Selective Loss of Human Leukocyte Class I Allospecificities and Staining of Melanoma Cells by Monoclonal Antibodies Recognizing Monomorphic Determinants of Class I Human Leukocyte Antigens

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ABSTRACT

Immunoperoxidase staining of frozen sections from surgically removed melanoma lesions showed that anti-human leukocyte antigen (HLA)-A2, A28 monoclonal antibody (mAb) stained keratinocytes, but did not stain melanoma cells in 21% of the 14 primary and 44% of the 9 metastatic lesions tested. The loss of HLA-A2 and/or A28 allospecificities did not affect the staining patterns with mAb recognizing monomorphic determinants of HLA Class I antigens, in terms of percentage of stained melanoma cells and intensity of staining. This finding is not likely to reflect the sensitivity of the immunoperoxidase technique, since cytofluorographic analysis detected no significant difference in the staining pattern by mAb to monomorphic determinants of HLA Class I antigens between a melanoma cell line and an autologous transplantant that had acquired HLA-A2 antigens following gene transfer. The results of the present study imply that the frequency of abnormalities in HLA Class I antigen expression by melanoma cells is higher than that described in the literature, since selective losses of HLA Class I allospecificities are not detected by staining of melanoma cells with mAb to monomorphic determinants of HLA Class I antigens. The latter reagents have been used in most of the published studies to characterize the expression of HLA Class I antigens in melanoma lesions. Furthermore, the present results provide a mechanism for the unexpected resistance to cytotoxic T-cell-mediated lysis and the unexpected poor clinical course of the disease in some patients despite a high expression of HLA Class I antigens as measured by staining of melanoma cells with mAb to monomorphic determinants.

INTRODUCTION

In melanoma as well as in other neoplastic diseases, malignant transformation of cells is often associated with changes in HLA Class I antigen expression (for review, see Ref. 1). The potential clinical significance of these changes is suggested by the role of HLA Class I antigens in the interaction of melanoma cells with host's immune system and by the statistically significant association between level of HLA Class I antigen expression and clinical course of the disease in patients with Stage II melanoma (2, 3).

A large number of surgically removed melanoma lesions have been tested for their expression of HLA Class I antigens utilizing polyclonal and monoclonal xenonabodies to monomorphic determinants shared by the gene products of HLA-A, -B, and -C loci. A recent review of the literature (4) has shown that HLA Class I antigens are not expressed by melanoma cells in about 30% of surgically removed lesions. Although HLA Class I allospecificities have been shown to be expressed by melanoma cells in about 30% of surgically removed lesions, the loss of HLA-A2 and/or A28 allospecificities did not affect the staining patterns with mAb recognizing monomorphic determinants of HLA Class I antigens, in terms of percentage of stained melanoma cells and intensity of staining. This finding is not likely to reflect the sensitivity of the immunoperoxidase technique, since cytofluorographic analysis detected no significant difference in the staining pattern by mAb to monomorphic determinants of HLA Class I antigens between a melanoma cell line and an autologous transplantant that had acquired HLA-A2 antigens following gene transfer. The results of the present study imply that the frequency of abnormalities in HLA Class I antigen expression by melanoma cells is higher than that described in the literature, since selective losses of HLA Class I allospecificities are not detected by staining of melanoma cells with mAb to monomorphic determinants of HLA Class I antigens. The latter reagents have been used in most of the published studies to characterize the expression of HLA Class I antigens in melanoma lesions. Furthermore, the present results provide a mechanism for the unexpected resistance to cytotoxic T-cell-mediated lysis and the unexpected poor clinical course of the disease in some patients despite a high expression of HLA Class I antigens as measured by staining of melanoma cells with mAb to monomorphic determinants.

