Establishment of a Quantitative Assay of Abnormal Glycolipid Expression in Endometrial Cells, and Its Diagnostic Value for Endometrial Carcinoma

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ABSTRACT

We developed a new quantitative method for detecting abnormal glycolipid expression in endometrial cells using a monoclonal antibody (MSN-1) and analyzed the glycolipid antigen recognized by MSN-1 in 173 clinical endometrial cell samples (66 normal endometria, 39 endometrial hyperplasias, and 68 endometrial adenocarcinomas). The mean glycolipid antigen levels in normal endometrium, endometrial hyperplasia, and endometrial carcinoma were 0.42 ± 1.37, 2.13 ± 3.84, and 19.4 ± 25.8 (mean ± SD) units, respectively. If the cutoff rate of this assay was fixed at 1.8 units, the positivity rates for patients with normal endometrium, endometrial hyperplasia, and endometrial carcinoma were 6.1% (4/66), 28.2% (11/39), and 76.5% (52/68), respectively. In 35 endometrial carcinoma patients, endometrial smears were simultaneously performed, and there were 22 positive smears (62.9%). When the cytological diagnosis was combined with our assay, 94.3% (33/35) of the carcinomas were detected. Thus, this assay seems to be a supplementary diagnostic method for endometrial carcinoma.

INTRODUCTION

Cell surface glycoconjugates are related to the recognition, growth, and differentiation of cells, and it is well known that the sugar chains of these glycoconjugates show structural alterations in association with malignant transformation (1). Therefore, it is anticipated that tumor-specific monoclonal antibodies targeting glycoconjugate antigens may be of diagnostic value for cancer. For example, NA 19–9, which recognizes the sialosyl-Lea antigen, is already used as a tumor marker for the diagnosis of colorectal and pancreatic cancer (2).

With regard to endometrial carcinoma of the uterus, several investigators have reported on the structural changes of cell surface glycoconjugates associated with malignant transformation (3, 4). There are some blood group-related antigens which are specifically expressed in endometrial cancer tissue (5, 6). However, most of these antigens are detected by immunohistochemical techniques and not by quantitative assays. Quantitative analysis should theoretically make it simple to distinguish endometrial carcinoma from normal endometrium in an easy and objective manner.

For this purpose, we prepared an anti-endometrial carcinoma monoclonal antibody, MSN-1. This monoclonal antibody has been proved to recognize a glycolipid antigen on the surface of tumor cells (7–9). In immunohistochemical studies, MSN-1 rarely reacted with normal endometrial tissue, but reacted with >80% of the endometrial carcinoma samples (7). In the present study, MSN-1 was used for TLC and immunostaining to quantitatively analyze the amount of glycolipid antigen recognized, and the expression of the target antigen was assayed in specimens of normal endometrium, endometrial hyperplasia, and endometrial carcinoma.

The establishment and the diagnostic value of this new quantitative assay are reported here.

MATERIALS AND METHODS

Materials

Monoclonal Antibody MSN-1. The antihuman uterine endometrial adenocarcinoma antibody MSN-1 was derived from a BALB/c mouse immunized with a human uterine endometrial adenocarcinoma cell line (SNG-II; Ref. 7). The immunoglobulin class of this antibody was IgM, and the epitope was the Lea-related glycolipid sugar moiety (8). In immunohistochemical studies, MSN-1 seldom reacted with normal endometrial tissue, but reacted frequently and strongly with endometrial carcinoma tissue (7). Thus, MSN-1 was shown to be a specific monoclonal antibody for endometrial carcinoma.

Clinical Samples. We obtained a total of 172 endometrial cell samples from outpatients of Keio University Hospital and several related hospitals from 1990 to 1992. All of the endometrial cell samples were obtained with Endosearch (Lion Co., Tokyo, Japan), which is one of the commercially available devices in Japan for cytological examination of the endometrium (10). Most samples were obtained from the outpatients, and some samples of endometrial carcinoma were obtained under general anesthesia before tumor resection.

Among these 172 specimens, 66 were normal endometria, 39 were endometrial hyperplasias, and 67 were endometrial carcinomas. The patients with normal endometrium had no gynecological malignancies. The histological diagnosis was made by endometrial curettages performed simultaneously with endometrial sampling. In the normal groups, 37 patients had proliferative endometrium and 29 had secretory endometrium.

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2 To whom requests for reprints should be addressed.
The patients with endometrial hyperplasia also had no gynecological malignancies. Histological diagnosis was done in the same manner. Among these patients, 6 had CGH, 24 had ADH, and 9 had ATH.

