Association between Sialyl Lewis\textsuperscript{a} Expression and Tumor Progression in Melanoma

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ABSTRACT

Twenty-three primary and 27 metastatic melanoma lesions and 17 pigmented nevi lesions were tested utilizing the immunoperoxidase reaction with anti-sialyl Lewis\textsuperscript{a} (sLe\textsuperscript{a}) and anti-sLe\textsuperscript{b} mAbs. sLe\textsuperscript{a} was expressed in 9, 25, and 5 lesions and sLe\textsuperscript{b} was expressed in 6, 11, and 2 of these lesions, respectively. Expression of sLe\textsuperscript{a} in melanocytic tumors is associated with tumor progression. Serum levels of sLe\textsuperscript{a} and sLe\textsuperscript{b} were analyzed by a sandwich assay using mAbs in 25 melanoma patients. Only 2 patients at stage 4 showed higher levels of sLe\textsuperscript{a} and sLe\textsuperscript{b} than did normal control subjects. Moreover, sLe\textsuperscript{a} and sLe\textsuperscript{b} were expressed in 1 and 2 of 5 human melanoma cell lines, respectively, and expression of sLe\textsuperscript{a} and sLe\textsuperscript{b} was not modulated by cytokines. These findings suggest that the expression of sLe\textsuperscript{a} in melanocytic tumors is correlated with disease progression.

INTRODUCTION

sLe\textsuperscript{a} and sLe\textsuperscript{b}, which are the ligands for the adhesion molecules E-selectin and P-selectin, respectively, have been found to be expressed on various types of carcinomas. Expression of sLe\textsuperscript{a} and sLe\textsuperscript{b} has been shown to be correlated with poor prognosis in patients with colorectal and lung carcinoma (1–6). This association may reflect the potential role of sLe\textsuperscript{a} and sLe\textsuperscript{b} in the metastatic process of carcinoma cells. To the best of our knowledge, the expression of sLe\textsuperscript{a} and sLe\textsuperscript{b} in melanoma has been analyzed on only a few cell lines in long term culture (7). Since sLe\textsuperscript{a} and sLe\textsuperscript{b} are likely to play a role in the interaction of melanoma cells with endothelial cells, we have investigated the expression of sLe\textsuperscript{a} and sLe\textsuperscript{b} in benign and malignant lesions of melanocytic origin. Furthermore, we have measured the serum level of sLe\textsuperscript{a} and sLe\textsuperscript{b} in patients with malignant melanoma, since these antigens have been reported to be useful markers to monitor the clinical course of the disease in patients with lung, gastric, and colorectal cancer (8, 9).

MATERIALS AND METHODS

Cell lines. Cultured human melanoma cell lines Colo38 (10), MeWo (10), WM164 (11), SK-MEL19 (12), and G361 (a strain from the American Type Culture Collection, Rockville, MD) were grown in DMEM supplemented with 10% FCS and 2 mM l-glutamine.

Melanocytic Lesions. Benign and malignant melanocytic lesions were obtained from patients who underwent surgery in the Department of Dermatology, Kumamoto University School of Medicine, Kumamoto, Japan. Tissues were processed within 15 min following surgical removal. Each tissue was divided into two parts, one of which was fixed in 10% buffered formaldehyde and processed for routine histopathology while the other was snap-frozen in liquid nitrogen and stored at −80°C until use. Four-μm-thick cryostat sections were dried and fixed in absolute acetone for 1 min. Under these conditions, cryostat sections could be stored for at least 3 months at −20°C without loss of reactivity with mAb.

Sera. Sera obtained from patients with malignant melanoma were stored at −20°C for at least 6 months.

mAbs and Conventional Antisera. The anti-sLe\textsuperscript{a} mAb KM231 and the anti-sLe\textsuperscript{b} mAb KM93 were developed as described elsewhere (9, 13–15). mAbs were purified from ascitic fluid by sequential precipitation with caprylic acid and ammonium sulfate (16).

Fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin xenoantibodies, biotinylated anti-mouse IgM xenoantibodies, and the VECTASTAIN avidin-biotin complex kit were purchased from Vector Laboratories (Burlingame, CA), respectively.

Cytokines. Recombinant human IFN-γ and recombinant TNF-α were purchased from R&D Systems (Minneapolis, MN) and Amersham (Amerham, Buckinghamshire, England), respectively.