MATERIALS AND METHODS

Cell Lines and Melanoma Lesions. Cultured human melanoma cells FO-1 that do not express HLA Class I antigens because of an abnormality of B2m gene (12) were grown in RPMI 1640 supplemented with 10% Serum Plus (Hazelton Biologies, Inc., Lenexa, KS) and 2 mm L-glutamine. Cells were harvested by vigorous pipetting with phosphate-buffered saline supplemented with 1 mm EDTA. Melanoma lesions were obtained from patients who underwent surgery in the Department of Dermatology, Kumamoto University Medical School, Kumamoto, Japan. The diagnosis of type of melanoma was based on the anatomic site of lesions and on their clinical and histopathological characteristics. Tissues were processed within 15 min following surgical removal. Each tumor tissue was divided into 2 parts. One half was fixed in 10% buffered formaldehyde and processed for routine histopathology. The other half of the specimen 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 fixation conditions, cryostat sections could be stored for at least 3 months at -20°C without loss of reactivity with mAb.

mAb and Conventional Antiserum. The mAb W6/32 to a monomorphic determinant expressed on β2-μ-associated HLA Class I heavy chains (13); the mAb TP25.99 to a determinant expressed on both β2-μ-associated and β2-μ-free HLA Class I heavy chains (14); the mAb F4/72 to a determinant restricted to HLA-A antigens; the mAb SA24/23 to a determinant restricted to HLA-B antigens; the anti-HLA-A2, A28 mAb CR11-351 (15), HO-3, HO-4, and KS-1 (16); the anti-HLA-A2, A28 mAb PA2.1 (17); the anti-HLA-A2, B17 mAb HO-2; and the anti-human β2-μ mAb NAMB-1 (18) were developed as described. mAbs were purified from ascitic fluid by sequential precipitation with caprylyc acid and ammonium sulfate (19).
Fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin xenoantibodies and the Vectastain avidin-biotin complex kit were purchased from Jackson ImmunoResearch Laboratories, Inc. (Avondale, PA) and from Vector Laboratories (Burlingame, CA), respectively.

Cytokine. Recombinant human interferon-γ was obtained from Hoffman-LaRoche, Inc., Nutley, NJ.

Plasmids. Plasmid pRSVneo, which carries the neomycin resistance gene; plasmid pHLLA-A2, which carries the entire HLA-A2 gene, including its regulatory sequences, and the neomycin resistance gene; and plasmid pBl2 ml3, which carries a functional β2m gene, have been described previously (20-22).

Serological Assays. 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 (23). The percentage of stained tumor cells in each section and the staining intensity were estimated independently by 2 observers. 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 by incubating cells (5 × 10⁵) with an excess of mAb for 30 min at 4°C. Then cells were washed and incubated for 30 min at 4°C with an appropriate amount of fluorescein isothiocyanate-anti-mouse immunoglobulin xenoantibodies. Cells were analyzed utilizing a fluorescence-activated cell sorter analyzer (Becton Dickinson, Mountain View, CA). Results are expressed as log of fluorescence intensity.

DNA-mediated Gene Transfer. Exponentially growing FO-I cells were transfected using the calcium phosphate precipitation technique (24) either with pRSVneo, with pRSVneo and pBl2 ml3, or with pBl2 ml3 and pHLLA-A2 plasmids. Neomycin-resistant clones from each of the 3 transfections were selected in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and the neomycin analogue G418 (Gibco Life Technologies, Inc., Grand Island, NY) at the concentration of 800 µg/ml. G418-resistant clones, which became visible 2 to 3 weeks after transfection, were pooled to minimize problems in the interpretation of the results caused by interclonal variability and were expanded. The population of G418-resistant cells transfected with pRSVneo plasmid was designated FO-1-neo. The G418-resistant cell population transfected with pRSVneo and pBl2 ml3 plasmids was subjected to fluorescence-activated cell sorting, utilizing the anti-HLA Class I mAb W6/32. The resulting subpopulation, which expresses HLA Class I antigens, was designated FO-1-β2. The G418-resistant cell population transfected with pBl2 ml3 and pHLLA-A2 plasmids was subjected to fluorescence-activated cell sorting, using the anti-HLA-A2, Aw69 mAb PA2.1. The resulting subpopulation that expresses HLA-A2 antigens besides endogenous HLA Class I antigens was designated FO-1-A2.