In the patients with endometrial carcinoma, the histological diagnosis was confirmed postoperatively. Patients who had endometrial carcinoma associated with endometrioid carcinoma of the ovary and patients in whom the primary site could not be defined clinically or histologically were excluded from this study. Histological differentiation was assessed according to the International Federation of Gynecology and Obstetrics classification, with grade 1 being well-differentiated, grade 2 being moderately differentiated, and grade 3 being poorly differentiated carcinoma. Forty-one patients had grade 1, 20 patients had grade 2, and 6 patients had grade 3 lesions. In 49 patients, the surgical pathological staging according to the International Federation of Gynecology and Obstetrics classification (1988) was well documented. Ten patients had stage Ia, 19 patients had stage Ib, 1 patient had stage Ic, 10 patients had stage IIb, 4 patients had stage IIIa, 4 patients had stage IIIc, and 1 patient had stage IVa disease (Table 1).

**Detection of the Glycolipid Antigen for MSN-1**

**TLC Immunostaining.** Endometrial cells were obtained directly from the uterine cavity using Endosearch, and an endometrial cell suspension was made by washing the device with 2 ml physiological saline. After lysis of erythrocytes with EDTA (GIBCO, Grand Island, NY), the specimens were frozen at −80°C for 2 h and lyophilized overnight. The dry weight of the specimens was then determined. Total cell surface glycolipids were extracted with chloroform/methanol (1:1, v/v) and stored at −20°C until used. Glycolipids extracted from 100 μg specimen (dry weight) were applied to thin-layer silica gel plates (Polygram; Macherey Nagel, Duren, Germany) and developed with chloroform/methanol/water (65:35:8, v/v/v). The TLC plates were incubated with a blocking buffer (1% polyvinyl pyrrolidone, 1% ovalbumin, and 0.02% Na2S in PBS) at 37 for 1 h, and then incubated with the monoclonal antibody MSN-1 (diluted to 1:250 with PBS) at 37°C for 1 h. After washing the plate five times with 0.1% Tween 20 in PBS, the antibodies bound to the plate were detected with the indirect immunoperoxidase method, using incubation with hors eradish peroxidase-labeled antimouse IgG + IgM antisera for 1 h (KPL Inc., Gaithersburg, MD; diluted 1:500 with PBS) and the enzyme substrates (H2O2 and 4-chloro-1-naphthol).

**Densitometry.** The reactivity of MSN-1 staining was measured densitometrically with a dual wavelength TLC densitometer (CS-9000; Shimadzu Co. Kyoto, Japan). The analytical and control wavelengths for 4-chloro-1-naphthol-positive spots were set at 550 and 700 nm, respectively. We initially measured the reactivity of MSN-1 with glycolipid extracts from 10, 20, 50, 100, and 200 μg SNG-II cells (dry weight) to analyze the relationship between reactivity of MSN-1 and the weight of the endometrial cell samples.

**Data Analysis.** Statistical comparisons were performed using the Mann-Whitney U nonparametric test, and p < 0.05 was regarded as statistically significant. The cutoff level of this assay was also determined. In the endometrial carcinoma cases in which the endometrial smear was performed simultaneously in our hospital, the relationship between the reactivity of MSN-1 and the result of the endometrial smear was also analyzed.

**RESULTS**

The reactivity of MSN-1 with the glycolipid extracted from SNG-II cells was analyzed by TLC immunostaining, and a single spot was detected (Fig. 1a). When this spot was analyzed densitometrically, the density of the spot increased in proportion to the weight of the cell sample (Fig. 1b). Thus, it was shown that the amount of the glycolipid antigen recognized by MSN-1 was directly proportional to the dry weight of the cells. Using these data as a standard curve, it was possible to quantitate the amount of gSNAg recognized by MSN-1 densitometrically. This standard curve was determined for every plate on which clinical samples were simultaneously spotted.

The reactivity of MSN-1 with the glycolipids of endometrial samples was quantitated as follows; reactivity for the spot produced by 100 μg SNG-II cells (dry weight) was defined as 1 unit, and the reactivity of 1 mg of a specimen (dry weight) was expressed in terms of this unit.

The reactivity of MSN-1 with 172 endometrial specimens is shown in Fig. 2 and Table 2. In 31 specimens of proliferative endometrium and 23 specimens of secretory endometrium, no reactivity with MSN-1 was detected. Another three specimens of proliferative endometrium and three of secretory endometrium showed relatively low reactivity (<1 unit). The reactivity of the remaining cases (three specimens of proliferative endometrium and three of secretory endometrium) showed more than 1 unit. The mean gSNAg level in proliferative and secretory endometrium was 0.29 ± 0.10 and 0.67 ± 1.84 (mean ± SD) units, respectively, and the mean gSNAg level for the normal endometrium was 0.42 ± 1.37 units. There was no significant difference between proliferative and secretory endometrium.

