] and Masao Iwamori[4, 5]

[1]Department of Obstetrics and Gynecology, School of Medicine, Keio University, 35 Shinano-machi, Shinjuku-ku, Tokyo 160-8582, [2]Department of Obstetrics and Gynecology, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki, Kanagawa 211-0061,[3]Ishiwata Gynecological Hospital, 1-4-21 Kamimito, Mito, Ibaraki 310-0041 and [4]Department of Biochemistry, Faculty of Science and Technology, Kinki University, 3-4-1 Kowakae, Higashiosaka, Osaka 577-8502

Transformation-associated expression of Le^b (Lewis antigen-b) or Le^Y in human colorectal carcinomas has been well described. To examine the expression of glycosphingolipids (GSLs) bearing Lewis-phenotypes in human gynecological carcinoma-derived cells, we determined the concentrations of all GSLs. Although neither Le^b nor Le^Y was present in HEC-108 cells established from the poorly differentiated type of endometrial adenocarcinoma, other cell lines from moderately or well-differentiated types expressed either Le^b or Le^Y, or both, at concentrations of 0.01 to 0.03 μg per mg of dry cells, which comprised 0.3 to 1.3% of the total GSLs. In the cervical and ovarian carcinoma-derived cell lines, Lewis phenotypes tended to be carried by nLc_4Cer, which was accumulated in the cells without sialylation or fucosylation. These results indicated that expression of Le^b- or Le^Y-phenotypes was strongly dependent on the metabolic ability to supply the precursor GSLs. Both Le^b and Le^Y were successfully detected by monoclonal antibody MSN-1, which was a useful probe for the simultaneous detection of Le^b and Le^Y. On application of MSN-1, either Le^b or Le^Y was detected in tissues from patients with well- and moderately differentiated types of endometrial adenocarcinoma at concentrations of 0.01 to 0.04 μg per mg of dry tissues, but not in the tissues of poorly differentiated type. Normal endometria at the follicular and luteal phases also contained the antigens, but the concentrations and the frequency of antigen expression were lower than those in the well- and moderately differentiated types of endometrial adenocarcinoma.

Key words: Glycosphingolipid — TLC-immunostaining — Fast atom bombardment mass spectrometry — Uterine endometrial adenocarcinoma — Lewis antigen

The plasma membrane of nearly all mammalian cells bears a glycocalyx layer, consisting of glycolipids, glycoproteins and proteoglycans, whose carbohydrate structures have been well defined in cells at various stages of proliferation and differentiation to determine the histo-blood group antigenicity and several carbohydrate-mediated functions.[1)] During the transformation of cells, alterations in the cell surface carbohydrates have been frequently observed, mainly due to the aberrant expression of glycosyl transferases, and among the transformation-associated carbohydrates, CA19-9, with a sialylated lacto-*N*-fucopentaose (sialyl Le^a) structure, has been successfully applied for the clinical diagnosis of cancer patients.[2, 3)] In addition, on the basis of the finding that sialylated or sulfated Lewis carbohydrates are involved in the ligands for selectins,[4, 5)] cancer cells expressing sialylated or sulfated Lewis structures have been shown to exhibit high dissemination and metastatic potentials through selectins, and the concentrations of these molecules in cancer cells and tissues, and in sera of patients have provided useful clues for predicting metastasis.[6, 7)] Also, the expression of Le^b, Le^Y, H and mucin core antigen-1 has been reported to be related to the grade of dysplasia and malignancy in cancer patients, and can be used to predict prognosis.[8, 9)] However, such carbohydrate structures applicable to clinical diagnosis are synthesized through multi-step reactions by several glycosyltransferases, the activities of which are influenced by availability of the respective sugar nucleotides, acceptor carbohydrate chains, and other factors including divalent cations, indicating that tumor-associated carbohydrates are dependent upon various epigenetic factors. To understand the metabolic alterations causing the expression of histo-blood group antigens in cancer cells, quantitative determination of all molecular species of carbohydrates including metabolic precursors is necessary. For this purpose, analysis of the glycosphingolipid (GSL) composition should provide useful information because GSLs with carbohydrate moieties of different chain lengths can be readily separated by means of a convenient procedure. In our previous study, human uterine

[5] To whom correspondence and reprint requests should be addressed.
E-mail: iwamori@chem.kindai.ac.jp

endometrial carcinoma-derived cells were shown to express high specific activity of β1,3-galactosyltransferase for the synthesis of lacto-*N*-tetraosyl ceramide as an important precursor of Lewis-GSL.[10] We have now extended this study to determine the entire GSL compositions in various gynecological cancer-derived cells and to compare the expression of Lewis-GSLs in tissues from patients with various grades of endometrial carcinomas.

MATERIALS AND METHODS

Materials Glycolipids were purified from various sources in our laboratory: I^3SO_3-GalCer, GM2, GD3, GM1 and GD1a from bovine brain, GlcCer, LacCer, Gb_3Cer, Gb_4Cer, GM3 and IV^3NeuAc-nLc_4Cer from human erythrocytes, Lc_4Cer, IV^3NeuAc-Lc_4Cer, Le^a (Lewis antigen-a) (III^4Fuc-Lc_4Cer) and Le^b (IV^2Fuc, III^4Fuc-Lc_4Cer) from human meconium, Le^X (III^3Fuc-nLc_4Cer) and Le^Y (IV^2Fuc, III^3Fuc-nLc_4Cer) from rectal carcinoma tissues, and II^3SO_3-LacCer and II^3SO_3-Gg_3Cer from human kidneys. Asialo GM1 and nLc_4Cer were prepared by treatment with *Arthrobacter ureafaciens* sialidase as reported previously,[11] and *N*-stearoyl derivatives of GlcCer, LacCer and Gb_3Cer were synthesized by acylation of glucosyl, lactosyl and globotetraosyl sphingosine, prepared with sphingolipid ceramide *N*-descylase (*Pseudomonas* sp. TK4),[12] with stearoyl chloride.[13]