RESULTS

Twenty-five primary and 13 metastatic melanoma lesions were stained with anti-HLA Class I mAb TP25.99, with anti-β2-m mAb NAMBl-1, with anti-HLA-A mAb F4/72, with anti-HLA-B mAb SA24/23, and with anti-HLA-A2,A28 mAb CR11–351, HO-3, and HO-4 utilizing the immunoperoxidase reaction. Representative results of the staining patterns are shown in Figs. 1 and 2. The HLA phenotype of the patients from whom the melanoma lesions had been removed was not known and could not be determined since patients' lymphocytes were not available. Therefore, staining with anti-HLA-A2,A28 mAb of keratinocytes surrounding melanoma cells was used as a control to monitor the presence of the HLA-A2 and/or A28 allospecificities in the patients investigated. The lesions could be divided into 2 major groups according to the concordant or discordant reactivity patterns of melanoma cells and autologous keratinocytes with the 3 anti-HLA-A2,A28 mAb. In the group with concordant staining patterns, 4 reactivity patterns could be identified. The 3 anti-HLA-A2,A28 mAb stained both melanoma cells and keratinocytes in 8 primary and in 4 metastatic lesions and stained neither melanoma cells nor keratinocytes in 6 primary and in 4 metastatic lesions. mAb CR11–351 and HO-4 stained both melanoma cells and keratinocytes, whereas mAb HO-3 stained neither type of cells in one

primary lesion. In the latter lesion, cells are likely to express HLA-A2 and/or A28 antigens, but to lack the determinant recognized by mAb HO-3. mAb CR11–351 stained both melanoma cells and keratinocytes, although neither mAb HO-3 nor mAb HO-4 stained the 2 types of cells in 4 primary lesions. The staining pattern of the latter 4 lesions is likely to reflect the expression of HLA-A9 antigens and not of HLA-A2 and/or A28 antigens, since mAb CR11–351 cross-reacts with HLA-A9 antigens (16), whereas the other 2 anti-HLA-A2,A28 mAb do not.

Table 1 summarizes the results obtained with lesions with discordant reactivity patterns of melanoma cells and autologous keratinocytes with the 3 anti-HLA-A2,A28 mAb. mAb CR11–351, HO-3, and HO-4 stained keratinocytes, but did not stain melanoma cells in 3 primary and in 4 metastatic lesions. Furthermore, mAb CR11–351 stained both melanoma cells and keratinocytes, whereas mAb HO-3 and HO-4 stained only keratinocytes in one primary and in one metastatic lesion. mAb CR11–351 stained only keratinocytes, whereas mAb HO-3 and HO-4 stained neither melanoma cells nor keratinocytes in one primary lesion. Keratinocytes in the latter lesion are not likely to express HLA-A2 and/or A28 antigens, but to express the HLA-A9 allospecificity that has been lost by melanoma cells. Finally, mAb HO-4 stained only keratinocytes in one primary lesion, whereas mAb CR11–351 and HO-3 stained both melanoma cells and keratinocytes. It is noteworthy that melanoma cells without detectable reactivity with 1, 2, or all 3 anti-HLA-A2,A28 mAb were stained by anti-HLA Class I mAb TP25.99, by anti-HLA-A mAb F4/72, and by anti-β2-m mAb NAMBl-1 in all but 2 primary lesions. In the latter 2 lesions, melanoma cells were stained neither by anti-HLA-A mAb F4/72 nor by anti-HLA-B mAb SA24/23, but were stained by anti-HLA Class I mAb TP25.99 and by anti-β2-m mAb NAMBl-1. These reactivity patterns suggest that in these 2 lesions melanoma cells do not express the gene products of HLA-A and -B loci, but express HLA-C antigens.