Serological Assays. Indirect immunoperoxidase staining of frozen tissues with mAb was performed utilizing the Vectastain kit following the manufacturer’s instructions. The procedure has been described in detail elsewhere (17). The percentage of stained tumor cells in each section and intensity of staining were estimated independently by 2 observers. The staining intensity was graded as: −, no staining detectable; ±, staining faint or barely detectable; +, staining clearly positive; and ++, staining very strong. Variations in the percentage of stained cells enumerated by the 2 investigators were within a 10% range. The mean percentage, rounded off to the nearest multiple of 10, was used to express the results.

Indirect immunofluorescence staining of cells was performed as described. Briefly, cells (1 × 10⁶ cells) were incubated with purified mAbs (10 μg/ml) for 30 min at 4°C. Cells were washed in PBS and FITC-labeled goat anti-mouse IgG (H+L) antibodies were added as a second antibody. After 30 min, immunoreactivity was analyzed by flow cytometry (EPICS Elite; Coulter Corporation, Hialeah, FL). Results are expressed as log of fluorescence intensity.

The solid-phase immunoradiometric sandwich assay to measure serum level of sLe\textsuperscript{a} and sLe\textsuperscript{b} was performed utilizing RIA kits prepared by Otsuka Assay Laboratories (Tokushima, Japan) following the manufacturer’s instructions. Briefly, polystyrene beads coated with anti-sLe\textsuperscript{a} mAb Ca19-9 (18) and anti-sLe\textsuperscript{b} mAb FH6 (19, 20) were added to 96-well microtiter plates. Serum was then added to each well, and following a 3-h incubation at 37°C, plates were washed 3 times with PBS. \(^{125}\)I-Labeled mAb Ca19-9 and/or mAb FH6 were added, and incubation was continued for an additional 3 h at room temperature. Radioactivity in wells was then counted using a scintillation counter.

HPTLC to Analyze Glycolipids Expressed by Melanoma Cells. Acidic glycolipid fractions were obtained from cell lines utilizing the method described previously (9). Briefly, homogenized cells were extracted with chloroform/methanol (2/1, v/v), followed by three partitions with H₂O to obtain the Folch upper phase. The upper phase was evaporated to a small volume and freed from salt with a C₁₈ silica gel column. Samples were spotted onto high performance thin layer chromatography (HPTLC) plates (Whatman International, Ltd., Maidstone, England) and developed with chloroform/methanol/H₂O (50/40/10) 0.25% CaCl₂. The separated glycolipids were visualized by staining with iodine and then exposed to a thin-layer chromatography scanner.

Statistical Analysis. The differences in the expression of sLe\textsuperscript{a} and/or sLe\textsuperscript{b} in terms of presence or absence in melanocytic tumors were analyzed using the \(X^2\) test.

RESULTS

Twenty-three primary melanoma lesions were obtained from 10 male and 13 female patients with an average age of 67.5 years (range, 30 to 93 years): 8 had stage 1; 10 had stage 2; 4 had stage 3; and 1 had stage 4. Tumor staging was based on the histopathological tumor-nodes-metastasis classification system (21). The mean thickness of the
Twenty-seven metastatic melanoma lesions were obtained from 12 male and 15 female patients with an average age of 60.1 years (range, 14 to 93 years). Twenty-two lesions were obtained from skin metastasis and the remaining 5 lesions were from lymph nodes.

Seventeen pigmented nevi lesions were obtained from 7 male and 10 female patients with an average age of 43.3 years (range, 8 to 81 years). Four lesions were compound type and 13 were intradermal type.

