原 著

Flow cytometric trials to detect tumor cells expressing tumor marker gp 160 in pleural fluids and ascites

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Expression of tumor-associated antigen gp 160 in 23 specimens of body fluids was analyzed by flow cytometry with the use of a monoclonal antibody 5 E 8. Lymphocytes (the negative control) had a mode fluorescence intensity of channel 10 ; 95% of the cells consistently fell below channel 40. Cells with fluorescence intensity below channel 40 were designated as gp 160 negative and all cells with fluorescence intensity above channel 40 were designated as gp 160 positive. Cultured cells derived from a lung large cell carcinoma, C-1509 (the positive control) had an adjusted mode fluorescence intensity of channel 60 ; the rate of gp 160 positive cells was 81.7%. All cytologically malignant specimens from patients with lung cancer and stomach cancer showed more than 50% gp 160 positive cells and a wide fluorescence distribution pattern (8/8) . It may be useful to detect lung cancer or stomach cancer cells in body fluids by flow cytometry with the monoclonal antibody 5 E 8.

Key words : Body fluid—Glycoprotein—Tumor-associated antigen—Monoclonal antibody—Flow cytometry

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胸腹水中の腫瘍マーカー gp 160 陽性腫瘍細胞のフローサイトメトリによる検出

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I. Introduction

Flow cytometric methodology represents a powerful analytical tool for screening and detecting abnormal and malignant cells, for

Table 1 A Channel of peak cell count after immunofluorescence reaction with the monoclonal antibody 5 E 8 ; percentage of gp 160 positive cells in pleural fluid specimens, estimated by flow cytometry

Case	Clinical diagnosis	Papanicolaou's cytological classification	Peak channel	Distribution pattern of fluorescence	% of gp 160 positive cells
1	Lung cancer	5	120	Wide	98.6
2	Lung cancer	5	60	Wide	84.1
3	Lung cancer	5	40	Wide	78.5
4	Lung cancer	5	40	Wide	61.4
5	Lung cancer	5	40	Wide	54.2
6	Lung cancer	2	20	Gaussian	42.7
7	Lung cancer	2	30	Gaussian	35.1
8	Lung cancer	2	10	Gaussian	2.7
9 ^a	Tuberculosis	2	40	Gaussian	45.9
10 ^b	Lymphocytes		10	Gaussian	1.5
11 ^c	C-1509		60	Gaussian	81.7

a : Pericardial fluid, b : negative control, c : positive control

Table 1 B Channel of peak cell count after immunofluorescence reaction with the monoclonal antibody 5 E 8 ; percentage of gp 160 positive cells in ascites specimens, estimated by flow cytometry

Case	Clinical diagnosis	Papanicolaou's cytological classification	Peak channel	Distribution pattern of fluorescence	% of gp 160 positive cells
12	Stomach cancer	5	80	Wide	92.5
13	Stomach cancer	5	30, 80	Wide	57.7
14	Stomach cancer	5	30, 65	Wide	53.5
15	Stomach cancer	1	10	Gaussian	17.4
16	Breast cancer	5	30	Gaussian	24.8
17	Hepatoma	5	20	Gaussian	35.1
18	Hepatoma	1	25	Gaussian	30.7
19	Hepatoma	1	10	Gaussian	5.0
20	Pancreas cancer	5	10	Gaussian	33.2
21	Pancreas cancer	4	20	Gaussian	27.3
22	Renal cancer	1	10	Gaussian	1.3
23	Mixed Müllerian tumor	3	30, 80	Wide	60.4
24	Ovarian cancer	5	40	Wide	82.4
25	Ovarian cancer	5	15	Gaussian	6.3

subclassifying malignancies beyond conventional morphologic type and grade, and for transcending light-microscope features by providing more biologically meaningful information. Flow cytometric techniques for analyzing DNA content and elevated tumor proliferation activity are now widely used¹⁾. Although flow cytometry is considered to be a potential tool for measuring and detecting tumor-associated

markers, only a few studies have been reported²⁻⁵⁾. In order to apply the flow cytometric method to screening for malignant cells, it is essential to find appropriate tumor associated markers which are useful for flow cytometry.

The monoclonal antibody 5 E 8, originally raised against a lung epidermoid carcinoma cell line⁶⁾, has been shown to react with malignant

cells of other primary tumors including those arising in the colon, stomach and breast. This antibody recognizes an epitope contained within a 160,000 dalton non-disulfide-bonded glycoprotein^{7,8)}. This molecule, dubbed gp 160, was identified on a broad spectrum of human lung tumors but was undetectable on normal human lung tissue. Because gp 160 has been identified as a plasma membrane-associated glycoprotein, it holds promise as a detector of relatively free tumor cells in pleural fluids or ascites in conjunction with flow cytometry. This paper reports on our investigation of this possibility.

