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Subcellular Localization of Galactosyltransferase Associated with Tumors in Endometrial and Ovarian Cancer Cells

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Frozen or resin sections of normal human endometrium, endometrial adenocarcinoma, and ovarian adenocarcinoma were examined with two monoclonal antibodies for galactosyltransferase associated with tumor (GAT) by immunofluorescence and immunoelectron microscopy. One antibody (MAb8628) stained the *trans*-cisternae of the Golgi apparatus, the *trans*-Golgi network, and the intracytoplasmic vesicles more intensely in cancer cells than in normal endometrial glandular cells. The other antibody (MAb8513) stained intracytoplasmic vesicles more intensely in cancer cells than in normal cells. MAb8513 also showed moderate staining of the *trans*-cisternae of the Golgi apparatus and *trans*-Golgi network in cancer cells, but only faint staining of these structures in normal glandular cells, indicating that GAT was overexpressed in the cancer cells. An immunofluorescence study using serial semithin cryosections (1 μm) demonstrated that the staining pattern of each antibody was different inside a single cancer cell, conforming to the two patterns mentioned above. The reason for this difference in staining remains unclear. GAT is a soluble form of β1,4-galactosyltransferase, so a difference in the cleavage site of the membrane-bound peptide may cause changes in immunoreactivity after its conversion to the soluble form (i.e., GAT) in endometrial or ovarian adenocarcinoma cells.

Key words: galactosyltransferase associated with tumor, β1,4-galactosyltransferase, immunoelectron microscopy, endometrial cancer, ovarian cancer

I. Introduction

Recently, changes in the carbohydrate structures of various glycoconjugates constituting the plasma membrane or other intracellular membranes have been demonstrated to occur in association with malignant transformation of cells [8, 18, 41]. N-acetylglucosaminide β1,4-galactosyltransferase (GalT, EC 2. 4. 1. 22) is one of the glycosyltransferases that participate in the elongation of carbohydrate chains [21]. GalT has been investigated due to the ease of its purification by affinity column chromatography using α-lactoalbumin from body fluids such as serum and milk, which contain high levels of a soluble form of GalT [9]. Some studies have identified different biochemical characteristics of GalT from malignant tumor cells and normal cells [29, 30], but it is not clear whether the localization of various forms of GalT is similar or not in malignant cells and normal cells [1, 5, 11, 17, 20, 23, 32, 39, 40].

There have been several reports about the elevation of serum GalT levels in patients with gynecologic malignancies, especially ovarian cancer [24, 26–28, 36]. In 1992, Uemura *et al.* [37, 38] prepared and characterized several mouse monoclonal antibodies (MAbs) against human GalT purified from ovarian tumor fluid, and they identified several oligomeric bands as well as a monomeric band by non-denaturing polyacrylamide gel electrophoresis. Since the GalT polymer corresponding to these oligomeric bands showed very low levels in GalT from the serum of healthy

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individuals, it was designated as "galactosyltransferase associated with tumor (GAT)". A new two-antibody sandwich enzyme immunoassay kit for GAT was subsequently developed using MAb8628 and MAb8513, taking advantage of the fact that MAb8628 reacts with both GalT polymer and GalT monomer, while MAb8513 only reacts with GalT polymer 25, 26, 38]. Using this assay, we found that the serum GAT level was significantly elevated in patients with malignancy compared with normal controls 26, 27]. Our findings also suggested that GAT may be a promising marker for gynecologic tumors, especially ovarian cancers, due to its high specificity. In the present study, we used immunofluorescence and immunoelectron microscopy to determine the subcellular localization of the antigens recognized by MAb8628 and MAb8513, in order to investigate the reason for the increased serum GAT level in patients with ovarian and endometrial cancer.

II. Materials and Methods

Monoclonal antibodies

Several different mouse MAbs directed against GAT were prepared by immunization of mice with β1-4 galactosyltransferase (GalT) purified from human ovarian tumor fluid by Uemura *et al.* 37, 38]. We used two of these Mabs (MAb8628 and MAb8513), which were reported to recognize epitopes on the GalT protein and not on the carbohydrate moiety, since these Mabs showed immunoreactivity with recombinant GalT expressed in *Escherichia coli.* MAb8628 and MAb8513 were IgG_1 and IgM antibodies, respectively.

Tissue preparation

Immunofluorescence staining was performed on 11 specimens of endometrial adenocarcinoma (9 well-differentiated, 1 moderately differentiated, and 1 poorly differentiated adenocarcinoma), 12 specimens of normal endometrium (6 proliferative and 6 secretory), and 4 specimens of ovarian carcinoma (1 serous cystadenocarcinoma, 1 mucinous cystadenocarcinoma, 1 endometrioid adenocarcinoma, and 1 clear cell adenocarcinoma). The specimens of normal endometrium were obtained by curettage. The endometrial adenocarcinoma specimens were obtained during surgery in patients with stage I or II cancer according to the classification of the International Federation of Gynecology and Obstetrics (FIGO) 1988 13]. Ovarian carcinoma specimens were obtained at operation in patients who ranged from stage I to III according to the FIGO classification (1987) 12].

Electron microscopic immunostaining was performed on 21 specimens obtained from 7 patients with endometrial adenocarcinomas (6 well-differentiated, and 1 moderately differentiated), 12 specimens obtained from 4 subjects with normal endometrium (2 in the proliferative phase and 2 in the secretory phase), and 18 specimens obtained from 3 patients with ovarian carcinomas (1 serous adenocarcinoma, 1 mucinous adenocarcinoma, and 1 endometrioid adenocarcinoma). Specimens were obtained after each patient gave informed consent, and permission was granted by the Ethics Committee of School of Medicine, Keio University.

Immunostaining with MAb8628 and MAb8513

Specimens were fixed with 4% formaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4) for 6 hr at 4°C. After being washed with 0.01 M sodium phosphate-buffered saline (PBS; 0.14 M NaCl, pH 7.2), the specimens were sequentially immersed in 10%, 15%, and 20% sucrose in PBS for 20 min each. Then the specimens were embedded in O.C.T. compound (Miles Scientific, Naperville, IL, U.S.A.) and immersed in liquid nitrogen. Cryostat sections (6 μm in thickness) were prepared, placed onto glass slides that had been pretreated with Neopren 16] (Nisshin EM Co. Ltd., Tokyo, Japan), and immediately transferred into PBS without air-drying for the immunostaining procedures.

Some specimens were immersed in 2.3 M sucrose in PBS for 6 hr after fixation and washing in PBS. Then these specimens were trimmed to 1–2 mm in size, attached to the ultramicrotome holder, and immediately immersed in liquid nitrogen. Semithin sections (1 μm) were cut with a Porter-Blum MT-2B ultramicrotome (Ivan Sorvall Inc., Newton, CT, U.S.A.) and a cryosection coldtome CM-41 (Sakura Ltd., Tokyo, Japan), and were placed on ethanol hydrochloride pretreated glass slides.

These tissue sections were stained by the indirect immunofluorescence method. The sections were first blocked with 5% normal rabbit serum (Dako Japan Co. Ltd., Kyoto, Japan) in PBS for 10 min and then incubated with MAb8628 (35 mg/ml) or MAb8513 (32 mg/ml) for 2 hr at 4°C. After being washed with PBS, the sections were incubated for 1 hr at 4°C with biotinylated rabbit anti-mouse IgG and IgM (Jackson, Baltimore, MD, U.S.A.) at a concentration of 12 μg/ml. Then the sections were washed with PBS and incubated with Texas Red-labeled streptavidin (Amersham, U.K; 1:80 dilution) for 30 min at room temperature. In some cases, 0.5 μg/ml of 4',6-diamidino-2-phenylindole (DAPI; Boehringer-Mannheim, Germany) was used for counterstaining 34]. The tissue sections were washed in PBS, mounted in 90% glycerol-0.1 M Tris-HCl buffer (pH 8.5) containing 0.5 mM *p*-phenylenediamine 14], and examined under a Nikon Microphot-FX microscope equipped with epifluorescence. Photomicrographs were taken with Fuji Fujichrome 400D film.

Electron microscopy with MAb8628 or MAb8513

Endometrial and ovarian specimens were fixed with 4% formaldehyde and 0.1–0.5% glutaraldehyde in 0.1 M PB (pH 7.4) for 4 hr at 4°C. Several endometrial adenocarcinoma and ovarian carcinoma specimens were fixed with 2.5% glutaraldehyde in 0.1 M PB (pH 7.4) for 4 hr at 4°C to examine the ultrastructural features.

After being washed with PBS, some of the fixed blocks of tissue were immersed sequentially in 10%, 15%, and 20%

sucrose-PBS (4 hr at each concentration) and then frozen in liquid nitrogen for pre-embedding staining. Sections that were 10 μm thick were attached to glass slides pretreated with Neopren. The sections were first treated with 0.3% H_2O_2 for 30 min to quench endogenous peroxidase activity and then were blocked with 5% normal goat serum in PBS for 10 min. Next, the sections were immersed in 1% BSA-PBS containing saponin (ICN Pharma. Inc., Cleveland, OH, U.S.A.) at a concentration of 0.5 mg/ml for 30 min, and incubated with MAb8628 at a concentration of 10 μg/ml in 1% BSA-PBS containing 0.5 mg/ml saponin for 14 hr at 4°C. After washing with PBS, the sections were incubated with horseradish peroxidase (HRP)-conjugated goat $F(ab')_2$ anti-mouse IgG and IgM (Bio Source International Inc., Camarillo, CA, U.S.A.) at a concentration of 100 mg/ml in 1% BSA-PBS containing saponin for 10 hr at 4°C. Subsequently, the sections were rinsed with PBS and fixed in 1.0% glutaraldehyde in 0.1 M PB for 10 min to better preserve the ultrastructure and immobilize the HRP-conjugates. Then the sections were rinsed in PBS, immersed in 3,3' diaminobenzidine (0.2 mg/ml)-H_2O_2 (0.005%) for 10 min, rinsed with distilled water, and osmicated for 1 hr. After this, the sections were dehydrated by passage through a graded ethanol series and embedded in Epon 812 on glass slides. Finally, ultrathin sections were cut, stained with lead citrate, and examined with a JEM-100CX transmission electron microscope (JEOL, Tokyo, Japan) at 80 kV.

For post-embedding staining, some fixed tissue blocks were rinsed with PBS, dehydrated with N,-N,dimethylformamide, and embedded in Lowicryl K4M (Chemische Werke Lowi, Germany) that was subsequently polymerized by UV irradiation (380 nm) at 4°C for 1 hr according to the rapid embedding method 2]. Ultrathin sections were cut, mounted on nickel grids, and floated on a drop of 5% normal rabbit serum in PBS for 10 min. Next, the sections were sequentially incubated on droplets of MAb8628 or MAb8513 at a concentration of 9 or 8 μg/ml, respectively, in 1% BSA-PBS at room temperature for 1 hr, and then in PBS for 15 min. This was followed by incubation with biotinylated rabbit anti-mouse IgG and IgM at a concentration of 12 μg/ml in 1% BSA-PBS at room temperature for 30 min. After washing with PBS for 15 min, the grids were incubated in 1% BSA-PBS for 30 min at room temperature with streptavidin-colloidal gold in 0.1% BSA-PB glycerol (the gold particles were 15 nm in diameter and were prepared in our laboratory as described previously 15]). Finally, the sections were washed briefly with PBS and distilled water, stained with lead citrate and uranyl acetate, and examined under a transmission electron microscope.