Carbohydrate-specific antibodies Rabbit polyclonal antibodies toward GM1, asialo GM1 and asialo GM2 were generated by immunizing rabbits intradermally with an emulsion prepared with 1 mg of purified glycolipid and 0.5 mg of BSA (bovine serum albumin) in 1 ml of PBS (phosphate-buffered saline), and 1 ml of Freund's complete adjuvant (Sigma, St. Louis, MO), and by monitoring the antibody titer by means of an enzyme-linked immunosorbent assay, the titers being 1:100 000 for anti-GM1, 1:600 000 for anti-asialo GM1, and 1:300 000 for anti-asialo GM2 antibodies. No cross reaction with structurally related glycolipid derivatives of individual antigens was observed.[14] Human monoclonal anti-Lc_4Cer (HMST-1),[15] mouse monoclonal anti-Le^b plus Le^Y (MSN-1),[16] and anti-GM2 (YHD-06)[17] antibodies were established in our laboratory. Monoclonal antibodies against Le^X (NCC-LU-279) and Le^Y (NCC-ST-433 and H18A) were kindly donated by Dr. S. Hirohashi, National Cancer Center (Tokyo). Monoclonal anti-Le^X (73-30), anti-sialyl Le^a (2D3), and sialyl Le^X (KM-93) were obtained from Seikagaku Co. (Tokyo).

Cell lines derived from human gynecological cancers The cell lines used in this experiment were established from patients with the following cancers: SNG-II, HHUA and Ishikawa cells, SNG-M cells, and HEC-108 cells from the well-, moderately and poorly differentiated types of uterine endometrial adenocarcinoma, respectively, SKG-II, SKG-IIIa and TCS cells from the non-keratinizing type of uterine cervical carcinoma, and MCAS and HMKOA cells, HAC-2, HUOAC and RMG-1 cells, and HTBOA cells from a mucinous cystadenocarcinoma, clear cell carcinoma and undifferentiated carcinoma of the ovary, respectively.[18–25]

Human tissues Cancerous tissues from patients suffering from uterine endometrial adenocarcinomas and normal endometria were obtained from the Departments of Pathology, and Obstetrics and Gynecology, Keio University Hospital, and National Saitama Hospital after pathological examination, and were used according to the guidelines of the Committee for Informed Consent.

Separation and quantitation of GSLs The cultured cells were harvested with a scraper and then lyophilized. Total lipids were extracted from the lyophilized cells with chloroform/methanol/water (20:10:1, 10:20:1 and 1:1, by vol.), and then the combined extracts were fractionated into neutral and acidic lipids on a DEAE-Sephadex column (A-25, acetate form; Pharmacia, Uppsala, Sweden). The neutral GSLs were separated from unabsorbed neutral lipid fractions by acetylation, separation of the acetylated GSLs, deacetylation and desalting, whereas the acidic GSLs were prepared from the absorbed acidic lipid fractions by cleavage of the ester-containing lipids, followed by dialysis.[26] The acidic and neutral GSLs thus obtained were developed on TLC (thin-layer chromatography) plates with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, by vol.) and chloroform/methanol/water (65:35:8, by vol.), respectively, and then visualized with orcinol-H_2SO_4 reagent. The density of spots was determined at an analytical wavelength of 420 nm and a control wavelength of 710 nm using a dual-wavelength TLC densitometer (CS-9000; Shimadzu, Kyoto). Standard curves were essentially obtained by using the same GSLs as those detected in the cells: 0.1 to 1.5 μg of GalCer (18:0), LacCer (18:0), Gb_3Cer (18:0), Gb_4Cer, Gg_4Cer, Lc_4Cer, nLc_4Cer, Le^X, Le^Y, Le^b, II^3SO_3-LacCer, II^3SO_3-Gg_3Cer, GM3, IV^3NeuAc-nLc_4Cer, GM2, GD3, GM1 and GD1a, on the same plate. The concentration of lipid-bound sialic acid was also determined densitometrically after visualization of the spots with resorcinol-HCl reagent, and the density of the spots was determined at an analytical wavelength of 580 nm and a control wavelength of 710 nm.

TLC-immunostaining The neutral and acidic GSLs prepared from cultured cells and total lipid extracts from the cancerous tissues were applied on plastic-coated TLC plates, which were then developed successively with *n*-hexane/diethyl ether (4:1, by vol.) and chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, by vol.). Each plate was incubated with a blocking buffer (1% PVP (polyvinylpyrrolidone) and 1% ovalbumin in PBS) at 4°C overnight and then with anti-carbohydrate antibodies in 3% PVP in PBS at 37°C for 2 h. Mouse monoclonal antibodies were used at the concentration of approximately

0.2 μg/ml, and rabbit antisera were usually diluted to 1: 500 (by vol.). Afterward, the plates were washed 5 times with 0.1% Tween 20 in PBS, and the bound antibodies were detected using peroxidase-conjugated anti-rabbit IgG+M or anti-mouse IgG+M antibodies (Cappel Laboratories, Cochranville, PA), diluted 1:1000 (by vol.) with 3% PVP in PBS, and with enzyme substrates H_2O_2 and 4-chloro-1-naphthol, as described previously.[27] The density of spots was also determined using 10 to 100 ng of the respective GSLs as standards for quantitation with a TLC-densitometer as described above, and the limit of detection was 5 ng of GSLs.