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*Table 1* Number of the endometrial carcinoma patients in each histological grade and surgical stage

<table>
<thead>
<tr>
<th>Histological grade</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1†</td>
<td>41</td>
</tr>
<tr>
<td>G2</td>
<td>20</td>
</tr>
<tr>
<td>G3</td>
<td>6</td>
</tr>
<tr>
<td>Surgical stage</td>
<td></td>
</tr>
<tr>
<td>Ia</td>
<td>10</td>
</tr>
<tr>
<td>Ib</td>
<td>19</td>
</tr>
<tr>
<td>Ic</td>
<td>1</td>
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<td>IIIa</td>
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</tr>
<tr>
<td>IIIc</td>
<td>4</td>
</tr>
<tr>
<td>IVa</td>
<td>1</td>
</tr>
</tbody>
</table>

†, grade 1 adenocarcinoma; G2, grade 2 adenocarcinoma; G3, grade 3 adenocarcinoma.

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The abbreviations used are: CGH, cystic glandular hyperplasia; ADH, adenomatous hyperplasia; ATH, atypical endometrial hyperplasia; gSNAg, SNG-II glycolipid antigen; Pap, Papanicolaou.
Among the 39 specimens of endometrial hyperplasia, gISn-Ag was not detected in 20 and another 5 showed relatively low levels (<1 unit). The remaining specimens showed higher reactivity in comparison to normal endometrium. The mean levels in CGH, ADH, and ATH specimens were 0.24 ± 0.58, 2.18 ± 2.91, and 3.27 ± 6.42 (mean ± SD) units, respectively. There was no significant difference among CGH, ADH, and ATH, and the mean gISn-Ag level for endometrial hyperplasia was 2.13 ± 3.84 units.

The gISn-Ag level of endometrium carcinoma specimens was much higher than that of normal endometrium or endometrial hyperplasia. The mean gISn-Ag level for grade 1, grade 2, and grade 3 carcinoma was 24.5 ± 29.3, 19.8 ± 23.3, and 8.2 ± 14.5 (mean ± SD) units, respectively, and there were no significant differences among these three carcinoma grades. The mean gISn-Ag level for endometrial adenocarcinoma was 19.4 ± 25.8 units. The mean gISn-Ag level for stages Ia, Ib, IIb, IIIa, and IIIc were 14.6 ± 16.2, 28.0 ± 28.2, 12.6 ± 12.5, 38.2 ± 29.3, and 19.5 ± 25.5 (mean ± SD) units, respectively. There were no significant differences among these groups, and also no significant differences among stage I, II, and III disease (P > 0.05).

Comparison of normal endometrium, endometrial hyperplasia, and endometrial carcinoma specimens showed significant differences of gISn-Ag expression among these three groups (P < 0.001 in all cases).

When we set a cutoff level of 1.8 units (approximately the mean + 1 SD for normal endometrium), the positivity rate of this assay for normal endometrium, endometrial hyperplasia, and endometrial carcinoma was 6.1% (4/66), 28.2% (11/39), and 76.5% (52/68), respectively. In the case of endometrial carcinoma, sensitivity was 82.9% for grade 1 tumors, 75% for grade 2 tumors, and 50.0% for grade 3 tumors (Table 3). Thus, gISn-Ag was more frequently and more strongly expressed by grade 1 carcinoma than by grade 2 or grade 3 carcinoma. In each stage of the endometrial carcinoma cases, the sensitivity was 80.0% (24/30) for stage I, 80.0% (8/10) for stage II, and 75.0% (6/8) for stage III disease, and there was no relationship between the positivity rate of this assay and the surgical staging (P > 0.05). The sensitivity and specificity of this assay was 76.5% (52/68) and 85.7% (90/105), and the positive predictive value and negative predictive value of this assay were 77.6% (52/67) and 87.4% (90/103).

There were 35 endometrial carcinoma patients who had endometrial smears performed simultaneously, and the results were as follows: 3 smears were negative, 10 smears were suspicious, and 22 smears were positive. Thus, the positivity rate of the cytological diagnosis was 62.9% (22/35). In two of the negative smears and nine of suspicious smears, the gISn-Ag level was above 1.8 units. However, in five positive smears, the gISn-Ag level was below 1.8 units. Thus, if the results of our assay were combined with the endometrial smear data, the positivity rate increased to 94.3% (33/35; Table 4).