For the ultrastructural examination of endometrial adenocarcinoma and ovarian carcinoma, specimens were fixed with 2.5% glutaraldehyde, embedded in Epon 812, sectioned, and observed under a transmission electron microscope.

Control experiments for immunocytochemistry

Specimens were incubated with normal mouse IgG or IgM (ICN ImmunoBiologicals, Lisle, France), or with 0.1% BSA-PBS alone instead of the primary antibody, and then were subjected to immunofluorescence microscopy and/or immunoelectron microscopy. Nonspecific staining was verified by incubating the sections with Texas Red-labeled streptavidin alone for immunofluorescence staining and with streptavidin-colloidal gold alone during post-embedding staining for immunoelectron microscopy.

III. Results

Light microscopic findings

All 12 specimens of normal endometrium were weakly positive by the immunofluorescent staining with MAb8628, while all 11 specimens of endometrial adenocarcinoma and all 4 specimens of ovarian carcinoma were strongly positive. The MAb8628 binding sites did not differ between normal and malignant endometrial or ovarian glandular cells. Both the normal and malignant glandular cells were positive in the apical supranuclear area, although only the malignant glandular cells sometimes showed positive granular spots in this area. The plasma membrane was essentially negative for staining. Using semithin cryosections, the supranuclear staining was observed to be "worm-like" and was more sharply outlined (Fig. 1).

When immunofluorescence staining was done with MAb8513, only 2 specimens of normal endometrium were weakly positive, whereas all 11 specimens of endometrial adenocarcinoma and all 4 specimens of ovarian carcinoma were strongly positive. The staining pattern of MAb8513 was quite different between normal and malignant endometrial glandular cells. Only a few of the normal endometrial glandular cells showed a weak positive reaction of small granular spots in the cytoplasm at a high magnification (×1,000, Fig. 2a). In contrast, the malignant glandular cells (including ovarian adenocarcinoma cells) showed a strong positive granular reaction in the cytoplasm on 6 μm-sections, and these granules were better defined on semithin cryosections (Fig. 2b). There were 1 to 5 granules in the cytoplasm of the malignant cells, whereas 0 to 1 granules were observed in the normal glandular cells. The location of the granules was often in the supranuclear region of the cytoplasm, but sometimes granules were observed in the basal or lateral areas of the nuclei in malignant glandular cells. The supranuclear U-shaped or worm-like positive areas detected with MAb8628 (Fig. 1) were not found with MAb8513, but the plasma membrane was negative, as was the case with the other antibody.

When immunofluorescence staining with MAb8628 and MAb8513 was done using serial semithin cryosections from the same block of ovarian endometrioid adenocarcinoma, the intracytoplasmic distribution of antigens for the two antibodies was markedly different. MAb8628 stained the supranuclear area in a worm-like or U-shaped pattern (Fig. 3a), whereas MAb8513 stained granular spots in the supranuclear area and sometimes in the subnuclear region (Fig. 3b).

Fig. 1. Localization by Texas Red of antigens reactive with MAb8628 in semithin cryosections (1 μm) of endometrial tissue on immunofluorescence microscopy. Nuclei were counterstained with DAPI. **a:** Glandular cells from normal proliferative endometrium show a positive reaction with a U-shaped pattern (arrowheads in "**a**") in the apical supranuclear area. **b:** Cancer cells show a stronger positive reaction with a worm-like pattern (arrowhead in "**b**") in the apical supranuclear area in a specimen of well-differentiated endometrial adenocarcinoma. The plasma membrane is negative. Original magnification: ×1,000 (**a**), ×1,000 (**b**). Bar=5 μm.

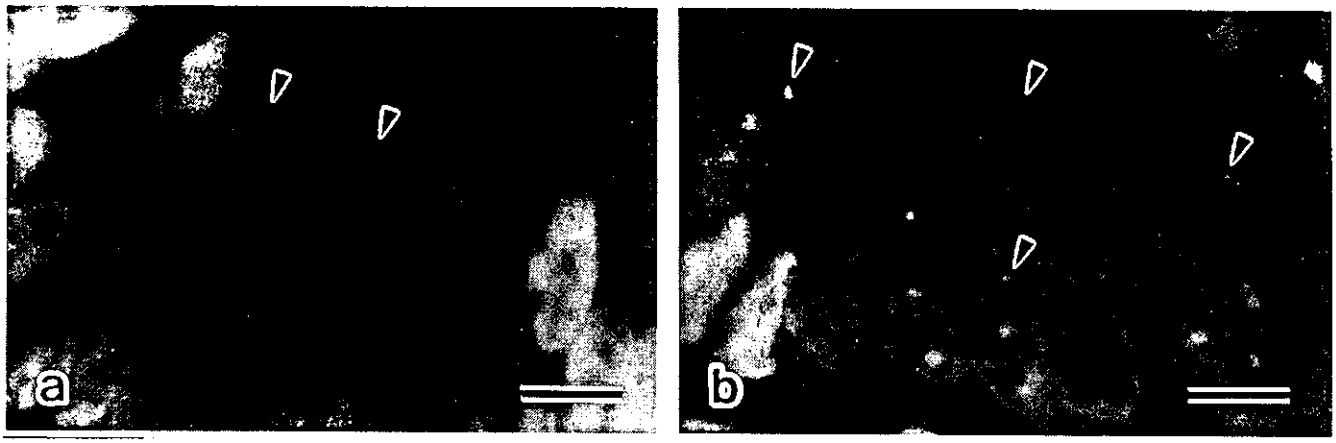

Fig. 2. Localization by Texas Red of antigens reactive with MAb8513 in semithin cryosections (1 μm) of endometrial tissue on immunofluorescence microscopy. Nuclei were counterstained with DAPI. **a:** Only a few normal endometrial glandular cells show a weak positive reaction, which appears as small granular spots (arrowheads) in the cytoplasm. **b:** Malignant glandular cells show strong positivity of granules (arrowheads) in the cytoplasm, with 1–2 granules per cell being detected. The plasma membrane is negative. Original magnification: ×1,000 (**a**), ×1,000 (**b**). Bar=5 μm.

Non-specific binding of Texas Red-labeled streptavidin was not detected when normal mouse IgG or IgM or 0.1% BSA-PBS was applied instead of the primary antibody, or when specimens were incubated with Texas Red-labeled streptavidin alone without the primary or secondary antibodies (data not shown). In our previous immunohistochemical study [33], there were also no nonspecific reactions due to these same secondary antibodies. Therefore, the positive reactions described above were considered to be specific for MAb8628 and MAb8513.

Electron microscopic findings

In the normal endometrial glandular cells, MAb8628 (Fig. 4a) and MAb8513 (Fig. 4b) reacted with antigens in the *trans*-cisternae of the Golgi apparatus on electron microscopy. In endometrial adenocarcinoma cells, both the *trans*-cisternae and the *trans*-Golgi network of the Golgi apparatus showed positivity for Mab8628 (Fig. 4c), as well as the intracytoplasmic granules, but the main positive sites were the *trans*-cisternae and the *trans*-Golgi network. On post-embedding staining, the number of gold particles in the trans-cisternae of the Golgi apparatus was greater in the endometrial adenocarcinoma cells than in normal endometrial glandular cells (Fig. 4a, c). In endometrial adenocarcinoma cells, MAb8513 reacted with the *trans*-cisternae, the *trans*-Golgi network of the Golgi apparatus, and the intracytoplasmic granules. In contrast to the other antibody, the main positive sites were the intracytoplasmic

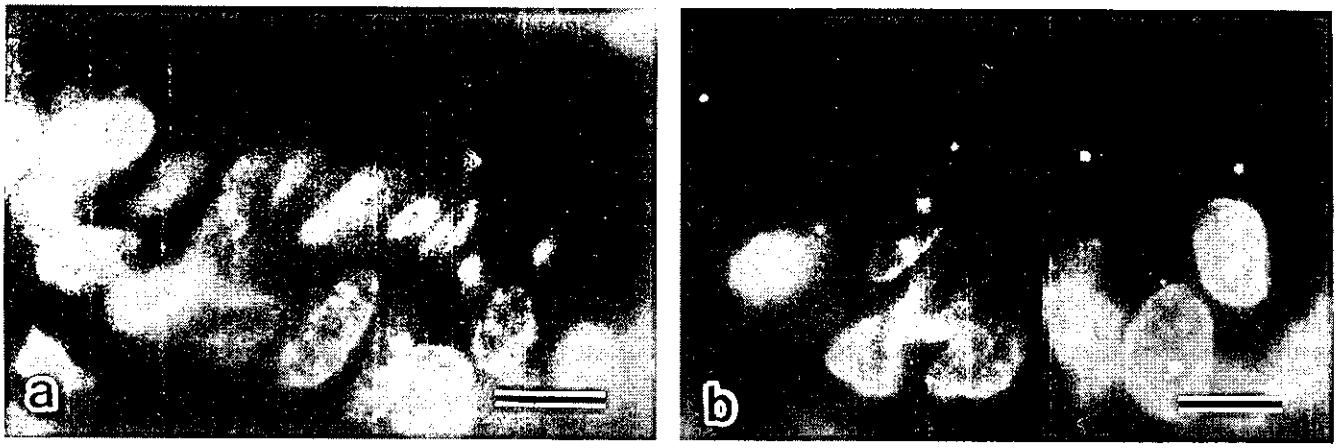

Fig. 3. Immunofluorescence localization by Texas Red of antigens reactive with MAb8628 (**a**) and MAb8513 (**b**) in semithin cryosections (1 μm) cut from the same block of ovarian endometrioid adenocarcinoma tissue. Nuclei were counterstained with DAPI. The intracytoplasmic localizations of staining by the two antibodies differs in the same cancer cell. **a:** MAb8628 reacts with the supranuclear area, producing worm-like or U-shaped staining. **b:** MAb8513 reacts with granular spots in the supranuclear areas. Original magnification: ×1,000 (**a**), ×1,000 (**b**). Bar=5 μm.