Structural analysis and quantitation of GSLs The individual GSLs were purified using a silica gel (Iatrobeads 6RS8060; Iatron Lab., Tokyo) column, with gradient elution with chloroform/isopropyl alcohol/water (85:15:0.2 and 40:60:2, by vol.) for mono- to trihexaosyl ceramides in the neutral GSLs and for sulfatides in the acidic GSLs, followed by a gradient of chloroform/methanol/water (70:30:4 and 10:90:4, by vol.) for polar GSLs. Then, characterization and quantitation of individual GSLs were performed as follows. Ceramide monohexosides: Purified ceramide monohexosides were chromatographed on a borate-impregnated plate with chloroform-methanol-water-15 *M* ammonia (280:70:6:1, by vol.) to separate and quantitate GlcCer and GalCer. Ceramide trihexosides: Gb_3Cer closely migrated with Lc_3Cer, but was separated from Gg_3Cer on a silica gel column, and after quantitation of Gb_3Cer plus Lc_3Cer by TLC-densitometry, Lc_3Cer was removed by enzymatic hydrolysis with *N*-acetyl β-glucosamidase (bovine kidney; Sigma), followed by determination of the product, LacCer from Lc_3Cer, and the remaining Gb_3Cer as above. Gg_3Cer was determined by TLC-immunostaining with anti-Gg_3Cer antibodies. Ceramide tetrahexosides: By silica gel column chromatography, Gb_4Cer was separated from the mixture of Lc_4Cer and nLc_4Cer, in which the concentration of Lc_4Cer was determined by TLC-immunostaining with anti-Lc_4Cer antibodies, and the amount of nLc_4Cer was calculated by subtraction of the amount of Lc_4Cer from that of the mixture. On the other hand, Gg_4Cer migrated in the region of ceramide pentahexosides and was quantitated by TLC-immunostaining with anti-Gg_4Cer antibodies. Le^b and Le^Y: Since SNG-II cells, which we used for analysis of the epitope structure of monoclonal antibody MSN-1, preferentially expressed Le^b, we were able to detect Le^b alone in our previous study.[16] Our recent study, however, showed that MSN-1 reacted with Le^Y with a similar affinity to that with Le^b. Accordingly, in this experiment we utilized MSN-1 antibodies for simultaneous detection of Le^b and Le^Y, and NCC-ST-433 or H18A antibodies for Le^Y. Quantitation of Le^b and Le^Y together with MSN-1, and of Le^Y with NCC-ST-433 or H18A was performed by TLC-immunostaining with known amounts of Le^b and Le^Y as standards, and the amount of Le^b was calculated from the two values. Sulfated GSLs: The structures of II^3SO_3-LacCer and II^3SO_3-Gg_3Cer were characterized by negative ion FABMS (JMS-700TKM; JEOL Ltd., Tokyo) with triethanolamine as a matrix solvent,[28] and their amounts were determined by TLC-densitometry as described above. IV^3NeuAc-Lc_4Cer, IV^3NeuAc-nLc_4Cer and GM2: These GSLs migrated together on a TLC plate, as well as on a silica gel column, and therefore after isolation of the band containing these GSLs, sialidase (*Vibrio cholerae*; Calbiochem, San Diego, CA) treatment, which was able to remove sialic acid from IV^3NeuAc-Lc_4Cer and IV^3NeuAc-nLc_4Cer, but not from GM2, was performed to separate Lc_4Cer plus nLc_4Cer from GM2 by TLC. Then their amounts were determined by TLC-densitometry and TLC-immunostaining with known amounts of nLc_4Cer and GM2, respectively, followed by determination of Lc_4Cer in the Lc_4Cer plus nLc_4Cer-band by TLC-immunostaining with anti-Lc_4Cer antibodies. Gangliosides: GM3 and GD3 were characterized by comparison of their mobilities on a TLC plate with those of standard GSLs before and after sialidase treatment. In a similar way, GM1 and GD1a were characterized by TLC-immunostaining with anti-GM1 antibodies before and after sialidase (*V. cholerae*) treatment. Thereafter, quantitation of gangliosides was carried out by TLC-densitometry with known amounts of individual GSL as standards.

RESULTS

GSLs in human gynecological cancer-derived cell lines To determine the precise concentrations of GSLs in the cultured cells, standard curves for quantitation by TLC-densitometry and TLC-immunostaining were prepared using the same purified GSLs as those detected in the cell lines, as described under "Materials and Methods." The results are compiled in Table I. Cholesterol was present in various cell lines at concentrations of 36.7 μg (64.8 nmol) to 41.0 μg (105.9 nmol) per mg of dry weight, and total GSLs amounted to 3 to 4 μg in eight out of 14 cell lines, corresponding to approximately 4 mol% of GSLs with respect to cholesterol. Fig. 1 shows TLC chromatograms of neutral and acidic GSLs in 0.5 mg of dried cells. On chromatograms, at least 10 neutral and 7 acidic GSLs, which were separated according to the differences in the carbohydrate moieties, were detected, but the relative amounts of individual GSLs were distinct among the cells. In no case was an identical GSL composition found in two lines. The ubiquitous GSLs present at concentrations of more than 0.02 μg per mg of dry weight were GlcCer, LacCer, Gb_3Cer, Lc_3Cer, Gb_4Cer and GM3, indicating that the metabolic pathways leading to Gb_4Cer and GM3 are always active in cell lines established from various human gynecological cancers.

Table I. Glycosphingolipid Compositions in Human Gynecological Carcinoma-derived Cells

No.	Cell line	GlcCer	LacCer	Gb_3-Cer	Lc_3-Cer	Gg_3-Cer	Gb_4Cer	Gg_4-Cer	Lc_4-Cer	nLc_4-Cer	Le^x	Le^a	Le^y	Le^b	II^3SO_3-LacCer	II^3SO_3-Gg_2Cer	GM3	GM2	IV^3-Neu-Ac-Lc_4-Cer	IV^3-Neu-Ac-nLc_4-Cer	GM1	GD3	GD1a
1	HHUA	0.26±0.01	0.20±0.01	0.9	0.2	—	0.06±0.02	—	—	0.05	—	t.r.	0.02	—	—	—	0.74±0.02	t.r.	—	0.16	0.06	—	—
2	SNG-M	0.42±0.01	0.18±0.01	1.19	0.25	—	0.24±0.02	—	0.20	0.04	—	0.10	0.01	0.02	0.28±0.09	0.22±0.03	0.26±0.07	t.r.	—	0.11	0.02	—	—
3	HEC-108	1.06±0.03	0.06±0.01	1.29	0.55	—	0.20±0.01	—	0.31	0.02	—	0.12	—	—	0.22±0.07	0.06±0.01	0.60±0.06	—	0.15	0.07	—	—	—
4	SNG-II	1.68±0.05	0.26±0.01	0.18	0.11	—	0.12±0.02	—	0.15	0.05	—	0.02	—	0.03	1.94±0.06	2.04±0.05	0.34±0.05	t.r.	0.07	0.16	—	t.r.	—
5	Ishikawa	1.34±0.04	0.28±0.02	0.12	0.08	—	0.14±0.01	—	—	0.02	0.03	—	0.02	0.02	—	—	0.56±0.02	—	0.3	0.19	—	0.01	—
6	TCS	0.10±0.01	0.06±0.01	0.08	0.02	—	0.08±0.01	—	—	—	—	—	0.02	—	—	—	0.06±0.02	—	0.05	0.04	—	—	—
7	SKG-IIIa	5.65±0.02	0.12±0.02	2.17	0.75	—	3.77±0.03	t.r.	—	—	t.r.	—	t.r.	—	—	—	0.46±0.02	t.r.	—	1.25	1.29	1.16	2.34
8	SKG-II	0.06±0.01	0.04±0.01	0.2	0.06	—	0.44±0.02	0.1	—	1.40	—	—	—	—	—	—	1.30±0.06	—	—	—	—	—	—
9	MCAS	0.78±0.03	0.08±0.02	0.3	0.12	—	0.20±0.05	—	—	0.11	0.05	—	0.13	—	—	—	2.24±0.03	—	—	0.08	0.06	—	—
10	HMKOA	1.66±0.05	0.22±0.02	0.13	0.05	0.08	0.32±0.03	—	—	0.02	0.02	—	0.01	—	—	—	0.16±0.04	t.r.	—	0.22	0.02	0.02	0.08
11	HUOCA	0.84±0.03	0.62±0.02	0.35	0.11	0.07	0.64±0.03	—	—	t.r.	0.02	—	0.07	—	—	—	0.16±0.05	—	—	0.38	0.06	0.10	0.10
12	RMG-1	0.72±0.02	1.44±0.02	1.4	0.82	—	1.42±0.02	—	0.05	t.r.	0.30	0.02	0.01	0.02	—	—	0.20±0.05	—	—	0.06	0.08	—	0.10
13	HTBOA	0.60±0.02	0.28±0.01	0.31	0.03	0.07	0.34±0.03	—	—	t.r.	—	—	—	0.02	—	—	0.20±0.05	0.96	0.13	0.31	—	—	0.30
14	HAC-2	1.88±0.01	1.00±0.01	5.77	0.02	—	0.96±0.07	—	—	0.02	—	—	—	0.02	0.28±0.05	—	0.02±0.02	—	—	0.02	—	—	—