II. Materials and methods

1. Histological/clinical assessment

The samples studied were obtained from fresh body cavity fluids submitted for conventional diagnostic cytology. A total of 23 samples of body cavity fluids from unselected patients were analyzed by conventional cytopathology and flow cytometry.

The fluid specimens were applied to slides and processed for conventional cytological assessment. The specimens, to be used for gp 160 testing, were fixed in formol saline for the examination of gp 160. The cytological specimens were classified on the basis of cytological diagnosis in the context of clinical presentation.

2. Monoclonal Antibodies

The hybridoma secreting monoclonal antibody specific for gp 160 was produced⁹⁾. Hybridoma cells were propagated *in vivo* in pristane-treated BALB/c mice. Ascites fluid was harvested and precipitated by half-saturated ammonium sulfate. The γ -globulin precipitates were dissolved in borate-buffered saline (pH 8), and after extensive dialysis the mouse immunoglobulin was bound to an affinity col-

umn containing rabbit anti-mouse γ 1-specific antibodies coupled to Sepharose 4 B. The mouse immunoglobulin was eluted from the column with 0.2 M glycine-HCl, pH 2.8. The antibody was stored at -20°C after extensive dialysis.

3. Immunocytochemical staining

The cells of each body fluid were fixed in formol saline and stored at 4°C until they could be stained. Prior to staining, the fixed cells were washed twice with 0.01 M phosphate buffered saline, pH 7.2 (PBS), and then treated with PBS supplemented with 1% swine serum for 30 minutes. After being washed, the cells were incubated with monoclonal antibody 5 E 8 for 30 minutes, washed twice with PBS, and then incubated with fluorescent isothiocyanate (FITC)-conjugated swine anti-mouse immunoglobulin (DAKO Immunoglobulins A/S Copenhagen, Denmark) at room temperature for 30 minutes.

Cell line C-1509, which was derived from a human lung large cell carcinoma, was used as a positive control and lymphocytes from normal human blood as a negative control.

4. Flow cytometry analysis

The suspension was filtered through 40 μm pore size nylon mesh to remove large clumps before analysis. An Ortho 50 H flow cytometer (Ortho Diagnostic Systems, Westwood, Massachusetts) linked to an Ortho Model 2150 digital computer was used for analysis. The excitation wave length of the argon ion laser (Spectra-Physics 164-03) was 488 nm with a 500 mw output. Emissions were measured at 530-570 nm by using a band-pass filter. The flow cytometer was standardized using 2.00 μm Lactec particles (Polyscience, Warrington, Pennsylvania). The samples stained with 5 E 8 were analyzed on the green fluorescence channel using logarithmic amplification.

Gates were set on the forward and right-

Fig. 1 Flow cytometry of cultured cells C-1509 as a positive control. The X-axis represents forward light scatter and the Y-axis 90° scatter (A). Fluorescence intensity of cells that reacted with 5 E 8 (circled in A) (B)

angle light scatter to exclude debris, erythrocytes and clumps (Fig. 1 A) and the percentage of antibody-bound cells was determined from a fluorescence histogram (Fig. 1 B).

III. Results

Intense fluorescence was observed in pleural fluids with lung cancer cells (Fig. 2 A and 2 B), and in ascites with stomach cancer cells after staining with the monoclonal antibody 5 E 8.

Lymphocytes, the negative control, had a mode fluorescence intensity of channel 10; 95% of the cells consistently fell below channel 40 and many were below channel 20. Because of this, cells with fluorescence intensity weaker

than channel 40 were designated as gp 160 negative and cells with fluorescence at any value above channel 40 were regarded as gp 160 positive. The C-1509 cultured tumor cells, the positive control, showed a mode fluorescence intensity of channel 60 as adjusted and gp 160 positive cells were counted at 81.7% of the sample cells (Fig. 1 B). In this control the tumor marker gp 160 exhibited Gaussian distribution with a sharp peak.

Specimens were measured with the same sensitivity to fluorescence as the positive and the negative controls. The results are shown in Tables 1 A and 1 B.

Among the carcinomas judged positive by flow cytometry, two different labeling patterns were observed. Cultured tumor cells showed uniformly intense staining (Fig. 1 B), while nine tumors.....five lung cancers, three stomach cancers and an ovarian cancer.....showed a wide spectrum of fluorescence intensity, from low channel to high channel, without a sharp peak (Fig. 2 C and 2 D).