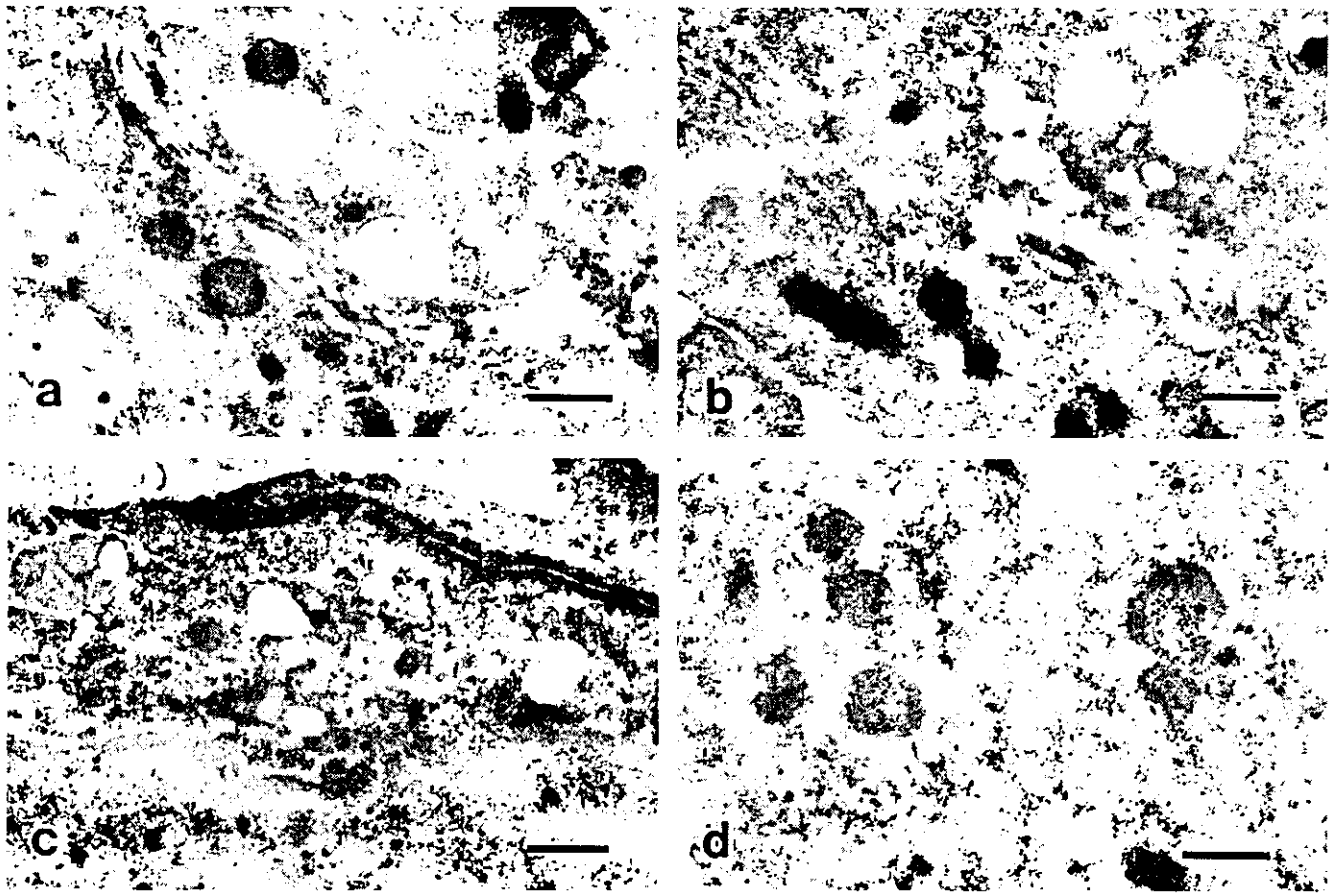

Fig. 4. Electron micrographs of endometrial tissues stained with MAb8628 (**a, c**) and MAb8513 (**b, d**). Colloidal gold labeling by the post-embedding method. **a:** MAb8628 reacts with the *trans*-cisternae of the Golgi apparatus in normal endometrial glandular cells. **b:** MAb8513 shows a similar pattern of reactivity. **c:** In well-differentiated endometrial adenocarcinoma cells, the *trans*-cisternae and the *trans*-Golgi network of the Golgi apparatus are positive. **d:** MAb8513 reacts with intracytoplasmic granules in well-differentiated endometrial adenocarcinoma cells. The plasma membrane is negative for staining with MAb8628 (**a, c**) and MAb8513 (**b**). Original magnification: ×6,600 (**a**), ×15,000 (**b**), ×13,000 (**c**), ×12,000 (**d**). Bar=0.5 μm.

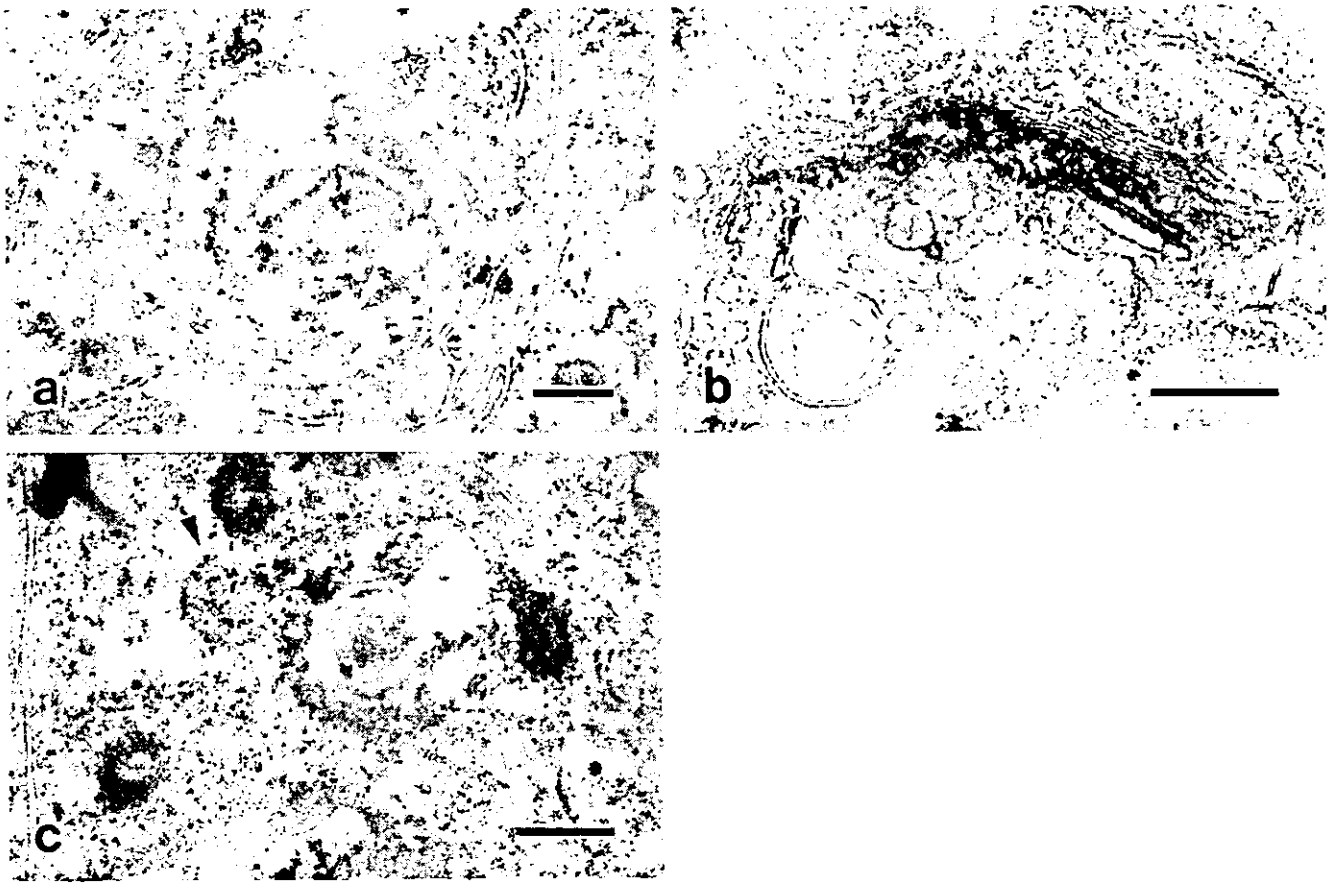

Fig. 5. Electron micrographs of ovarian endometrioid adenocarcinoma stained with MAb8628 (**a, b**) and with MAb8513 (**c**). Colloidal gold labeling by the post-embedding method (**a, c**) or HRP labeling by the pre-embedding method (**b**). **a, b:** MAb8628 reacts with the *trans*-cisternae and the *trans*-Golgi network of the Golgi apparatus. **c:** MAb8513 reacts with intracytoplasmic granules (arrowhead). The plasma membrane is negative for MAb8628 (**a, b**) and MAb8513 (**c**). Original magnification: ×10,000 (**a**), ×12,000 (**b**), ×10,000 (**c**). Bar=0.5 μm.

granules (Fig. 4d) rather than the *trans*-Golgi areas. The granules showed central, rather than peripheral, reactivity with MAb8513 (Fig. 4d).

In ovarian adenocarcinoma cells, MAb8628 or MAb8513 reactivity was found at the same sites as in endometrial adenocarcinoma cells (Fig. 5a–c).

Ultrastructural examination of well-differentiated endometrial adenocarcinoma and ovarian endometrioid adenocarcinoma (using specimens fixed with 2.5% glutaraldehyde and embedded in Epon 812) revealed abundant granular structures of various sizes near the Golgi apparatus and in the cytoplasm that were similar to the granular structures seen after post-embedding staining of Lowicryl K4M sections (not shown).

Nonspecific binding of streptavidin-colloidal gold was not detected when normal mouse IgG, IgM, or 0.1% BSA-PBS was applied instead of the primary antibody, or when sections were incubated with streptavidin-colloidal gold without the primary and secondary antibodies (not shown). In our previous immunohistochemical study 33], we also found no nonspecific reactions due to the same secondary antibodies. Therefore, the positive reactions described above were thought to be specific for MAb8628 and MAb8513.

IV. Discussion

Using immunofluorescence microscopy and immunoelectron microscopy, we examined the subcellular and suborganellar localizations of the antigens recognized by two monoclonal antibodies (MAb8628 and MAb8513) raised against GAT 37, 38], a form of GalT that is increased in the serum and ascites of patients with gynecologic malignancies 24, 26, 27]. We demonstrated that staining of the Golgi apparatus by MAb8628 was more intense in endometrial adenocarcinoma than in normal endometrium, while staining of intracytoplasmic granules by MAb8513 was more intense in endometrial adenocarcinoma than in normal endometrium (Figs. 1, 2).

Several immunohistochemical and biochemical studies have found the elevation of GalT in malignant tissues 10, 19, 22, 24, 26–28, 36, 42]. Nozawa *et al.* 26] found an increase of GAT in the serum of patients with endometrial or ovarian adenocarcinoma using a two-antibody enzyme immunoassay with MAb8628 and MAb8513. Nozawa

reported that the GAT-positive rate was 52.9% for ovarian cancer, 19.5% for endometrial cancer, 20.5% for cervical cancer, and only 5.7% for benign ovarian cysts and 6.6% for endometriosis when a cut-off value of 15 U/ml was employed. This suggested that GAT might be useful tumor marker for ovarian carcinoma due to its high specificity, since the positive rates for benign disease were extremely low.

In the present study, both MAbs showed stronger immunoreactivity with adenocarcinoma cells than with normal glandular cells. It seems that more GAT is produced by adenocarcinoma cells than by normal glandular cells based on the intense positivity for MAb8628 and MAb8513 of the *trans*-region of the Golgi apparatus and cytoplasmic granules, respectively, on immunoelectron microscopy. Therefore, the increase of serum GAT, shown by the two-antibody enzyme immunoassay with both of these MAbs 24, 26, 27, 37], in patients with endometrial or ovarian adenocarcinoma seems to be caused by the overproduction and secretion of GAT in malignant tissues.

The membrane-bound form of GalT has been reported to be localized in the trans-cisternae or trans-Golgi network of the Golgi apparatus 3, 5, 17, 23, 31, 35, 39], where GalT has a role in the glycosylation of proteins. Many studies have demonstrated that GalT also exists in the plasma membrane of some cells 7, 19, 22, 32, 42]. In the present study, however, we were unable to detect any GalT immunoreactivity of the plasma membrane. When performing a histochemical study of GalT, the specificity of the antibody can be a problem. In order to investigate the specific immunolocalization of GalT, it is necessary to exclude the possibility of crossreaction with carbohydrate structures on the enzyme, as suggested by Childs *et al.* 6] and Berger *et al.* 4]. Both MAbs used in this study have been shown to be protein-specific, since both reacted with recombinant GalT (produced by *Escherichia coli* transfected with GalT cDNA) in a two-antibody enzyme immunoassay 37].

Using serial semithin cryosections, we clearly demonstrated a difference in the staining patterns of these two MAbs. The 1-μm semithin section allowed us to clearly visualize the staining pattern as effectively (Fig. 3) as confocal laser scanning microscopy, without the problem of insufficient or uneven penetration of the antibody through a thick section. This difference in the immunofluorescence staining pattern suggested the possibility of different subcellular targets of the two MAbs at the ultrastructural level.