The percentage of melanoma cells stained by anti-HLA Class I mAb TP25.99 and by anti-HLA-A mAb F4/72 and the intensity of staining of lesions with selective loss of reactivity with anti-HLA-A2,A28 mAb were not different from those of lesions in which melanoma cells were stained by anti-HLA-A2,A28 mAb. These results suggest that the selective loss of HLA Class I allospecificities by melanoma cells may not be detected by analyzing the staining patterns obtained with mAb recognizing monomorphic determinants of HLA Class I antigens or determinants restricted to the gene products of one of the HLA Class I loci. To corroborate this possibility, additional experiments were performed with cultured human melanoma cells FO-1, which do not express HLA Class I antigens because of a deletion of the 5' region and a portion of the coding sequence of B2m gene (12). Following cotransfection with pRSVneo and a wild type B2m gene, FO-1 cotransfectants, referred to as FO-1-β2, expressed HLA Class I antigens as they were stained in indirect immunofluorescence by anti-HLA Class I mAb TP25.99 and W6/32 (Fig. 3). The staining is specific, since neither mAb stained FO-1 cells transfected only with pRSVneo gene, which are referred to as FO-1-neo. Following cotransfection with a wild type B2m gene and a HLA-A2 gene, FO-1 cotransfectants, referred to as FO-1-A2, expressed HLA-A2 antigens, as they were stained by anti-HLA-A2,A28 mAb CR11–351 and KS1 and by anti-HLA-A2,B17 mAb HO-2. Representative results are shown in Fig. 3. The staining is specific, since the 3 anti-HLA-A2 mAb stained neither FO-1-β2 nor FO-1-neo cells. It is noteworthy that the intensity of staining by anti-HLA Class I mAb TP25.99 and W6/32 of FO-1-A2 cells, which express HLA-A2 antigens besides the endogenous HLA-A25 and B8 allospecificities, is similar to that of FO-1-β2 cells, which express only the endogenous HLA Class I allospecificities (Fig. 3). Incubation for 72 h with interferon-γ (final
Discussion

Immunohistochemical staining of surgically removed melanoma lesions has shown that loss of HLA-A2 and/or A28 allospecificities does not cause any significant change in the staining patterns of melanoma cells by mAb recognizing monomorphic determinants shared by HLA-A, -B, and -C antigens or restricted to the gene products of HLA-A loci. This phenomenon is likely to reflect a compensatory increase in the level of the expressed HLA Class I allospecificities, since cytofluorometric analysis detected no significant difference in the staining by mAb recognizing monomorphic determinants of HLA Class I antigens on FO-1-β2 and FO-1-A2 cells. The latter 2 cell lines differ only in the expression of the HLA-A2 allospecificity. The results of the cytofluorometric analysis of transfected melanoma cells argue against the limited sensitivity of immunoperoxidase staining as the mechanism underlying the lack of detectable effects of the selective loss of HLA Class I allospecificities on the staining patterns of melanoma lesions by mAb defining monomorphic determinants of HLA Class I antigens.

A recent review of the literature (4) has indicated that about 18% and 44% of surgically removed primary and metastatic melanoma lesions, respectively, are not stained by mAb recognizing monomorphic determinants of HLA Class I antigens. These values are likely to be an underestimate of the frequency of occurrence of abnormalities in HLA Class I antigen expression, since the present study strongly suggests that immunohistochemical staining with mAb to monomorphic determinants of HLA Class I antigens does not detect a selective loss of HLA Class I allospecificities by melanoma cells. The latter phenomenon may account for the resistance to lysis by cytotoxic...
Fig. 2. Immunoperoxidase staining with anti-HLA Class I mAb TP25.99 (A and C) and with anti-HLA-A2, A28 mAb CR11–351 (B and D) of metastatic melanoma lesions from Patients 29 (A and B) and 31 (C and D). mAb TP25.99 and CR11–351 stained melanoma cells with a similar intensity in the lesion from Patient 29. While mAb TP25.99 stained both melanoma and normal epidermal cells in the lesion from Patient 31, mAb CR11–351 stained only normal epidermal cells.