In mesothelial cells and gp 160 negative cases, fluorescence was distributed only in low channels with a high peak, even when the gate was narrowed to the range only of large cells, which were assumed to be cancer cells (Fig. 3).

Comparison of the positive rate by cytometry and by cytology

Some samples without gp 160-positive cells showed positive fluorescence. When the data of flow cytometry and cytological diagnosis were compared, five out of 15 cytologically malignant specimens showed gp 160 positive in more than 70% of the cells, another five out of 15 cytologically malignant specimens showed gp 160 positive in 50% to 70% of the cells, one of two cytologically borderline specimens showed 40%-50% of the cells positive for gp 160, and in five out of six cytologically non-malignant specimens, gp-160 positive cells were

Fig. 2 A pleural effusion of adenocarcinoma of the lung (Case 1). 5 E 8-FITC immunofluorescence reaction (A) ; Papanicolaou's staining, $\times 190$ (B) ; flow cytometry of forward light scatter in X-axis and 90° scatter in Y axis (C) ; fluorescence intensity of cells reacted with 5 E 8 (circled in C) (D)

counted at less than 40%. In body fluid specimens with lung cancer or stomach cancer cells, cytologically malignant specimens showed a gp 160 positive rate of more than 50% and a wide fluorescent distribution pattern (8/8).

IV. Discussion

Immunofluorescent staining with the monoclonal antibody 5 E 8 followed by flow cytometry analysis detected gp 160 in body fluid specimens from patients with lung cancer, stomach cancer and ovarian cancer. Many cases of

gp 160-positive tumor cells in body fluids showed varying degrees of stain intensity without a sharp peak and thus differed from the cultured tumor cells, which maintained a uniformly expressed gp 160. Such specimens might contain some cells other than tumor cells but still show a wide range of fluorescence intensity. These results are similar to those of image-analysis studies on the distribution of progesterone receptors and estrogen receptors in breast carcinomas^{9,10}.

The cellular mechanism responsible for heterogeneous gp 160 staining reactions is not known

Fig. 3 Ascites of adenocarcinoma of the breast (case 16). Papanicolaou's staining, $\times 190$ (A); flow cytometry of forward light scatter in X-axis and 90° scatter in Y axis, narrower gate than cases in Figs. 1 and 2, (B); fluorescence intensity of cells reacted with 5 E 8 (circled in B) (C)

at the present time. Staining heterogeneity could be explained by the existence of varying levels of expression of the genes coding for gp 160, rather than by a complete loss of the gene. Further studies are necessary to elucidate the mechanism responsible for the variability in gp 160 expression.

Setting an arbitrarily dividing line between positive and negative populations is not desirable for practical use because some non-malignant cells show nonspecific fluorescence above channel 40. One resolution of this problem is to compare data for the same specimen reacted with both non-immune mouse IgG and the monoclonal antibody in order to make a better estimation of the positive rate. After the setting of a match range for the two histograms, the negative histogram (tumor cells stained with non-immune mouse IgG) is subtracted channel by channel from the positive histogram (anti gp 160 antibody). This allows dimly stained cells which would be disregarded in the arbitrary line method to be counted as positive^{2,4)}. However, in the present study we could not compare with the negative control because the amount of each specimen was insufficient to stain it with non-immune mouse IgG.

More than 50% of the cells in the cytologically positive specimens with lung cancer or stomach cancer (8/8) were estimated as gp-160 positive by flow cytometry using the monoclonal antibody 5 E 8. Therefore, 5 E 8 may be useful in detecting lung cancer or stomach cancer cells in body fluids, although further study is necessary to put this technique to practical use for cancer screening.

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要 約

モノクローナル抗体5E8を使用して、フローサイトメーターによる体腔液中の腫瘍関連抗原 gp 160 陽性腫瘍細胞の検出を試みた。陰性対照としてのリンパ球はチャンネル10に蛍光ピークがあり、95%の細胞がチャンネル40以下であった。チャンネル40より蛍光強度の弱い細胞は gp 160 陰性とし、チャンネル40より蛍光強度の高い細胞を gp 160 陽性とした。陽性対照としての肺の大細胞癌由来の培養細胞株 C-1509 の蛍光ピークをチャンネル60に感度調整したとき、gp 160 陽性細胞は81.7%であった。肺癌および胃癌患者からの細胞診陽性体腔液検体では、全例で50%以上の細胞が gp 160 陽性で広範な蛍光強度分布を示した(8/8)。フローサイトメトリーによる体腔液中の肺癌または胃癌細胞の検出にモノクローナル抗体5E8は使用可能と考えられた。

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