The results of immunohistochemical staining of 23 primary lesions, 27 metastatic lesions, and 17 pigmented nevi lesions stained with anti-sLea mAb KM231 and anti-sLe* mAb KM93 are summarized in Table 1. Representative staining patterns are shown in Fig. 1. The following points are noteworthy: (a) The anti-sLea mAb KM231 stained 9 of the 23 primary lesions, 25 of the 27 metastatic lesions, and 5 of the 17 pigmented nevi lesions. The percentage of stained metastatic lesions was significantly \( P = 0.0001, 0.0001 \) higher than those of primary lesions and pigmented nevi lesions. However, the difference between the percentage of primary melanoma lesions and pigmented nevi lesions was not statistically significant; (b) the percentage of tumor cells stained by mAb KM231 was less than 20% in primary lesions and in pigmented nevi lesions with weak intensity; (c) mAb KM231 stained only 4 of 16 primary lesions less than 3 mm thick but stained 5 of 7 primary lesions at least 3 mm thick. The expression of sLea in primary lesions less than 3 mm thick is significantly lower than in primary lesions at least 3 mm thick \( P = 0.035 \) and in metastatic lesions \( P = 0.0001 \) (Fig. 2a); (d) the anti-sLe* mAb KM93 stained 6 of the 23 primary lesions, 11 of the 27 metastatic lesions, and 2 of the 17 pigmented nevi lesions. The percentage of stained metastatic lesion was significantly \( P = 0.040 \) higher than that of pigmented nevi lesions; however, results for other groups were not statistically significant; (e) the mean percentage of melanoma cells stained by mAb KM93 was 37.8 ± 38.3 and 30.1 ± 23.6 in metastatic lesions stained with strong and weak intensity, respectively. In contrast, the percentage of cells stained by mAb KM231 was less than 20%; (f) mAb KM93 stained only 1 of the 16 primary lesions less than 3 mm thick and 5 of the 7 primary lesions at least 3 mm thick. The expression of sLe* in primary lesions less than 3 mm thick is significantly lower than in primary lesions at least 3 mm thick \( P = 0.035 \) and in metastatic lesions \( P = 0.014 \) (Fig. 2b); (g) the relationship of expression of sLe* and/or sLe* in primary melanoma lesions with disease free interval and/or survival time could not be analyzed, since only 4 of 23 patients investigated suffered from recurrence of the disease and only 1 died.

The distribution of sLea in primary and metastatic melanoma lesions resembles that of ICAM-1. Since the latter is susceptible to modulation by cytokines, additional experiments tested the effect of IFN-γ and TNF-α on the expression of sLea and sLe* by melanoma cells. Cultured human Colo38, MeWo, WM164, SK-MEL19, and G361 melanoma cells were utilized for these experiments. WM164 cells were stained in IIF by anti-sLea mAb KM231 and weakly stained by sLe* mAb KM93, while MeWo cells were weakly stained only by mAb KM93. Colo38, SK-MEL19, and G361 cells were not stained by either mAb (Fig. 3). To test the modulation of sLea and sLe* by cytokines, WM164 and MeWo cells were incubated for 48 h at 37°C with IFN-γ (final concentration, 1 to 1000 units/ml) or TNF-α (final concentration, 1 to 1000 units/ml). Cells were then stained with mAb KM93 and KM231 in IIF. No effect was detected on the reactivity of the two cell lines with both mAbs.

Table 1 Level of expression of sLe* and sLe* in surgically removed benign and malignant lesions of melanocytic origin

<table>
<thead>
<tr>
<th>mAb</th>
<th>Specificity</th>
<th>Lesions</th>
<th>% of stained cells</th>
<th>Lesions</th>
<th>% of stained cells</th>
<th>Lesions</th>
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<td></td>
<td></td>
<td>sLe*</td>
<td>1 ± 0</td>
<td>14 38.8 ± 29.0</td>
<td>20 73.1 ± 19.7</td>
<td>71.7 ± 10.3</td>
<td>0 5 12 14 14 0 5 10</td>
</tr>
<tr>
<td>KM231</td>
<td>sLe*</td>
<td>1 ± 0</td>
<td>14 38.8 ± 29.0</td>
<td>16 40.0 ± 5.6</td>
<td>31.4 ± 21.9</td>
<td>0 2 15 10</td>
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</tr>
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</table>

\* Number of lesions.
\* Mean ± SD.
Sialyl Lewis* expression and tumor progression in melanoma

HPTLC staining with mAb KM231 and mAb KM93 revealed no reaction with gangliosides extracted from the cultured human melanoma cell lines, Colo38, MeWo, WM164, SK-MEL19, and G361.