On immunoelectron microscopy, both antibodies recognized the *trans*-cisternae of the Golgi apparatus in normal

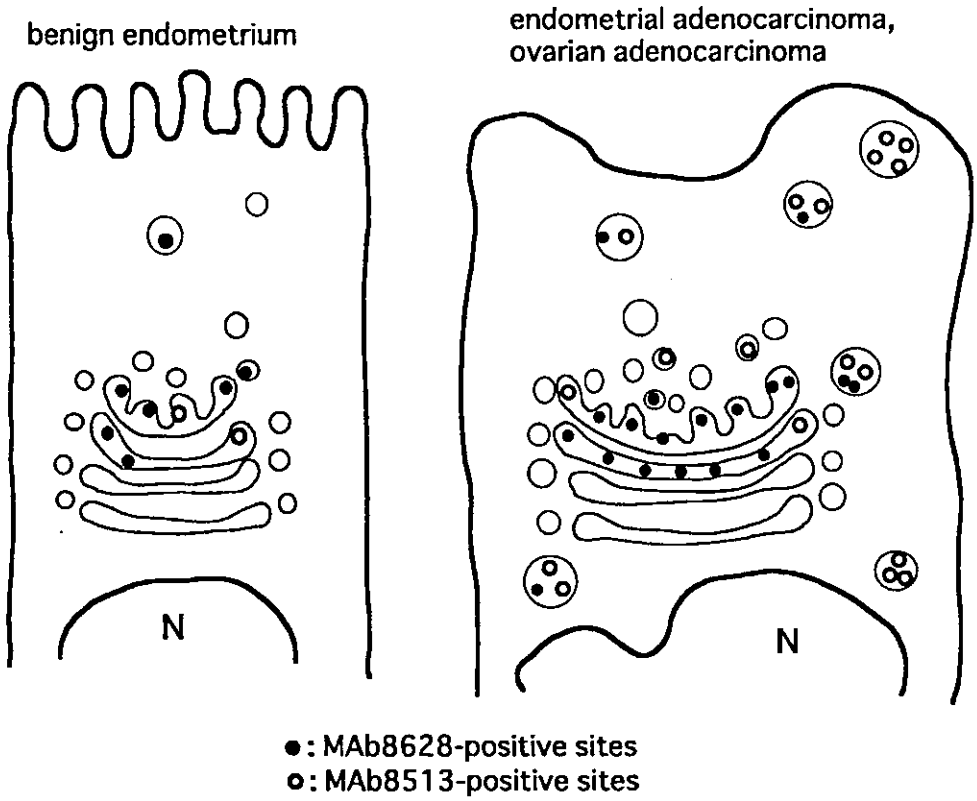

Fig. 6. Diagrams showing the results of immunoelectron microscopy. In normal glandular cells, MAb8628 and MAb8513 were found to react with the *trans*-cisternae of the Golgi apparatus. In adenocarcinoma cells, MAb8628 and MAb8513 reacted with the *trans*-cisternae and the *trans*-Golgi network of the Golgi apparatus, as well as with intracytoplasmic granules. However, the main localization of each antibody was different, since MAb8628 mainly reacted with the *trans*-cisternae and the *trans*-Golgi network of the Golgi apparatus, whereas MAb8513 primarily stained the intracytoplasmic granules.

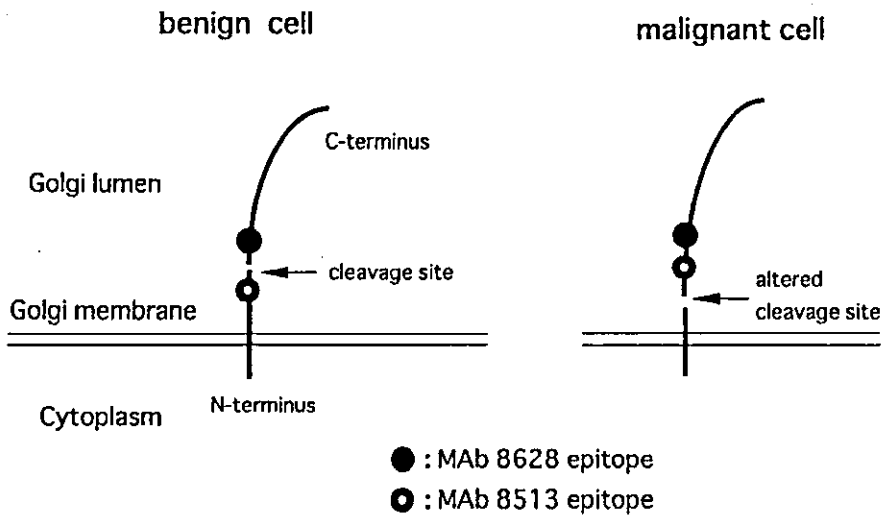

Fig. 7. Diagram showing the creation of GalT and GAT in the Golgi apparatus. Both MAbs recognize the stem region of the membrane-bound form of GalT. The membrane-bound enzyme is cleaved at a certain point within the stem region, which converts it to the soluble form containing the MAb8628 epitope. The MAb8513 epitope is closer to the NH_2-terminus than to the cleavage site and thus remains with the membrane-bound portion after cleavage occurs. In malignant cells, aberrant proteolytic cleavage occurs at a different site closer to the NH_2-terminus than it does in benign cells, so the aberrant soluble form of GAT has a longer peptide chain that includes the MAb8513 epitope.

glandular cells (Fig. 4a, b). In adenocarcinoma cells, however, MAb8628 mainly showed a reaction with the *trans*-Golgi network and the *trans*-cisternae of the Golgi apparatus (Fig. 4c), whereas MAb8513 primarily reacted with granules rather than with the *trans*-Golgi areas (Fig. 4d). The intracellular localization of the targets of these two MAbs is shown as a diagram in Fig. 6. These ultrastructural findings agreed with the above-mentioned results of the immunofluorescence study.

Why did the immunohistochemical localizations differ when these two MAbs targeted epitopes on the same peptide chain? According to Uejima *et al.* 37], these MAbs both recognize the stem region of the membrane-bound form of GalT. The membrane-bound form of the enzyme is cleaved at a point within the stem region, specifically at amino acid residues 78 and 79, to release the soluble form of the enzyme that contains the MAb8628 epitope. This epitope is located closer to the COOH-terminus than to the proteolytic cleavage site, while the epitope of MAb8513 is located closer to the NH_2-terminus and therefore remains with the membrane-bound portion after cleavage (Fig. 7). In malignant cells, Uejima *et al.* 37] proposed that aberrant proteolytic cleavage may occur at a different site, closer to the NH_2-terminus than to the usual site that is cleaved to generate the soluble form of GalT in benign cells. As a result, the aberrant soluble GalT form may consist of a longer peptide chain bearing the epitopes of both MAb8628 and MAb8513 (Fig. 7).

Uemura *et al.* 38] reported that GAT readily forms aggregates in human body fluids and is detected primarily in the dimer form by non-denaturing polyacrylamide gel electrophoresis. Such structural alterations may influence the antigenicity of the epitopes for the two MAbs.

In the present study, we could not determine which enzyme, the membrane-bound or soluble form or the GAT monomer or polymer, was actually recognized by the MAbs. A biochemical study by Uemura *et al.* 38] showed that MAb8628 could react with both the monomer and polymer of soluble GalT, while MAb8513 could only react with the polymer of soluble GAT; but they did not confirm reactivity for the membrane-bound form of GalT biochemically. We also were unable to clarify this issue in the present immunohistochemical study. However, MAb8513-reactive antigens tended to be localized in granular structures corresponding to the downstream part of the secretory pathway where polymerization would be expected to have progressed further than in the *trans*-cisternae of the Golgi apparatus. The immunohistochemical data on MAb8513 binding sites were compatible with the biochemical data of Uemura *et al.* 38], since MAb8513 reacts with the polymerized soluble form of GAT. Furthermore, gold particles were centrally located on the intracytoplasmic granules after post-embedding staining with MAb8513 (Fig. 4d), indicating the existence of the soluble form rather than the membrane-bound form of GalT.

In conclusion, we demonstrated an increase in the expression of GAT in endometrial and ovarian epithelial adenocarcinoma cells by immunofluorescence and immunoelectron microscopy using MAb8628 and MAb8513. This is the first report on the histochemical examination of GAT and the first demonstration of a difference in the subcellular localization of the antigens for these two MAbs. When measured by two-antibody enzyme immunoassay, GAT seems to be a useful tumor marker for female genital tract malignancy. GAT appears to be created by some tumor-specific change in the secretory mechanism, but the reason for alteration of the proteolytic cleavage site still remains to be investigated.

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Suppression of gap junctional intercellular communication via 5′ CpG island methylation in promoter region of E-cadherin gene in endometrial cancer cells

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Previously, we demonstrated that connexins (Cxs) showed aberrant localization and expression in most endometrial hyperplasia and carcinoma samples, indicating that during endometrial carcinogenesis, loss of gap junctional intercellular communication (GJIC) may occur at relatively early stages. In the present study, we focused on the correlations between GJIC and the expression of the E-cadherin and its 5′ CpG island methylation in endometrial cancer cells and tissues to investigate their roles in the carcinogenesis and tumor progression of endometrial cancer. In this study, three of the 10 cell lines investigated, Ishikawa, RL-952 and KLE, in which both Cxs and E-cadherin mRNA were expressed, exhibited GJIC by scrape-loading/dye transfer. On the other hand, the other seven cell lines, in which either or both Cxs and E-cadherin mRNA were negative or weakly expressed, did not show GJIC. HEC-50, HEC-1B and HEC-108, in which Cxs were positively expressed but E-cadherin was negatively expressed, showed cytoplasmic localization of Cxs by immunohistochemistry. All five lines, which showed the weak expression of E-cadherin, had E-cadherin 5′ CpG island methylation. By immunohistochemistry of 56 endometrial carcinomas, 13 of 27 methylated samples showed weak expression of Cx26 and the other 14 showed diffuse localization in cytoplasm. On the other hand, of 29 unmethylated samples, two showed cell–cell localization, 25 weak expression and two diffuse localization. Furthermore, E-cadherin expression was revealed to be drastically down-regulated by E-cadherin antisense oligonucleotides that post-transcriptionally down-regulated E-cadherin expression and in the cell, the localization of Cxs were changed from the cell–cell borders to the cytoplasm, and GJIC also decreased. The results indicated that 5′ CpG island methylation, which caused loss of E-cadherin expression, indirectly caused the suppression of GJIC by aberrant localization of Cxs in endometrial carcinoma cells.

Introduction

Stimulation of the endometrium by estrogens without the differentiating effect of progestins is the primary etiological factor associated with the development of endometrial hyperplasia and adenocarcinoma. Although it is widely accepted that endogenous and exogenous sources of unopposed estrogen increase the risk of endometrial adenocarcinoma, and several molecular alterations have been identified, the molecular pathogenesis of endometrial cancer remains poorly understood (1).