t.r., trace amount; —, not detected.
Values with standard deviation were obtained by TLC-densitometry after visualization of the spots with orcinol-H_2SO_4 reagent, and those without standard deviation were obtained by TLC-immunostaining, as described under "Materials and Methods."

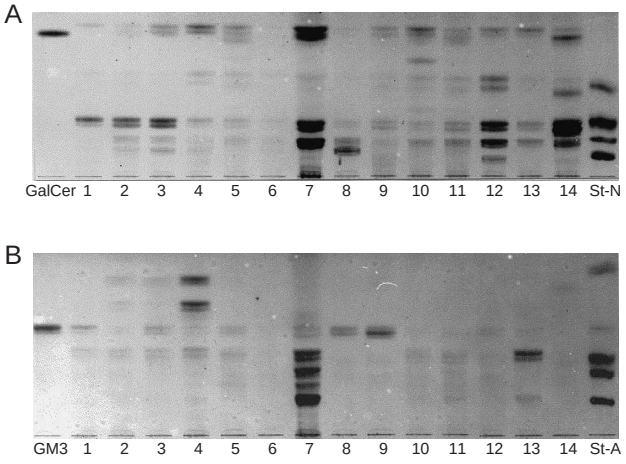

Fig. 1. TLC of neutral (A) and acidic (B) GSLs from human gynecological cancer-derived cell lines. GSLs, corresponding to 0.5 mg of dry weight, were chromatographed with chloroform/methanol/water (65:35:8, by vol.) for A and with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, by vol.) for B, and then the spots were visualized with orcinol-H_2SO_4 reagent. St-N, standard neutral GSLs, LacCer, Gb_3Cer, Gb_4Cer and Gg_4Cer from the top; St-A, standard acidic GSLs, I^3SO_3-GalCer, GM3, II^3NeuAc-nLc_4Cer, GM1 and GD1a from the top. 1, HHUA; 2, SNG-M; 3, HEC-108; 4, SNG-II; 5, Ishikawa; 6, TCS; 7, SKG-IIIa; 8, SKG-II; 9, MCAS; 10, HMKOA; 11, HUOCA; 12, RMG-1; 13, HTBOA; 14, HAC-2.

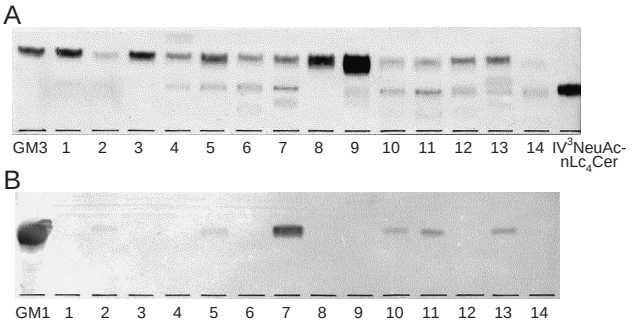

Fig. 2. TLC-immunostaining of acidic GSLs from human gynecological cancer-derived cell lines. Acidic GSLs, corresponding to 0.2 mg of dry weight, were chromatographed with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, by vol.), and then the spots were visualized by immunostaining with anti-GM3 (A), and anti≤GM1 (B) antibodies. The numbers for the cell lines are the same as in Fig. 1.

GSLs with trihexose or shorter carbohydrate chains

GlcCer was the major ceramide monohexoside and was maintained at a higher concentration than that of LacCer in all cells, probably due to the kinetic properties of β-galactosyl transferase as to the synthesis of LacCer. The subsequent metabolic steps from LacCer, involving sialyl, sulfo, α-galactosyl, β-*N*-acetylglucosaminyl and β-*N*-acetylgalactosaminyl transferases to afford GM3, II^3SO_3-LacCer, Gb_3Cer, Lc_3Cer and Gg_3Cer, respectively, determine the extension of the carbohydrate chains. Among them, Gb_3Cer, Lc_3Cer and GM3 were ubiquitous at concentrations of more than 0.02 μg per mg of dry weight, providing a sufficient supply of precursors for the globo-, lacto- and ganglio-series GSL pathways (Fig. 2). The ratios of GM3 to LacCer, indicating a gradient of GM3 synthetic potential with appropriate synthetic activity of LacCer as a substrate and consumption of GM3 for the

following ganglio-series GSLs in ovarian carcinoma-derived cells (6 out of 7 cell lines), were less than 1.0, but the endometrial and cervical carcinoma-derived cells exhibited ratios of higher than 1.0. The ability to sulfate LacCer for the synthesis of II^3SO_3-LacCer was observed in 3 endometrial carcinoma-derived cell lines and one ovarian carcinoma-derived cell line. Although the amount of II^3SO_3-LacCer was lower than that of LacCer in ovarian carcinoma-derived HAC-2 cells, the ratios of II^3SO_3-LacCer to LacCer were 2- to 7-fold in 3 endometrial-derived cell lines, all of which exhibited further synthesis of II^3SO_3-Gg_3Cer by GalNAc transferase (Fig. 1). Among the neutral trihexaosyl ceramides, Gb_3Cer and Lc_3Cer were present at concentrations of 0.02 to 5.77 μg in all cells, and the former was always at a higher concentration than the latter. Gg_3Cer was only detected in 3 ovarian carcinoma-derived cell lines.