Table 1  Selective loss of reactivity of melanoma lesions with anti-HLA-A2,A28 mAb in the immunoperoxidase reaction

<table>
<thead>
<tr>
<th>Specificity mAb</th>
<th>HLA-A,B,C</th>
<th>HLA-A</th>
<th>HLA-B</th>
<th>HLA-A2,A28</th>
</tr>
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<tbody>
<tr>
<td>Primary lesions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>NM b</td>
<td>100c</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>16</td>
<td>ALM</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>24</td>
<td>SSM</td>
<td>50</td>
<td>50</td>
<td>–/–d</td>
</tr>
<tr>
<td>8</td>
<td>ALM</td>
<td>80</td>
<td>80</td>
<td>–/–d</td>
</tr>
<tr>
<td>20</td>
<td>MUC</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>21</td>
<td>MUC</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Metastatic lesions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>NM</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>32</td>
<td>ALM</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>36</td>
<td>ALM</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>37</td>
<td>NM*</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>26</td>
<td>NM</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
</tbody>
</table>

* Patient number.
+ Sections tested were from acral lentigious melanoma (ALM), mucous melanoma (MUC), nodular melanoma (NM), and superficial spreading melanoma (SSM) lesions.
+ Percentage of stained melanoma cells.
+ The histopathological nature of the primary lesion could not be determined.

T-cells of melanoma cells without detectable abnormalities in their reactivity with mAb to monomorphic determinants of HLA Class I antigens, since certain HLA Class I allospecificities have been shown to function as restricting elements in the recognition of melanoma cells by cytotoxic T-cells (25–27). Furthermore, the same mechanism may underlie the unexpected poor clinical course of patients with a high level of HLA Class I antigens in their metastatic lesions (3), provided that lysis of melanoma cells by cytotoxic T-cells does indeed play a role in the clinical course of the disease.

Different mechanisms are likely to underlie the abnormal reactivity patterns of the tested melanoma lesions with mAb CR11–351, HO-3, and/or HO-4, which recognize spatially close, but distinct determinants of HLA-A2 and/or A28 antigens. The melanoma cells that are stained by 1 or 2 of the 3 anti-HLA-A2, A28 mAb tested are likely to express the HLA-A2 and/or A28 antigen bearing molecules, although with changes in their structural conformation that result in the loss of some of the polymorphic determinants. In contrast, the melanoma cells without detectable staining by the 3 anti-HLA-A2,A28 mAb are
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not likely to express the HLA-A2 and/or A28 antigen-bearing molecules. The latter cells resemble the melanoma cell lines M2 and M3, which have been shown to not be expressed and to express the HLA-A2 gene, but to express the gene products of the other loci (28).

Selective loss of HLA-A2 and/or A28 allospecificities occurred more frequently in metastatic than in primary lesions, since it was found in 21% of the primary and 44% of the metastatic lesions tested. This finding is consistent with the more frequent lack of reactivity of metastatic than of primary lesions with mAb to monomorphic determinants of HLA Class I antigens, as it has been found in melanoma (for review, see Ref. 4) and in various types of carcinomas (29). In view of the described role of HLA-A2 antigens as restricting elements in the recognition of melanoma cells by autologous T-cells, the lack of HLA-A2 allospecificity is likely to give metastatic cells a selective advantage, since they evade detection by cytotoxic T-cells recognizing peptides derived from melanoma-associated antigens and presented by HLA-A2 alloantigens. Furthermore, selective loss of HLA Class I allospecificities by melanoma cells may eliminate the potential beneficial effect that generation of cytotoxic T-cells, specific for melanoma-associated antigens, could have on the clinical course of the disease. This interpretation implies that active specific immunotherapy that aims at generating cytotoxic T-cells specific for melanoma-associated antigens, could have on the clinical course of the disease in patients with melanoma. Cancer Res., 48: 1019-1025, 1988.

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