Gap junctions are intercellular channels that directly connect the cytoplasm of the neighboring cells and allow exchanges of low molecular weight (< ~1000 Da) metabolites, inorganic ions and other small hydrophilic molecules between the cells in contact. Second messengers in signal transduction such as cyclic AMP, Ca^{2+} and inositol trisphosphate can pass through gap junction channels. Therefore, gap junctional intercellular communication (GJIC) is considered to play an important role in the control of cell growth, differentiation, the maintenance of homeostasis and morphogenesis. The gap junction channels are composed of hexagonal arrangements of oligomeric proteins called connexins (Cxs). It has been demonstrated that GJIC can be regulated by different factors such as growth factors, oncogenes, Ca^{2+}, pH and hormones (2,3). As carcinogenesis involves a disturbance of homeostasis and cancer cells show uncontrolled growth, it is considered that altered GJIC play an important role in carcinogenesis (4). Several lines of evidence suggest that a disturbance of GJIC facilitates the clonal growth of potential cancer cells and Cx genes may act as tumor suppressors (5–9). Several reports have demonstrated that Cx expression is decreased in pre-cancerous lesions (10–12) and mutated in some cancers (13,14). Other reports demonstrated that only when the Cxs are stably down regulated by a mutation/oncogene phosphorylation would it become a carcinoma (15). Recently, we demonstrated that the expression of Cx26 and Cx32 was suppressed according to cell proliferation in the normal endometrial epithelium in a hormone-dependent manner (16) and expression in most endometrial hyperplasia and carcinoma samples were suppressed, indicating that during endometrial carcinogenesis, loss of GJIC may occur at relatively early stages (17). In these studies, we found some samples that expressed Cx26 or 32 but showed cytoplasmic localization (Figure 1). However, it remains unclear why Cx26 and 32 show cytoplasmic localization.

E-cadherin is a Ca^{2+}-dependent adhesion molecule that, in association with α-, β- and γ-catenin, constitutes the major component of adherent junctions in vertebrates and this binding is essential for the establishment of tight physical cell–cell adhesion (2). Transcriptional inactivation of E-cadherin expression has been shown to occur frequently in tumor progression. The cadherin system interacts directly with products of oncogenes, e.g. c-erbB-2 protein (18) and the epidermal growth factor receptor (19), and of the tumor suppressor gene, adenomatous polyposis coli (APC) protein, through β-catenin (20), which may be important in signal transduction pathways contributing to the determination of the biological properties of human cancers (21). In conclusion, inactivation of the E-cadherin system by multiple mechanisms, including

Abbreviations: Cxs, connexins; GJIC, gap junctional intercellular communication; LY, lucifer yellow; MSP, methylation-specific PCR.

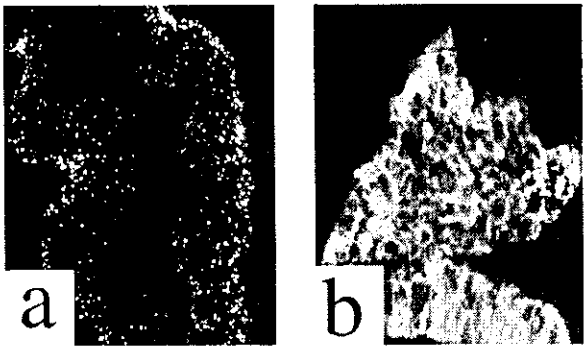

Fig. 1. Aberrant localization of Cx26 in endometrial cancers. Normally, Cxs are localized on the cell–cell border as spots (a); however, some endometrial cancer samples show cytoplasmic localization (b). (a) Normal endometrium in the secretory phase; (b) moderately differentiated endometrial cancer. Original magnification, ×400.

Table I. Specific primers for RT–PCR

Cx26	
Forward:	5′-TTGGTGTTTGCTCAGGAAGA-3′
Reverse:	5′-CTTTCCAATGCTGGTGGAGT-3′
Cx32	
Forward:	5′-CCTGCACAGACATGAGACCA-3′
Reverse:	5′-GTTCACGCCACTGAGCAAG-3′
E-cadherin	
Forward:	5′-AAACAGGATGGCTGAAGGTG-3′
Reverse:	5′-TCAGGATCTTGGCTGAGGAT-3′
β-catenin	
Forward:	5′-TTGATGGAGTTGGACATGG-3′
Reverse:	5′-CAGGACTTGGGAGGTATCCA-3′
GAPDH	
Forward:	5′-GAGTCAACGGATTTCGTCGT-3′
Reverse:	5′-GGTGCCATGGAATTTGCCAT-3′

both genetic and epigenetic events, plays a significant role in multistage carcinogenesis. It has recently become evident that loss of E-cadherin expression is also associated with aberrant 5′ CpG island methylation in various tumors (22–24). In endometrial carcinoma, aberrant promoter-region CpG island methylation has been reported in some genes such as estrogen receptor α (25) and progesterone receptor B (26). In a previous study, we analyzed the methylation status and immunohistochemical expression of E-cadherin in 142 endometrial tissues; 21 normal endometria, 17 endometrial hyperplasias and 104 endometrial carcinomas. In endometrial carcinoma, the positive ratio of methylation was higher and was associated with tumor dedifferentiation and myometrial invasion. This is the first report to analyze methylation of the E-cadherin gene promoter of endometrial carcinoma (27).

Considering this evidence, we focused on the correlations between GJIC and the expression of the E-cadherin and its 5′ CpG island methylation in endometrial cancer cells and tissues to investigate their roles in the carcinogenesis and tumor progression of endometrial cancer.

Materials and methods

Cell lines

Ten human endometrial adenocarcinoma cell lines, HEC-1A, HEC-1BE, HEC-50B, HEC-108, SNG-II, SNG-M, Ishikawa, SPAC-1L, KLE and RL-952, were used in this study. They were routinely cultured in minimum essential medium supplemented with 10% FCS in a humidified atmosphere at 37°C. All culture reagents were from Gibco (Paisley, UK). The cells were grown on Petri dishes or Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL). For demethylation experiments, cells were cultured in presence of 1 μM 5-aza-2′ deoxycytidine (AzaC; Sigma, St Louis, MO) for 3 days. HEC-1A, HEC-1BE, HEC-50B and HEC-108 were kindly given by Dr Kuramoto (Kitasato University, Japan), SNG-II and SNG-M were from Dr Nozawa (Keio University, Japan), Ishikawa was from Dr Nishida (Tsukuba University, Japan), SPAC-1L was from Dr Hirai (Cancer Institute Hospital, Japan) and KLE and RL-952 were provided by ATCC.

Methylation-specific PCR (MSP)

E-cadherin 5′ CpG island MSP was performed on sodium bisulfite-treated DNA according to Herman *et al.* (24). Previous studies demonstrated that MSP primers spanned the transcription start site of E-cadherin as island 3 and methylation in the regions targeted by those primer sets correlated best with loss of expression (24).

RNA isolation and RT–PCR analysis

To verify the presence of specific mRNAs of Cx26, Cx32, E-cadherin and β-catenin, we amplified them by RT–PCR and the GAPDH gene was amplified as a control. Total RNA of the tissues was extracted by a single-step technique with TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. The quality of the RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide, and 18S and 28S RNA bands were visualized under UV light. Five micrograms of total RNA was denatured at 65°C for 10 min and incubated at 36°C for 60 min in RT buffer containing random primers, deoxynucleotide triphosphates (dNTPs), RNAase inhibitors and avian myeloblastosis virus RT (Takara, Tokyo, Japan) in a final volume of 20 μl, followed by boiling for 5 min. One microliter of each RT reaction mixture was applied to 25 μl of PCR mixture, containing 2.5 U AmpliTaq DNA polymerase (Takara), 1.5 mmol/l $MgCl_2$, 1× *Taq* buffer and 0.2 mmol/l each of four dNTPs. The specific primers of Cx26, Cx32, E-cadherin and β-catenin and GAPDH used for PCR are shown in Table I. Thirty-eight cycles of PCR were carried out with a program of 30 s at 94°C, 1 min at 58°C and 1 min at 72°C. Aliquots of the PCR products were electrophoresed on 2.5% agarose gel. Mutation analysis of the exon 3 region of the β-catenin gene was analyzed according to the method described previously (2,28). Because, the APC protein down-regulates β-catenin levels by cooperating with glycogen synthase kinase 3 (GSK-3β), inducing phosphorylation of the serine-thereonine residues coded in exon 3 of the β-catenin gene (29,30) and its degradation through the ubiquitin–proteasome pathway, furthermore mutation in exon 3 of β-catenin results in stabilization of the protein, cytoplasmic and nuclear accumulation, and participation in signal transduction and transcriptional activation through the formation of complexes with DNA binding proteins (31).

Immunofluorescence

The cultured cells on chamber slides were fixed with cold acetone. The fixed tissue cells were pre-incubated with a blocking solution (PBS containing 5% skimmed milk) for 30 min at room temperature incubated with anti-Cx26 (Clone No, CX-12H10, Zymed Laboratories, San Francisco, CA) diluted 1:500, anti-Cx32 (Clone No., CX-2C2; Zymed Laboratories) diluted 1:500 and anti-E-cadherin (Clone No., HECD-1; Takara) diluted 1:500 for 2 h, and washed in PBS. FITC-conjugated anti-mouse immunogloblin diluted 1:200 in PBS was then added (Dakopatts, Copenhagen, Denmark) and the slides were incubated for 1 h. After incubation with secondary antibodies, the slides were washed in PBS, mounted in fluorescent mounting medium (Dakopatts) and examined by immunofluorescent microscopy (Nikon, Tokyo).

Measurement of GJIC

For measuring GJIC, we used the scrape-loading/dye transfer method with some modification (32). Endometrial carcinoma cells on 35 mm dishes were rinsed several times with PBS. Four lines were made around the center of the dish using a surgical blade and 2 ml of 0.05% Lucifer yellow CH (LY; Sigma) in PBS was added to the dishes after scraping. LY is a small molecule (457 Da) that can freely move through gap junctions from loaded cells to neighboring ones. Three minutes after the dye treatment, the cells were rinsed several times with PBS to remove excess dye. We immediately observed the intensity of LY transfer with an Olympus inverse microscope equipped with appropriate filters (Olympus, Tokyo, Japan), photographed five points per dish and the layers in which LY spread were counted. This procedure was repeated three times. Thus, 15 points were photographed and the average of the spread cell layer was calculated.

Treatment with E-cadherin antisense oligonucleotide

To examine the role of E-cadherin in Cx localization of endometrial carcinoma cells, we added E-cadherin antisense oligonucleotides that post-transcriptionally down-regulate E-cadherin expression. First, Ishikawa was seeded for 1×10^4 cells/ml on chamber slides (Lab-Tek, Nalge Nunc) and for 1×10^5 cells/ml on 3 cm culture dishes (Nalge Nunc). The antisense and control ologonucleotides were designed *BIOGNOSTIK* (Gottingen, Germany) and highly purified phosphorothioate oligonucleotides and applied to the cells without carrier. The cell was cultured in the presence of E-cadherin antisense oligonucleotides (A1, A2, A3), control oligonucleotide (C1), or in the culture medium alone (C2). Antisense oligonucleotides and controls directed to E-cadherin have been designed and manufactured by *BIOGNOSTIK*. The concentration of oligonucleotide used in this study is based on experimental protocol of the *BIOGNOSTIK* Antisense Oligonucleotides Application Notes. The cells were treated with 2 μM of E-cadherin antisense oligonucleotide or control oligonucleotide. After incubation for 48 h, these cells were used in immunohistochemistry, scrape-loading/dye transfer method and western blotting study.