GSLs with tetrahexose or longer carbohydrate chains In all cells, Gb_4Cer was present at concentrations of more than 0.06 μg per mg of dry weight, and extended structures of Gb_4Cer, such as *N*-acetylhexosamine-Gb_4Cer, hexose-Gb_4Cer and NeuAc-hexose-Gb_4Cer, were not detected at all on negative ion fast atom bombardment mass spectromentry (FABMS), indicating that Gb_4Cer is the terminal structure of the globo-series GSLs. However, the concentrations of Gb_4Cer, as well as the ratio of Gb_4Cer to Gb_3Cer, were different among the cells, probably due to the activity of Gb_3Cer:GalNAc-transferase, and were not correlated with the type of cancer. In contrast to the globo-series GSLs, further modification by fucosyl and sialyl transferases of Lc_4Cer and nLc_4Cer occurred in all the cell lines except SKG-II, in which nLc_4Cer was present as the terminal structure of the lacto-series GSLs at significantly high concentrations, and accordingly the concentrations of Lc_4Cer and nLc_4Cer in the other cells were less than one-tenth of that in SKG-II. Coincident with the previous finding that β1,3-Gal transferase involved in the synthesis of Lc_4Cer was frequently activated in endometrial carcinoma-derived cells,[10] SNG-II, SNG-M, HEC-108 and Ishikawa cells expressed Lc_4Cer and its sialylated derivatives (Fig. 3). Although IV^3NeuAc-Lc_4Cer was detected in 5 cell lines, IV^3NeuAc-nLc_4Cer was detected in 13 cell lines other than SKG-II. Also, the rate of sialylation of nLc_4Cer was higher than that of Lc_4Cer, when the ratios of sialylated derivatives to Lc_4Cer and nLc_4Cer were compared. On the other hand, expression of the ganglio-series GSLs, Gg_4Cer, GM1 and GD1a, was rare in human gynecological cancer-derived cells.

Expression of Lewis-phenotypes in human gynecological cancer-derived cells As shown in Fig. 1 and Table I, GSLs with longer carbohydrate chains, which were present at concentrations of more than 0.02 μg per mg of dry weight, comprised lacto-series GSLs, which were modified with fucose to confer the Lewis-phenotypes. Since the synthetic potential for Lc_4Cer was highly enhanced in the endometrial carcinoma-derived cells, as described above, Lc_4Cer-based Le^a or Le^b was detected in

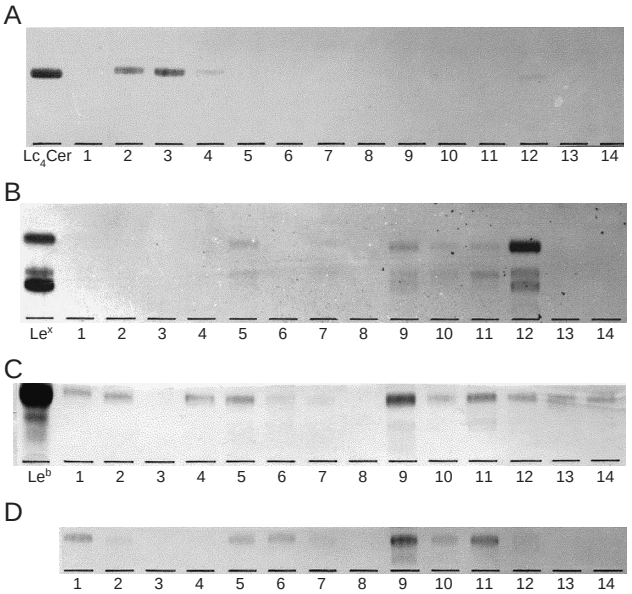

Fig. 3. TLC-immunostaining of neutral GSLs from human gynecological cancer-derived cell lines. Neutral GSLs, corresponding to 0.2 mg of dry weight, were chromatographed with chloroform/methanol/water (65:35:8, by vol.), and then the spots were visualized by immunostaining with anti-Lc_4Cer (A), anti-Le^X (B), anti-Le^b+Le^Y (C), and anti-Le^Y (D) antibodies. The numbers for the cell lines are the same as in Fig. 1.

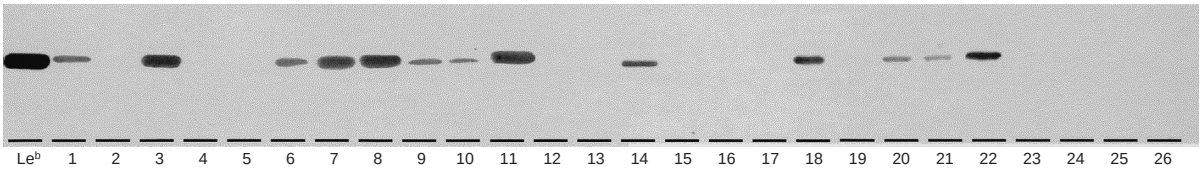

Fig. 4. TLC-immunostaining with monoclonal antibody MSN-1 of lipid extracts of tissues of patients suffering from gynecological carcinomas. Numbers under the TLC plate correspond to the numbers of cancer patients in Table II.