**DISCUSSION**

Endometrial carcinoma is the most common gynecological malignancy in Europe and America (11), and is also gradually increasing in Japan. The principle screening method is a Pap smear of endometrial cells, but it is occasionally very difficult to distinguish between benign endometrial cells and well-differentiated adenocarcinoma because the morphology of endometrial cells is influenced by many factors (12, 13). The nuclear chromatin pattern, size of the cells and nuclei, appearance of the nucleoli, and shape of cell aggregates have all been reported to be useful in distinguishing endometrial carcinoma from benign endometrial lesions (13–15). However, diagnostic accuracy of cytopathology for endometrial carcinoma is highly influenced by the subjective judgment of the individual screener or cytopathologist when compared with the assessment of cervical lesions (13). Thus, the number of suspicious results is relatively high for endometrial Pap smears, and these patients receive unnecessary endometrial curettage. Accordingly, the development of new objective diagnostic methods is urgently required.

The application of tumor-specific monoclonal antibodies to immunocytochemistry is a newly available method that is anticipated to supplement cytdiagnosis in many areas (16–18). Quantitative analysis of the antigens recognized by monoclonal antibodies MSN-1 developed on a TLC and 3.27 ± 6.42 (mean ± SD) units, respectively.
antibodies should allow the diagnosis of cancer to become more objective. Cell surface glycolipids are easily extracted, and the purified glycolipids are stable and maintain their antigenicity (20).

The reactivity of several monoclonal antibodies with endometrial carcinoma has already been reported (4, 19, 21-24), and MSN-1 is one of the most specific monoclonal antibodies available. Thus, to establish an objective diagnostic method for endometrial carcinoma, we used MSN-1 immunostaining and TLC to quantitate the expression of glSn-Ag.

Abnormal glycoconjugate expression in association with malignant transformation has been reported for endometrial carcinoma, but no detailed quantitative investigations have been performed. Our method for the quantitative analysis of glSn-Ag expression is unique in that it directly measures the amount of antigen in endometrial samples.

Since glSn-Ag is considered to be mainly a Leb antigen (8), the blood type of the patient may influence the reactivity of endometrial samples with MSN-1. Although we did not investigate the blood types of our patients, the influence of blood type is thought to be minimal because this is also supported by Poropatich et al. (25), who performed an immunohistochemical study which proved that the reactivity of MSN-1 was independent of patient blood type.

In our assay, the SD of each group is relatively high, especially in the endometrial carcinoma group. This is because the expression of glSn-Ag is varied widely in each case and each cell. In the immunohistochemical study, glSn-Ag was expressed very strongly in some cases and not so strongly in others (7), and in the same case, some cells expressed glSn-Ag and others did not. We recognize glSn-Ag as a tumor marker of the cell surface like other tumor markers in serum. Then it is reasonable that the SD in our assay became very high.

In our assay, the expression of glSn-Ag in endometrial carcinoma cases was independent of the surgical staging. Therefore, this assay may be useful in detecting early stage endometrial carcinoma. This fact may be an advantage for the screening method of endometrial carcinoma.
Our results indicate that MSN-1 showed a very high reactivity with grade 1 endometrial carcinoma. It is sometimes difficult to distinguish grade 1 carcinoma from benign endometrium, especially atypical endometrial hyperplasia, even using endometrial biopsy specimens (26). In our study, the glSn-Ag levels of atypical endometrial hyperplasia and well-differentiated endometrial carcinoma were 3.27 ± 6.42 and 24.5 ± 29.3, respectively (P < 0.01); therefore, glSn-Ag was much more strongly expressed by well-differentiated carcinoma. Thus, it is anticipated that this assay may become a supplementary differential diagnostic method for these lesions.

In the present study, the positivity rates for cytological diagnosis of endometrial carcinoma using the endometrial smear and for our assay were 62.9% and 76.5%, respectively. When this assay was used in combination with cytological diagnosis, the positivity rate increased to 94.3%. The positivity rate of grade 1 carcinoma was much higher than that of grade 3 carcinoma (82.9% versus 42.9%), and this can probably be explained by the finding that the antigen recognized by MSN-1 shows much higher expression in grade 1 than grade 3 carcinoma on histochemical analysis (7). On the other hand, grade 3 carcinoma is more easily diagnosed using the endometrial smear because of the characteristic cytological changes of the nucleus and nucleolus.

Schwartz et al. (27) have reported that patients with hyperplasia who had strongly expressed the antigen recognized by MSN-1 on immunohistochemical analysis had advanced endometrial cancer. In our study, three cases of adenomatous hyperplasia that expressed glSn-Ag at a relatively high level showed progression to endometrial carcinoma (data not shown). This suggests that MSN-1 may be useful in following up hyperplasia and in selecting high-risk endometrial hyperplasia, which may progress to endometrial cancer.

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Establishment of a quantitative assay of abnormal glycolipid expression in endometrial cells, and its diagnostic value for endometrial carcinoma.

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