Western blotting of E-cadherin

The cells cultured in serum-free William's medium E in the presence of E-cadherin antisense oligonucleotides, or control oligonucleotide, or in the culture medium alone were collected using a cell scraper, respectively. The sample was mixed with SDS electrophoresis sample buffer (10 mmol/l Tris–HCl, pH 7.8, 1 mmol/l EDTA, 3% sodium dodecyl sulfate, 5% glycerol, 10% mercaptoethanol), heated for 5 min at 95°C, run on 9% polyacrylamide electropheresis gels (Mini-Protein II, Bio-Rad, Richmond, CA), and then blotted onto a polyvinylidene difluoride membrane (Bio-Rad). Protein concentrations were determined for each sample using the bicinchoninic acid protein assay reagent kit (BCA) (Pierce, Rockford, IL) and 20 μg protein were applied respectively. The filters were blocked in 5% (w/v) dry milk in PBS containing 0.05% Tween 20 (T-PBS) and incubated for 2 h at room temperature in anti-E-cadherin (Clone No., HECD-1; Takara) diluted 1:1000. After four washes with 0.05% T-PBS, the blots were incubated for 1 h at room temperature with a horseradish-peroxidase anti-mouse antibody (Dakopatts) diluted 1:1000 in 5% (w/v) dry milk in T-PBS. They were then washed and treated with enhanced chemiluminescence western blotting detection reagents (Amersham, Little Chalfont, Buckinghamshire, UK) and exposed to blue-light-sensitive autoradiographic film (Hyperfilm-ECL, Amersham). In negative controls, normal mouse serum was used as the first antibody. The densities of the positive bands were measured using NIH-image.

Hypermethylation analysis and immunohistochemistry for Cx26 in endometrial carcinoma tissues

Samples of endometrial carcinoma tissues were obtained from 56 women who had undergone hysterectomy at the Sapporo Medical University Hospital. Biopsy samples were obtained according to institutional guidelines (University Hospital), and informed consent was obtained from patients. DNA was extracted from frozen samples kept at 80°C. Then 1 μg of the DNA was denatured using NaOH and treated with sodium bisulfite for 16 h according to Herman *et al.* (24). The frozen tissues were cut into slices 6 μm thick, mounted on albumin-coated slides and fixed with cold acetone. Each slide was stained with the monoclonal anti-Cx26 antibody as described previously (16). For each tissue sample, the intensity of immunostaining was graded weak/negative, positive on cell–cell border or diffuse in cytoplasm. Statistical analyses were performed using the Mann–Whitney test.

Results

GJIC in the endometrial carcinoma cells

To examine the existence of GJIC in the endometrial carcinoma cells, scrape-loading/dye transfer was performed. As shown in Table II, seven of the 10 cell lines, HEC-1A, HEC-50B, HEC-1BE, SNG-II, SPAC-1L, SNG-M and HEC-108, did not show GJIC. The dye spread was one or two cells thick (Figure 2a). The other three cell lines, Ishikawa, RL-952 and KLE, showed GJIC. In RL-952 and KLE, the dye reached 6.3 ± 0.5 (SE) and 5.2 ± 0.3 cells, respectively, from the scrape line (Figure 2b) and in Ishikawa it reached > 10 cells (Figure 2c).

RT–PCR of E-cadherin, β-catenin Cx26 and Cx32

To evaluate mRNA expression of E-cadherin, β-catenin Cx26 and Cx32, RT–PCR was performed. As shown in Figure 3 and Table II, of the 10 cell lines, Ishikawa, RL-952, KLE, SNG-II and SNG-M showed strong expression of E-cadherin mRNA, whereas HEC-1A, HEC-50B, HEC-1BE, SPAC-1L and HEC-108 showed weak or negative expression. Cx26 mRNA was weakly detected in HEC-1A, HEC-50B, SNG-II, SPAC-1L and SNG-M, whereas Ishikawa, HEC-1BE, RL-952, KLE and HEC-108 showed intensive expression. In HEC-50B, Cx32 was normally detected. In other cells Cx32 was expressed coordinately with Cx26. The mRNA expression of β-catenin was similarly detected in all cells. No mutation in the exon 3 region of the β-catenin gene was found in any cells (data not shown).

Subcellular localization of Cx26, Cx32 and E-cadherin

Subcellular localization of Cx26, Cx32 and E-cadherin was analyzed by immunohistochemistry for the cultured endometrial carcinoma cells. The results are shown in Table III. Of these 10 cell lines, E-cadherin was localized on the cell–cell border in five, in Ishikawa (Figure 4a), RL-952, KLE, SNG-II and SNG-M. However, HEC-1A and HEC-50B, E-cadherin was localized on the cell–cell border but quite weakly expressed and in HEC-1BE (Figure 4b), SPAC-1L and HEC-108, E-cadherin was not detected. Cx26 was detected as spots on the cell–cell border for three lines, Ishikawa (Figure 4c), RL-952 and KLE. The other seven showed aberrant localization and expression. In HEC-1A, HEC-1BE (Figure 4d), HEC-50B, SPAC-1L and HEC-108, which showed weak expression of E-cadherin, Cx26 was localized in the cytoplasm and, in SNG-II and SNG-M, positive spots of Cx26 were rarely detected in the cytoplasm or the cell–cell border. Cx32 was detected as spots on the cell–cell border in

Table II. GJIC, Cx26, Cx32 and E-cadherin mRNA expression and methylation of E-cadherin gene

Cell line	GJIC	mRNA expression of				Mutation of β-catenin	Methylation of E-cadherin gene
		Cx26	Cx32	β-catenin	E-cadherin		
HEC-1A	1.3 ± 0.1 (SE)	Weak	Weak	Positive	Weak	No	Methylated
HEC-50B	0.9 ± 0.1 (SE)	Weak	Positive	Positive	Weak	No	Methylated
HEC-1B	1.0 ± 0.1 (SE)	Positive	Positive	Positive	Negative	No	Methylated
Ishikawa	15.9 ± 1.2 (SE)	Positive	Positive	Positive	Positive	No	Unmethylated
RL-95–2	6.3 ± 0.5 (SE)	Positive	Positive	Positive	Positive	No	Unmethylated
SNG-II	1.2 ± 0.2 (SE)	Weak	Negative	Positive	Positive	No	Unmethylated
KLE	5.2 ± 0.3 (SE)	Positive	Positive	Positive	Positive	No	Unmethylated
SNG-M	1.0 ± 0.1 (SE)	Weak	Negative	Positive	Positive	No	Unmethylated
SPAC-1L	0.9 ± 0.1 (SE)	Negative	Negative	Positive	Negative	No	Methylated
HEC-108	0.9 ± 0.1 (SE)	Positive	Positive	Positive	Negative	No	Methylated

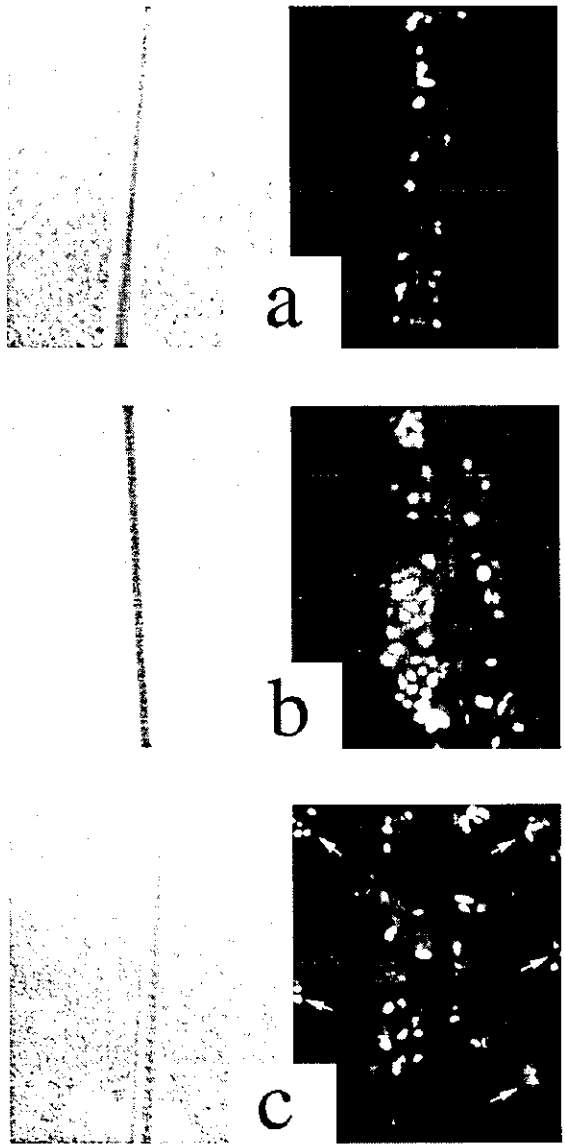

Fig. 2. Scrape-loading/dye transfer in endometrial carcinoma cell lines. In HEC-1A, HEC-50B, HEC-1BE, SNG-II, SPAC-1L, SNG-M and HEC-108, the dye spread is one or two cells thick (a). The other three cell lines, Ishikawa, RL-952 and KLE, show GJIC. In RL-952 and KLE, the dye reaches 6.3 ± 0.5 (SE) and 5.2 ± 0.3 cells from the scrape line (b), respectively, and in Ishikawa it reaches > 10 cells (arrow) (c). Original magnification, ×200.

three cell lines, Ishikawa, RL-952 and KLE. In HEC-1BE, HEC-50B, SPAC-1L and HEC-108, which showed the weak expression of E-cadherin, Cx32 was localized in the cytoplasm and, in HEC-1A, SNG-II and SNG-M, positive spots of Cx32 were rarely detected in the cytoplasm or the cell–cell border.

MSP of E-cadherin

E-cadherin 5′ CpG island MSP was performed on sodium bisulfite-treated DNA for the 10 cell lines. As shown in Table II and Figure 5a, five lines, HEC-1A, HEC-1BE, HEC-50B, SPAC-1L and HEC-108, which showed weak expression of E-cadherin, had E-cadherin 5′ CpG island methylation. The other five cell lines did not show DNA methylation of the E-cadherin gene. The five cell lines that had E-cadherin methylation were treated with 1 μM 5-aza-2′ deoxycytidine for 3 days, then their E-cadherin mRNA was partially recovered (Figure 5b).

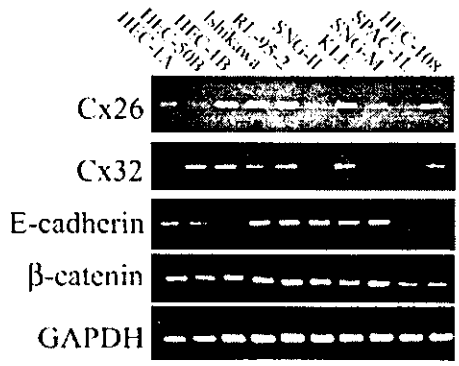

Fig. 3. RT–PCR of E-cadherin, β-catenin Cx26 and Cx32 in endometrial cancer cells. Of the 10 cell lines, Ishikawa, RL-952, KLE, SNG-II and SNG-M show strong expression of E-cadherin mRNA, whereas HEC-1A, HEC-50B, HEC-1BE, SPAC-1L and HEC-108 showed weak or negative expression. Cx26 mRNA is weakly detected in HEC-1A, HEC-50B, SNG-II, SPAC-1L and SNG-M, whereas Ishikawa, HEC-1BE, RL-952, KLE and HEC-108 show intensive expression. In HEC-50B, Cx32 mRNA is positively detected. In the other cell lines, Cx32 is expressed coordinately with Cx26. The mRNA expression of β-catenin is similarly detected in all cell lines.