Table II. Concentrations of Le^b plus Le^Y in Human Normal Endometria and Cancerous Tissues, as Detected with Monoclonal Antibody MSN-1

Tissue		Patient No.	Le^b plus Le^Y (μg/mg of dry weight)
Normal endometria	Follicular phase	N−1	0.01
		N−2	—
		N−3	t.r.
		N−4	—
		N−5	0.01
	Luteal phase	N−6	—
		N−7	t.r.
		N−8	—
		N−9	0.01
		N−10	0.01
		N−11	—
		N−12	—
		N−13	—
Endometrial adenocarcinoma	Well-differentiated	3	0.04
		5	—
		7	0.02
		8	0.03
		11	0.03
		14	0.01
		21	0.01
		22	0.04
		26	—
	Moderately differentiated	9	0.01
		10	0.01
		15	—
		18	0.02
	Poorly differentiated	2	—
		13	—
		16	—
		23	—
		24	—
		25	—
	Unknown	1	0.01
		4	t.r.
		17	—
		19	—
Endometrial carcinoma (clear cell)		20	0.01
Cervical carcinoma		6	0.01
Ovarian cancer		12	—

t.r., trace amount; —, not detected.

4 endometrial cell lines other than HHUA, whose Lewis-phenotype was preferentially on the nLc_4Cer backbone to express Le^Y. Only HEC-108 cells established from the poorly differentiated type of endometrial adenocarcinoma expressed neither Le^b nor Le^Y. Both of these phenotypes based on Lc_4Cer and nLc_4Cer were detected by monoclonal antibody MSN-1. On the other hand, the Lewis-phenotypes in cervical and ovarian carcinoma-derived cells were mainly on nLc_4Cer. In the case of SKG-II cells, Lewis-GSLs were scarcely detected, but a compensatory increase in nLc_4Cer was observed, probably due to the decreased activities of α1,3-fucosyltransferase, for Le^X, and α2,3-sialyltransferase, for IV^3NeuAc-nLc_4Cer. Thus, expression of Lewis-phenotypes largely depended on the synthesis of either Lc_4Cer or nLc_4Cer. In summary, $III^4\alpha$Fuc, $IV^2\alpha$Fuc-Lc_4Cer (Le^b), and $III^3\alpha$Fuc, $IV^2\alpha$Fuc-nLc_4Cer (Le^Y) were detected in 11 out of 14 cell lines at concentrations of 0.01–0.13 μg per mg of dry weight, and comprised 0.20–3.92% of the total GSLs. Also, monoclonal antibody MSN-1 was found to be a useful probe for the simultaneous detection of Le^b and Le^Y.

Expression of Le^b- and Le^Y-GSLs in the tissues of gynecological cancers By use of monoclonal antibody MSN-1, expression of Le^b and Le^Y in tissues from the patients was examined, as shown in Fig. 4, and antigens were found to be detected sufficiently with 0.2 mg dry weight. Antigens were present in normal endometria at the follicular and luteal phases and in the well- and moderately differentiated types of endometrial carcinoma, but not in the poorly differentiated type (Table II). The amounts of Le^b and Le^Y in the well-differentiated type (0.01–0.04 μg/mg of dry weight) were higher than those in the moderately differentiated type (0.01–0.02 μg/mg of dry weight) and normal endometria (less than 0.01 μg/mg of dry weight). Also, the frequency of antigen expression in the well- and moderately differentiated types of endometrial adenocarcinoma was higher than that in the normal endometria tested.

DISCUSSION

Transformation-associated carbohydrate antigens provide a useful means of diagnosis and prediction of the prognosis for patients suffering from cancers. However, their expression on cancerous cells and tissues is not always observed, and their intensity, as detected with specific monoclonal antibodies, is variable in the tissues of individual patients with the same type of cancer and in cell lines during the subculture process.[29] These phenomena are thought to be mainly due to the multi-step metabolism required for the synthesis of carbohydrate antigens, which are composed of plural carbohydrates in both lipid- and protein-conjugated forms. In fact, although all cell lines used in this experiment contained globo-series GSLs up to Gb_4Cer, the relative concentrations of GlcCer, LacCer, Gb_3Cer and Gb_4Cer were entirely different among the cell lines, indicating that the metabolic flow determining the expression of GSL-bearing epitopes is flexibly regulated

and can change rapidly. For example, the ratio of Gb_3Cer to Gb_4Cer, the substrate to product ratio for GalNAc transferase at the final step of Gb_4Cer synthesis, was not identical among the cells, as shown in Table I. Six cell lines exhibited values higher than 1.00, and the other 8 cell lines gave values lower than 1.00, probably due to not only differences in the expression of the GalNAc transferase gene and its enzymatic activity, but also the metabolic equilibrium throughout the synthetic pathways for the globo- and all other series GSLs, because LacCer is also utilized as a common precursor for lacto- and ganglio-series GSLs. In the case of the lacto-series GSLs, Lc_4Cer and nLc_4Cer were further converted to sialylated and fucosylated derivatives, and accordingly the concentrations of GSLs in the metabolic route seem to be regulated in complex ways. Actually, the amounts of GSLs belonging to the lacto-series were clearly different among the cell lines, as shown in Table I. Some cell lines contained sialylated and fucosylated nLc_4Cer-derivatives without precursor nLc_4Cer, suggesting metabolic potential for the complete conversion of nLc_4Cer to derivatives. In the case of SKG-II cells, which did not contain fucosylated or sialylated nLc_4Cer, precursor nLc_4Cer was found to be accumulated at a significantly high concentration, and this supports the idea that modification of nLc_4Cer is a major metabolic pathway in human gynecological carcinoma-derived cells. As for Le^Y and Le^b, they have been proven to be colorectal carcinoma-associated phenotypes useful as markers for diagnosis,[30–33] and to be synthesized by α1,2-fucosyltransferase from Le^X and Le^a, respectively.[34] Considering the enzymatic properties of α1,2-fucosyltransferase, which catalyzes the fucosylation of Le^X and Le^a together, if cancer cells possess the activity, the expression of either Le^Y- or Le^b-phenotypes should be determined by the supply of the precursor structure, Le^X or Le^a. Therefore, the best way to detect the transformation-associated alterations in the Lewis-phenotypes would be the simultaneous detection of Le^Y and Le^b, which could be performed by use of monoclonal antibody MSN-1. As shown in this paper, the MSN-1 antibody was successfully applied to discriminate Lewis-positive cells from negative ones, a distinction that is related to the differentiation types of endometrial adenocarcinomas. HHUA and SNG-II cells preferentially expressed Le^Y and Le^b, respectively, SNG-M and Ishikawa cells had both structures, and neither structure was expressed in HEC-108 cells, indicating that either Le^Y or Le^b is expressed in well- and moderately differentiated types of endometrial adenocarcinoma. The results obtained for the cell lines were similar to those for the cancerous tissues, either Le^Y or Le^b being expressed in the tissues of the well- and moderately differentiated types, but not in the poorly differentiated type. The mode of antigen expression in the cancerous tissues was also in accord with the previous observations using immunohistochemical and flow cytometric procedures with the MSN-1 antibody, whose reactivity was significantly higher in the well-differentiated type than in the poorly differentiated type.[35] From the findings that Lewis-GSLs in endometrial adenocarcinoma-derived cells comprised 0.7 to 3.4% of the total GSLs, and their concentrations were maintained despite the additional sulfating pathway from LacCer to give sulfated GSLs amounting to 7 to 53% of the total GSLs, the synthetic potential for Le^Y or Le^b was judged to be mainly governed by the supply of the precursor GSLs, resulting in the higher expression of Lewis-phenotypes in the well-differentiated types. Thus, to assess the metabolic background of the expression of tumor-associated Lewis-phenotypes in relation to clinical diagnosis, immunohistochemical detection of antigens, together with analyses of the genes for α1,3/4- and α1,2-fucosyltransferases are essential, and the MSN-1 antibody should provide a powerful tool for probing endometrial adenocarcinoma-associated antigens.