*sLe* and sLe* are present in serum and their level has been shown to be increased in patients with lung, gastric, and colorectal carcinoma (8, 9). Therefore, we have measured the serum levels of sLe* and sLe* in patients with malignant melanoma. Sera were obtained from 13 male and 14 female malignant melanoma patients with an average age of 62.0 years (range, 14 to 87 years). Four patients had stage 2 melanoma, 7 had stage 3, and 16 had stage 4. Sera from 26 healthy individuals with an average age of 47.7 years (range, 25 to 72 years) were used as controls. The serum level of sLe was increased in 2 patients with stage 4 (77 and 1500 units/ml; normal control was less than 37 units/ml). One of the patients had liver metastases and his skin metastases were stained by anti-sLe mAb KM231. The serum level of sLe was increased in 2 patients with stage 4 (95.4 and 45.1 units/ml; normal control less than 38 units/ml). Both of them had liver metastases and their skin metastases were stained by mAb KM93. It is noteworthy that the level of serum sLe and sLe was within normal limits in patients whose metastases were stained by mAb KM231 and KM93.

To investigate the shedding of sLe* and sLe* from melanoma cells and the effect of cytokines on the shedding of these antigens, spent medium was harvested from cultured human melanoma cells which had been incubated with IFN-γ (final concentration, 1 to 1000 units/ml) or TNF-α (final concentration, 1 to 1000 units/ml) for 48 h and from control melanoma cells incubated under the same experimental conditions, but without cytokines. Neither sLe* nor sLe* was detected in the spent medium harvested from cytokine treated and from control melanoma cells.

**DISCUSSION**

Immunohistochemical staining with mAb has shown for the first time that sLe* and sLe* are expressed in surgically removed benign and malignant lesions of melanocytic origin. The expression of sLe* is higher than that of sLe*, as measured by the percentage of lesions and cells stained by the corresponding mAb, provided that this difference does not reflect differences in the characteristics of the mAb used. Furthermore, the results of the present study suggest that sLe* is a progression marker in lesions of melanocytic origin, since its expression in primary melanoma lesions increases with the increase of their thickness and this antigen is expressed in about 90% of metastatic lesions. The high expression of sLe* in metastases is likely to reflect the role of this antigen in series of events leading to metastasis of melanoma cells, since sLe* is a ligand for P-selectin and is involved in adherence of melanoma cells to activated endothelium and subsequent hematogenous spreading. Whether sLe* is a prognostic marker in melanoma could not be determined in the present study, since the number of patients investigated is too small and the disease free interval and the survival time analyzed are too short. The expression of sLe* in colorectal cancer was also associated with recurrence and/or poor prognosis (3, 22); however, other studies showed no statistically significant association between them by multivariate analysis (23, 24). Thus, further studies are needed to determine whether the expression of sLe* in colorectal cancer correlates with prognosis.

IIF staining showed differential expression of sLe* and sLe* by five human melanoma cell lines. To the best of our knowledge, expression of sLe* and sLe* by human melanoma cell lines has been described only by Kunzendorf et al. (7). They tested four melanoma cell lines with anti-sLe* and anti-sLe* mAb and found that all of them were stained by anti-sLe* mAb but were not stained by anti-sLe* mAb. Whether the differences in the results obtained by Kunzendorf et al. (7) and by ourselves reflect the characteristics of the cell lines and/or of the mAb used in the two investigations remains to be determined. It is noteworthy that we did not detect sLe* and sLe* in the five human melanoma cell lines tested, utilizing HPTLC immunostaining. If not caused by differences in their sensitivity, the discrepancy between the results of IIF staining and HPTLC immunostaining may reflect the expression of sLe* and sLe* by melanoma cells as glycoproteins and not as glycolipids. This possibility is supported by the recent report that the carbohydrate chain of sLe* was present in human colon cancer cell line SW1116 cells in association with both lipids and proteins (25).

The distribution of sLe* in the lesions of melanocytic origin resembles those of ICAM-1 and vitronectin receptor (26–29). The latter two markers are barely detectable in pigmented nevi but have a high expression in metastatic lesions. sLe* is similar to vitronectin receptor in its lack of susceptibility to modulation by cytokines but is different
from ICAM-1 which is modulated by both IFN-γ and TNF-α (10, 30). An additional difference with ICAM-1 is represented by the fact that the level of serum sLeα is increased in only 20% of patients with stage 4 melanoma, while the level of serum ICAM-1 is increased in about 90% of patients with stage 4 melanoma (28).

REFERENCES


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