Table III. Immunohistochemical finding of E-cadherin, Cx26 and Cx32

Cell line	Subcellular localization of		
	Cx26	Cx32	E-cadherin
HEC-1A	Cytoplasm	Cytoplasm	Weak
HEC-50B	Cytoplasm	Cytoplasm	Weak
HEC-1B	Cytoplasm	Cytoplasm	Negative
Ishikawa	Cell–cell border	Cell–cell border	Cell–cell border
RL-95-2	Cell–cell border	Cell–cell border	Cell–cell border
SNG-II	Negative	Negative	Cell–cell border
KLE	Cell–cell border	Cell–cell border	Cell–cell border
SNG-M	Negative	Negative	Cell–cell border
SPAC-1L	Negative	Negative	Negative
HEC-108	Cytoplasm	Cytoplasm	Negative

Change of E-cadherin expression, Cx localization and GJIC by treatment with E-cadherin antisense oligonucleotide

To examine the role of E-cadherin in Cx localization and GJIC of endometrial carcinoma cells, we added E-cadherin antisense oligonucleotides that transcriptionally down-regulated E-cadherin expression, to Ishikawa, which had expression of E-cadherin, cell–cell localization of Cxs and had the most GJIC among the analyzed endometrial carcinoma cells. In this study we used three E-cadherin antisense oligonucleotides (A1, A2 and A3). The cells were cultured in the presence of E-cadherin antisense oligonucleotides, a control oligonucleotide (C1), or in the culture medium alone (C2). Western blotting revealed that, although in the cells treated with the control oligonucleotide E-cadherin expression was not changed compared with those in the culture medium alone, all of these antisense oligonucleotides drastically down-regulated E-cadherin protein expression (A1, 0.28; A2, 0.26; A3, 0.59 compared with C1) (Figure 6a). By immunohistochemistry, E-cadherin was detected on the cell–cell borders in C1 and C2 (Figure 6b); however, in A1, A2 and A3, it was quite weakly detected (Figure 6c). Cxs were also observed on the cell–cell borders in C1 and C2 (Figure 6d), whereas immunohistochemistry showed they were dispersed in the cytoplasm when the

anti-E-cadherin antisense oligonucleotides were added to the culture medium (Figure 6e). Then, to examine the change of GJIC in the endometrial carcinoma cells, scrape-loading/dye transfer was performed. In Ishikawa with C1 and in C2, the dye reached > 10 cells from the scrape line (Figure 6f). However, in the presence of E-cadherin antisense oligonucleotides, it drastically decreased and the dye spread was only one or two cells thick (Figure 6g).

Hypermethylation analysis and immunohistochemistry for Cx26 in endometrial carcinoma tissues

To determine the correlation between methylation of the E-cadherin 5′ CpG island and subcellular localization of Cx26 in endometrial carcinoma tissue, we analyzed the methylation of E-cadherin and performed immunohistochemistry for Cx26 in 56 samples of endometrial carcinoma. As shown in Table IV, of the 56 samples, 27 were methylated and the other 29 were unmethylated. Of the 27 unmethylated samples, 13 showed weak expression of Cx26 and the other 14 showed diffuse localization in cytoplasm. On the other hand, of the 29 unmethylated samples, two showed cell–cell localization, 25 weak expression and two diffuse localization ($P < 0.05$ compared with methylated samples).

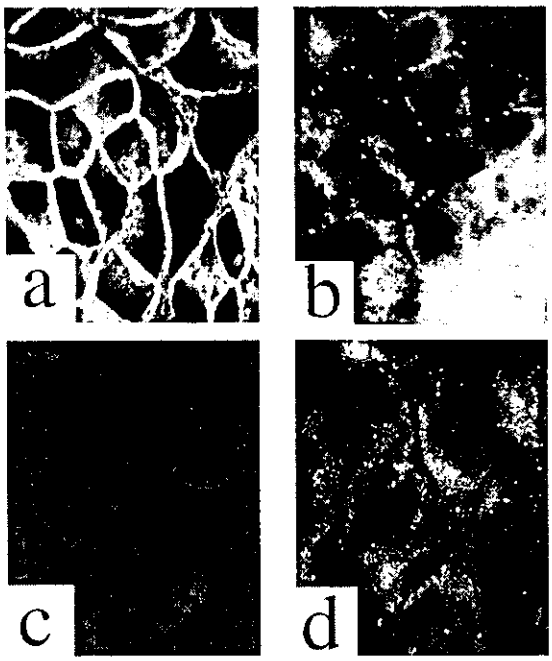

Fig. 4. Subcellular localization of E-cadherin and Cx26. In cell lines that express both E-cadherin and Cxs mRNA, such as Ishikawa, both subcellular localization of E-cadherin (a) and Cxs (b) is on the cell–cell border by immunohistochemistry. In cells that express Cxs but not E-cadherin mRNA, such as HEC-1BE, E-cadherin is not detected (c) and Cxs (d) is localized in the cytoplasm. (a and b) Ishikawa; (c and d) HEC-1BE; (a and c) E-cadherin; (b and d) Cx26. Original magnification, ×1000.

Discussion

As carcinogenesis involves a disturbance of homeostasis and cancer cells show uncontrolled growth, it is considered that altered GJIC play important roles in it. Several lines of evidence suggest that a disturbance of GJIC facilitates the clonal growth of potential cancer cells and Cx genes may act as a tumor suppressor (5,7,33,34). Several reports demonstrated that Cx expression was decreased in pre-cancerous legions. For example, in our previous study, pre-neoplastic and neoplastic lesions such as endometrial hyperplasia and carcinoma, showed obvious decreases in levels of Cx26 and Cx32 mRNA and immunohistochemically aberrant localization (17). However, little is known about the mechanism decreasing GJIC in the endometrium. In the present study, we analyzed a mechanistic connection between E-cadherin expression and Cx26/Cx32 localization/function and the correlation between methylation of the E-cadherin gene and GJIC in endometrial carcinoma cells.

Of the 10 endometrial cancer cell lines that we analyzed in this study, three had GJIC but seven did not. The three cell lines that had GJIC, showed cell–cell localization of Cx26 and 32 by immunohistochemistry and positive expression of Cx26 and 32 mRNA by RT–PCR; however, the other seven cell lines, which did not show GJIC, showed aberrant expression and localization of Cx26 and 32. Of the seven lines, five showed cytoplasmic localization of Cx26 by immunohistochemistry and the other two showed negative expression of Cx26 by RT–PCR. The results for Cx32 were similar to those for Cx26 by immunohistochemistry and RT–PCR. The results indicated that positive expression and localization in the

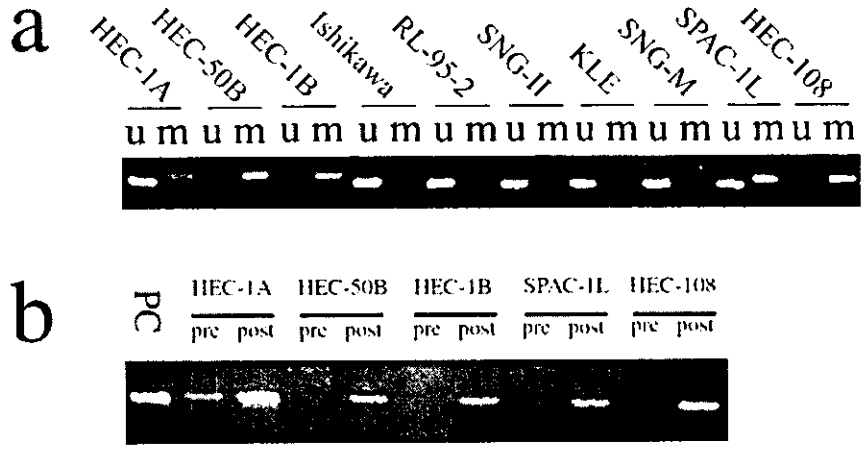

Fig. 5. E-cadherin 5′ CpG island methylation in endometrial cancer cells. (a) Five cell lines, HEC-1A, HEC-1BE, HEC-50B, SPAC-1L and HEC-108, which have weak expression of E-cadherin, have E-cadherin 5′ CpG island methylation. The other five do not show DNA methylation of the E-cadherin gene. (b) The five cell lines that had E-cadherin methylation were treated with 1 μM 5-aza-2′ deoxycytidine for 3 days, then their E-cadherin mRNA was partially recovered.

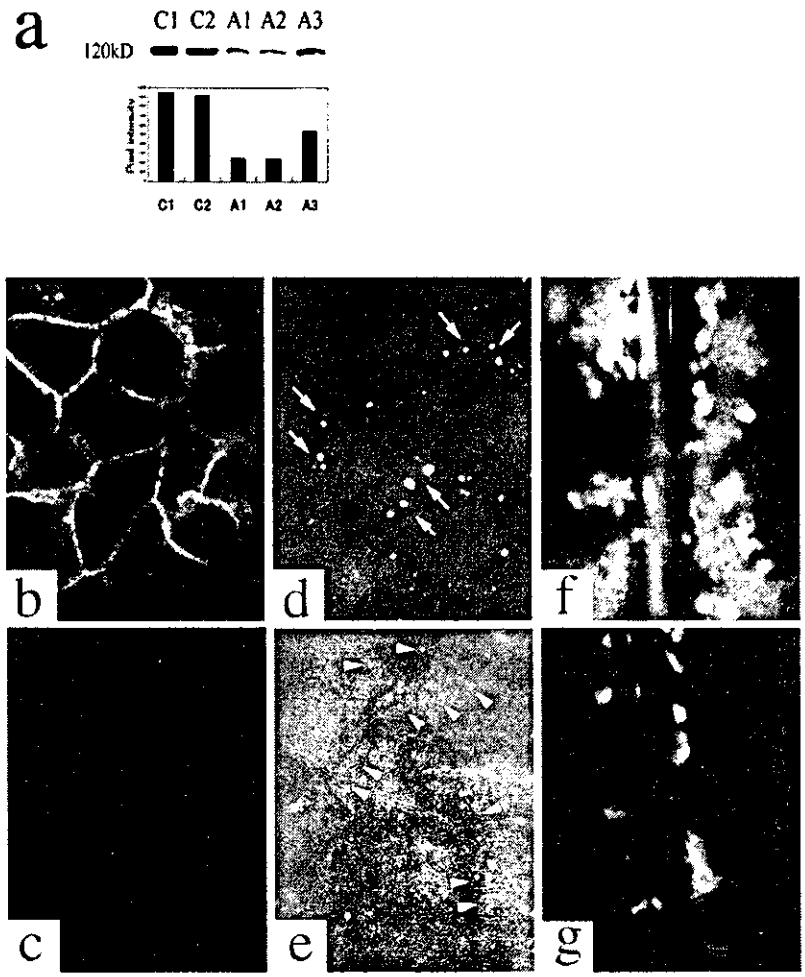

Fig. 6. Change of E-cadherin expression, Cx localization, and GJIC by treatment with E-cadherin antisense oligonucleotide. (a) By western blotting, although in the cells treated by the control oligonucleotide, E-cadherin expression was not changed compared with the culture medium alone, all of these antisense oligonucleotides were revealed to be drastically down-regulated E-cadherin protein expression (A1, 0.28; A2, 0.26; A3, 0.59 comparing with C1). (b–e) E-cadherin was detected on the cell–cell borders in C1 and C2 (b); however, in A1, A2 and A3, it was quite weakly detected (c). Cxs were also observed on the cell–cell borders in C1 and C2 (d, arrow), whereas they were dispersed in the cytoplasm when the anti E-cadherin antisense oligonucleotides were added to the culture medium (e, arrowhead) by immunohistochemistry. Original magnification, ×1000. (f and g) Ishikawa in C1 and in C2, the dye reached >10 cells from the scrape line (f). However, in the presence of E-cadherin antisense oligonucleotides, it drastically decreased and the dye spread was only one or two cells thick (g). Original magnification, ×200.