(Received June 20, 2002/Revised July 30, 2002/Accepted August 2, 2002)

REFERENCES

1) Roseman, S. Reflections on glycobiology. *J. Biol. Chem.*, **276**, 41527–41542 (2001).
2) Koprowski, H., Herlyn, M., Steplewski, Z. and Sears, H. F. Specific antigen in serum of patients with colon carcinoma. *Science*, **212**, 53–55 (1981).
3) Magnani, J. L., Nilsson, B., Brockhaus, M., Zopf, D., Steplewski, Z., Koprowski, H. and Ginsburg, V. A monoclonal antibody-defined antigen associated with gastrointestinal cancer is a ganglioside containing sialylated lacto-N-fucopentaose II. *J. Biol. Chem.*, **257**, 14365–14369 (1982).
4) Bevilacqua, M. P., Stengelin, S., Gimbrone, M. A., Jr. and Seed, B. Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. *Science*, **243**, 1160–1165 (1989).
5) Ohmori, K., Kanda, K., Mitsuoka, C., Kanamori, A., Kurata-Miura, K., Sasaki, K., Nishi, T., Tamatani, T. and Kannagi, R. P- and E-selectins recognize sialyl 6-sulfo Lewis X, the recently identified L-selectin ligand. *Biochem. Biophys. Res. Commun.*, **278**, 90–96 (2000).
6) Liu, F., Qi, H. L., Zhang, Y., Zhang, X. Y. and Chen, H. L. Transfection of the c-erbB2/neu gene upregulates the expression of sialyl Lewis X, α1,3-fucosyltransferase VII, and metastatic potential in a human hepatocarcinoma cell line. *Eur. J. Biochem.*, **268**, 3501–3512 (2001).
7) Aubert, M., Panicot, L., Crotte, C., Gibier, P., Lombardo,