Table IV. Methylation of E-cadherin gene and subcellular localization of Cx26

E-cadherin gene	Subcellular localization of Cx26		
	Normal	Weak	Diffuse
Methylated	0	13	14[a]
Unmethylated	2	25	2[a]

[a]$P < 0.05$.

cell–cell contact region of Cxs were necessary for GJIC in endometrial carcinoma cells.

The most convincing evidence for the involvement of aberrant GJIC during carcinogenesis has come from the fact that most, if not all, cancer cells have aberrant GJIC. The loss of GJIC in cancer cells was first demonstrated by the group of Loewenstein and Kanno (35). Many ensuing studies have confirmed that various cancer cells have lost or decreased GJIC capacity (2,5,6). However, it is also known that not all tumorigenic or transformed cells have decreased GJIC (36). For example, BALB/c 3T3 cells transformed by various carcinogens maintained their GJIC at levels similar to that of non-transformed counterparts; however, these transformed cells did not communicate with surrounding normal cells (37). Similar selective lack of GJIC was observed with tumorigenic and non-tumorigenic rat liver epithelial cells (38). From these results and others, it was postulated that, for cells to become cancerous, they may need to lose GJIC with their surrounding normal cells rather than losing their homologous GJIC (36). Therefore, HEC-1A, HEC-50B, HEC-1BE, SNG-II, SPAC-1L, SNG-M and HEC-108, which did not show GJIC, lose both homologous and heterogeneous GJIC but the other three cell lines, Ishikawa, RL-952 and KLE, might lose heterogeneous GJIC losing homologous GJIC. Furthermore, the observation that there exists two types of tumors, one that does not express any Cxs at the transcriptional level and the others that express Cxs but do not have functional GJIC. This could support the idea of Trosko *et al.* that there are two target cells for the carcinogenic process; the stem cell and the early differentiated cell (39).

Cadherins are a family of cell–cell adhesion molecules essential for tight connection between cells (40), and E-cadherin is the major cadherin molecule expressed in epithelial cells. The cadherin-mediated cell adhesion system is known to act as an 'invasion suppressor system' in cancer cells as non-invasive cells can be transformed into invasive ones when treated with antibodies to block the function of cadherin or with cadherin-specific antisense RNA (41,42), and transfection of human cancer cell lines with E-cadherin cDNA can reduce their invasiveness (43). In fact, immunohistochemical examination has revealed that decreased E-cadherin expression is associated with tumor dedifferentiation and progression in endometrial carcinoma (27,44) and many other tumors (45–47). In this study, three of the 10 cell lines, Ishikawa, RL-952 and KLE, in which both Cxs and E-cadherin mRNA were expressed, had GJIC in scrape-loading/dye transfer. On the other hand, the other seven lines, in which one or both Cxs and E-cadherin mRNA were negative or weakly expressed, did not show GJIC. HEC-50, HEC-1B and HEC-108, in which Cxs were positively expressed but E-cadherin was negatively expressed, showed subcellular localization of Cxs by immunohistochemistry. Furthermore, Cx26 and 32, which were localized on the cell–cell contact region in Ishikawa, RL-952 and KLE, were dispersed in cytoplasm after addition of the E-cadherin antibody. From these results, it was revealed that loss of E-cadherin expression caused aberrant localization of Cx26 and 32 in endometrial cancer cells and indirectly suppressed GJIC. This result was supported by the previous results that Ca^{2+}-dependent regulation of GJIC in mouse epidermal cells is directly controlled by the calcium-dependent cell adhesion molecule E-cadherin (48,49) and L-CAM (E-cadherin) transfection restored cell–cell adhesion and GJIC in sarcoma cells (50).

We also analyzed mRNA expression and the existence of mutation in the exon 3 region of β-catenin. β-Catenin is known to bind directly to the cytoplasmic domain of E-cadherin and supports cell–cell adhesion (51). The APC protein down-regulates β-catenin levels by cooperating with GSK-3β, inducing phosphorylation of the serine-thereonine residues coded in exon 3 of the β-catenin gene (30) and its degradation through the ubiquitin–proteasome pathway, furthermore mutation in exon 3 of β-catenin results in stabilization of the protein, cytoplasmic and nuclear accumulation, and participation in signal transduction and transcriptional activation through the formation of complexes with DNA binding proteins (31). If these cells have any abnormality in β-catenin, it may affect the E-cadherin mediated cell–cell adhesion. However, their mRNA expression was positive and there was not any mutation in these 10 cell lines.

DNA methylation in the promoter regions of many genes is associated with the regulation of gene expression; it results in transcriptional silencing of the gene either through a direct effect or via a change in the chromatin conformation that inhibits transcription (52). The transformation of normal mammary epithelial cells into carcinoma and the subsequent progression to invasion and metastasis involve the accumulation of numerous genetic 'hits', including the activation or amplification of dominant oncogenes and the deletion or inactivating mutation of key tumor suppressor genes (53). It has recently become evident that tumor suppressor genes may also be transcriptionally silenced in association with aberrant promoter-region CpG island methylation (54–56). Loss of E-cadherin expression has also been associated with aberrant 5′ CpG island methylation in various tumors (22–24). In this study, we analyzed E-cadherin expression by RT–PCR and 5′ CpG island methylation of the E-cadherin gene in 10 endometrial cancer cell lines and found that all five lines that showed negative or weak expression of E-cadherin mRNA had 5′ CpG island methylation. As described above, 5′ CpG island methylation, which caused loss of E-cadherin expression, indirectly caused the suppression of GJIC due to aberrant localization of Cxs.

In endometrial carcinoma, aberrant promoter-region CpG island methylation has been reported in some genes such as estrogen receptor α (25) and progesterone receptor B (26). In a previous study, we analyzed the methylation status and immunohistochemical expression of E-cadherin in 142 endometrial tissues, consisting of 21 normal endometria, 17 endometrial hyperplasias and 104 endometrial carcinomas. In endometrial carcinoma, the positive ratio of methylation was higher and was associated with tumor dedifferentiation and myometrial invasion (27). In another study, we demonstrated that the expression of Cx26 and Cx32 was suppressed in most endometrial hyperplasia and carcinoma samples, indicating that, during endometrial carcinogenesis, loss of GJIC may occur at relatively early stages (17) and we also found some samples that expressed Cx26 or 32 but showed cytoplasmic localization. To examine the correlation between methylation of the E-cadherin 5′ CpG island and the subcellular localization of Cx26 in endometrial carcinoma tissue, we analyzed the methylation of E-cadherin and performed immunohistochemistry for Cx26 in 56 samples of endometrial carcinoma. In a previous study, as Cx32 was almost co-localized with Cx26 (16,17), we analyzed only Cx26 in this study. Of the 56 samples, 27 were methylated and the other 29 were unmethylated. Of the 27 methylated samples, 13 showed weak expression of Cx26 and the other 14 samples showed diffuse localization in the cytoplasm. On the other hand, of the 29 unmethylated samples, two showed cell–cell localization, 25 weak expression and two diffuse localization. The results indicated that 5′ CpG island methylation, which caused loss of E-cadherin expression, indirectly caused suppression of GJIC by inducing aberrant localization of Cxs not only *in vitro* but also *in vivo*.

In the present study, we added E-cadherin antisense ologonucleotides that post-transcriptionally down-regulated E-cadherin expression to examine the role of E-cadherin in Cx localization of endometrial carcinoma cells. By western blotting, E-cadherin expression was revealed to be drastically down-regulated in the presence of these E-cadherin antisense oligonucleotides. In the cells, the localization of Cx26 was changed from the cell–cell borders to the cytoplasm, and GJIC also decreased. These results indicate that the transcriptional down-regulation of E-cadherin causes the suppression of GJIC via aberrant localization of CXs.

In our previous study, we analyzed the methylation status and immunohistochemical expression of E-cadherin in 104 endometrial carcinomas. In that study, methylation was detected in 15.6% of well-differentiated adenocarcinomas, in 50.0% of moderately differentiated carcinomas and in 81.8% of poorly differentiated carcinomas. For samples with no myometrial invasion 23.1% had methylation, whereas in those with invasion of half or more of the myometrium the figure was 55.6%. Of samples that did not have lymph node metastasis, 33.7% had methylation, whereas 60.0% of samples that had lymph node metastasis had methylation. Thus, it was concluded that hypermethylation in the promoter region of the

E-cadherin gene is associated with tumor dedifferentiation and myometrial invasion in endometrial carcinoma (27). The present findings suggest that methylation-induced changes in the expression of E-cadherin may result in impaired cell–cell adhesion and defective GJIC, and may be one of the key mechanisms through which changes toward dedifferentiation and progression of endometrial cancers are mediated. Although, both the decreased expression of E-cadherin by hypermethylation of its promoter region and the decreased GJIC to inhibit the mechanical adhesion of E-cadherin are already proved, these results help us to understand the endometrial carcinogenesis.

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Three-dimensional coculture of endometrial cancer cells and fibroblasts in human placenta derived collagen sponges and expression matrix metalloproteinases in these cells☆

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Abstract

Objective. Collagen gel constitutes a valuable tool for the study of cell–matrix interactions; however, it is sometimes difficult to use the gel, in which tumor and stromal cells are cocultured, for immunohistochemistry, because it is easily broken during the process of fixation and embedding in paraffin, especially after long-term culture.

Methods. To examine the interaction between endometrial cancer cells and fibroblasts in tumor invasion, we carried out three-dimensional (3-D) coculture of various endometrial cancer cell lines and fibroblasts in human placenta derived collagen sponges and analyzed the expression and localization of matrix metalloproteinases (MMP) and plasminogen activators (PA) in these cells by immunohistochemistry.

Results. After 4 weeks of culture on the collagen sponges, endometrial cancer cells composed stratiform or glandular structures on the layer of extracellular matrix, which was composed from fibloblasts and extracellular matrix. Compared to Ishikawa cells, which were rarely invasive, HEC-1A and HEC-1BE and cocultured fibroblasts showed high invasiveness and strong expression of some proteins. In cell line HEC-1BE, MMP-1, -7, and -9, MT1-MMP, tissue inhibitors of metalloproteinases 2, and uPA showed intensive staining in both cancer cells and fibroblasts by immunohistochemistry. HEC-1A cells and cocultured fibroblasts showed expression patterns similar to those of HEC-1BE.

Conclusion. These results suggested that expression of MMPs and uPA was accelerated in fibroblasts surrounded by cancer cells. We believe that our 3-D coculture system has merit in that the interaction between cancer cells and stromal cells can be visually analyzed by immunohistochemistry and that experiments for a long period, at least 2 weeks, are possible. Furthermore, it is expected that some animal, e.g., nude mouse, experiments can be replaced by experiments using this culture system.

Introduction

Matrix metalloproteinases (MMPs) are an important group of zinc enzymes that are responsible for degradation of extracellular matrix components such as collagen and proteoglycans in normal embryogenesis and remodeling and in many disease processes such as invasion of cancer [1–5]. Connective tissue cells can also produce tissue inhibitors of metalloproteinases (TIMPs), and the balance between the levels of MMPs and TIMPs is thought to be an important determinant of extracellular matrix breakdown in vivo [6]. These are generally secreted by the same cells that secrete the MMPs and are also transcriptionally controlled [7]. The plasminogen activating system also plays a key role in the cascade of tumor-associated proteolysis leading to extracellular matrix degradation and stromal invasion. Changes in the expression of this system, consisting of urokinase- and tissue-type plasminogen activators (uPA and tPA, respec-

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