D., Sadoulet, M. O. and Mas, E. Restoration of α(1,2) fucosyltransferase activity decreases adhesive and metastatic properties of human pancreatic cancer cells. *Cancer Res.*, **60**, 1449–1456 (2000).
8) Goupille, C., Marionneau, S., Bureau, V., Hallouin, F., Meichenin, M., Rocher, J. and Le Pendu, J. α1,2-Fucosyltransferase increases resistance to apoptosis of rat colon carcinoma cells. *Glycobiology*, **10**, 375–382 (2000).
9) Baldus, S. E., Hanisch, F. G., Putz, C., Flucke, U., Monig, S. P., Schneider, P. M., Thiele, J., Holscher, A. H. and Dienes, H. P. Immunoreactivity of Lewis blood group and mucin peptide core antigens: correlations with grade of dysplasia and malignant transformation in the colorectal adenoma-carcinoma sequence. *Histol. Histopathol.*, **17**, 191–198 (2002).
10) Yoshiki, J., Kubushiro, K., Tsukazaki, K., Udagawa, Y., Nozawa, S. and Iwamori, M. High expression of uridine diphosphate-galactose: Lc_3Cer β1-3 galactosyltransferase in human uterine endometrial cancer-derived cells as measured by enzyme-linked immunosorbent assay and thin-layer chromatography-immunostaining. *Jpn. J. Cancer Res.*, **88**, 669–677 (1997).
11) Iwamori, M., Ohta, Y., Uchida, Y. and Tsukada, Y. *Arthrobacter ureafaciens* sialidase isoenzymes, L, M1 and M2, cleave fucosyl GM1. *Glycoconj. J.*, **14**, 67–73 (1997).
12) Suzuki, H., Ito, M., Kimura, T., Iwamori, Y. and Iwamori, M. Inhibitory activity of sulphoglycolipid derivatives towards pancreatic trypsin. *Glycoconj. J.*, **17**, 787–793 (2000).
13) Kopaczyk, K. C. and Radin, N. S. *In vivo* conversions of cerebroside and ceramide in rat brain. *J. Lipid Res.*, **6**, 140–145 (1965).
14) Iwamori, M., Shimomura, J., Tsuyuhara, S., Mogi, M., Ishizaki, S. and Nagai, Y. Differential reactivities of fucosyl GM1 and GM1 gangliosides on rat erythrocyte membrane revealed. *J. Biochem. (Tokyo)*, **94**, 1–10 (1983).
15) Nozawa, S., Narisawa, S., Kojima, K., Sakayori, M., Iizuka, R., Mochizuki, H., Yamauchi, T., Iwamori, M. and Nagai Y. Human monoclonal antibody (HMST-1) against lactoseries type 1 chain and expression of the chain in uterine endometrial cancers. *Cancer Res.*, **49**, 6401–6406 (1989).
16) Iwamori, M., Sakayori, M., Nozawa, S., Yamamoto, T., Yago, M., Noguchi, M. and Nagai, Y. Monoclonal antibody-defined antigen of human uterine endometrial carcinomas is Le^b. *J. Biochem.*, **105**, 718–722 (1989).
17) Horikawa, K., Yamasaki, M., Iwamori, M., Nakakuma, H., Takatsuki, K. and Nagai, Y. Concanavalin A-stimulated expression of gangliosides with GalNAc β1-4(NeuAc α2-3)Galbeta structure in murine thymocytes. *Glycoconj. J.*, **8**, 354–360 (1991).
18) Morisawa, T. The results of primary culture of endometrial adenocarcinoma and characterization of its established cell line. *J. Jpn. Soc. Clin. Cytol.*, **26**, 433–442 (1987).
19) Ishiwata, I., Nozawa, S., Inoue, T. and Okumura, H. Development and characterization of established cell lines from primary and metastatic regions of human endometrial adenocarcinoma. *Cancer Res.*, **37**, 1777–1785 (1977).
20) Nozawa, S., Sakayori, M., Ohta, K., Iizuka, R., Mochizuki, H., Soma, M., Fujimoto, J., Hata, J., Iwamori, M. and Nagai, Y. A monoclonal antibody (MSN-1) against a newly established uterine endometrial cancer cell line (SNG-II) and its application to immunohistochemistry and flow cytometry. *Am. J. Obstet. Gynecol.*, **161**, 1079–1086 (1989).
21) Ishiwata, I., Ishiwata, C., Soma, M., Arai, J. and Ishikawa, H. Establishment of human endometrial adenocarcinoma cell line containing estradiol-17β and progesterone receptors. *Gynecol. Oncol.*, **17**, 281–290 (1984).
22) Nishida, M., Kasahara, K., Kaneko, M., Iwasaki, H. and Hayashi, K. Establishment of a new human endometrial adenocarcinoma cell line, Ishikawa cells, containing estrogen and progesterone receptors. *Jpn. J. Obstet. Gynecol.*, **37**, 1103–1111 (1985).
23) Ishiwata, I., Nozawa, S., Kiguchi, K., Kurihara, S. and Okumura, H. Establishment of human uterine cervical cancer cell line and comparative studies between normal and malignant uterine cervical cells *in vitro*. *Acta Obstet. Gynecol. Jpn.*, **30**, 731–738 (1978).
24) Nozawa, S., Udagawa, Y., Ohta, H., Kurihara, S. and Fishman, W. H. Newly established uterine cervical cancer cell line (SKG-III) with Regan isoenzyme, human chorionic gonadotropin β-subunit, and pregnancy-specific β1-glycoprotein phenotypes. *Cancer Res.*, **43**, 1748–1760 (1983).
25) Ishiwata, I., Ishiwata, C., Soma, M., Akagi, H., Hiyama, H., Nakaguchi, T., Ono, I., Hashimoto, H. and Ishikawa, H. Presence of human papillomavirus genome in human tumor cell lines and cultured cells. *Anal. Quant. Cytol. Histol.*, **13**, 363–370 (1991).
26) Iwamori, M., Sawada, K., Hara, Y., Nishio, M., Fujisawa, T., Imura, H. and Nagai, Y. Neutral glycosphingolipids and gangliosides of bovine thyroid. *J. Biochem. (Tokyo)*, **91**, 1875–1887 (1982).
27) Lin, B., Hayashi, Y., Saito, M., Sakakibara, Y., Yanagisawa, M. and Iwamori, M. GDP-fucose β-galactoside α1,2-fucosyltransferase, MFUT-II, and not MFUT-I or -III, is induced in a restricted region of the digestive tract of germ-free mice by host-microbe interactions and cycloheximide. *Biochim. Biophys. Acta*, **1487**, 275–285 (2000).
28) Iwamori, M., Kiguchi, K., Kanno, J., Kitagawa, M. and Nagai, Y. Gangliosides as markers of cortisone-sensitive and cortisone-resistant rabbit thymocytes: characterization of thymus-specific gangliosides and preferential changes of particular gangliosides in the thymus of cortisone-treated rabbits. *Biochemistry*, **25**, 889–896 (1986).
29) Kubushiro, K., Tsukazaki, K., Tanaka, J., Takamatsu, K., Kiguchi, K., Mikami, M., Nozawa, S., Nagai, Y. and Iwamori, M. Human uterine endometrial adenocarcinoma: characteristic acquirement of synthetic potentials for II^3SO_3-LacCer and ganglio series sulfoglycosphingolipids after transfer of the cancer cells to culture. *Cancer Res.*, **52**, 803–809 (1992).

30) Schoentag, R., Primus, F. J. and Kuhns, W. ABH and Lewis blood group expression in colorectal carcinoma. *Cancer Res.*, **47**, 1695–1700 (1987).
31) Kim, Y. S., Yuan, M., Itzkowitz, S. H., Sun, Q. B., Kaizu, T., Palekar, A., Trump, B. F. and Hakomori, S. Expression of Le^Y and extended Le^Y blood group-related antigens in human malignant, premalignant, and nonmalignant colonic tissues. *Cancer Res.*, **46**, 5985–5992 (1986).
32) Langkilde, N. C., Wolf, H. and Orntoft, T. F. Lewis antigen expression in benign and malignant tissues from RBC Le(a-b-) cancer patients. *Br. J. Haematol.*, **79**, 493–499 (1991).
33) Baldus, S. E., Hanisch, F. G., Putz, C., Flucke, U., Monig, S. P., Schneider, P. M., Thiele, J., Holscher, A. H. and Dienes, H. P. Immunoreactivity of Lewis blood group and mucin peptide core antigens: correlations with grade of dysplasia and malignant transformation in the colorectal adenoma-carcinoma sequence. *Histol. Histopathol.*, **17**, 191–198 (2002).
34) Yazawa, S., Nakamura, J., Asao, T., Nagamachi, Y., Sagi, M., Matta, K. L., Tachikawa, T. and Akamatsu, M. Aberrant $\alpha 1 \rightarrow 2$ fucosyltransferases found in human colorectal carcinoma involved in the accumulation of Le^b and Y antigens in colorectal tumors. *Jpn. J. Cancer Res.*, **84**, 989–995 (1993).
35) Nozawa, S., Sakayori, M., Ohta, K., Iizuka, R., Mochizuki, H., Soma, M., Fujimoto, J., Hata, J., Iwamori, M. and Nagai, Y. A monoclonal antibody (MSN-1) against a newly established uterine endometrial cancer cell line (SNG-II) and its application to immunohistochemistry and flow cytometry. *Am. J. Obstet. Gynecol.*, **161**, 1079–1086 (1989).