Association of Antigen-Processing Machinery and HLA Antigen Phenotype of Melanoma Cells with Survival in American Joint Committee on Cancer Stage III and IV Melanoma Patients

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Abstract

Because changes in the expression level of antigen-processing machinery (APM) components and HLA class I and II antigens in melanoma cells are expected to affect their interactions with the immune system of the host, we assessed the clinical relevance of quantitative variations in the expression of these molecules in melanoma lesions. Short-term (<10 in vitro passages) melanoma cell lines isolated from 85 American Joint Committee on Cancer (AJCC) stage III and IV patients were stained with APM component and HLA class I antigen-specific and HLA class II antigen-specific monoclonal antibodies and analyzed by flow cytometry. The phenotype of all tumors was characterized by intertumor and intratumor heterogeneity in the expression of all the markers and by significant correlations in the level of expression of markers belonging to the HLA class I antigen-processing and presentation pathway. Hierarchical clustering of the mean fluorescence intensity data defined two main clusters of tumors. The corresponding groups of patients differed significantly in the overall survival but not in other relevant clinical variables, including AJCC stage and therapy received after surgery. Cox regression analysis showed that β2-microglobulin and HLA class II antigen expression were significantly associated with patients’ survival. This evidence was corroborated by the immunohistochemical analysis for HLA class II antigen expression of melanoma lesions from an unrelated group of 52 AJCC stage III and IV patients. These results suggest that quantitative variations in APM component and HLA expression were significantly associated with patients’ survival.

Introduction

Tumor-associated antigens (TAA) expressed by neoplastic cells can be recognized by CD8+ and CD4+ T cells. CD8+ T cells recognize cell surface–bound complexes generated by the loading of β2-microglobulin (β2M)–associated HLA class I heavy chains with antigen-derived peptides (1). The generation of these complexes and their transport to the cell membrane depends on well-balanced interactions among the antigen-processing machinery (APM) components (2). The peptides generated mostly, but not exclusively, from endogenous proteins by proteasome are transported by TAP to the lumen of the endoplasmic reticulum (ER), where they are loaded on the newly synthesized β2M-associated HLA class I heavy chains with the assistance of the chaperones calnexin, calreticulin, ERp57, and tapasin (2). The resulting trimeric complex then travels to the cell membrane and presents peptides to CTLs.

CD4+ T cells recognize complexes of MHC class II molecules with peptides generated from degradation of exogenous or endogenous proteins (3). A wide array of TAAs (see ref. 4 for update) is naturally processed in neoplastic cells and to yield peptides that associate with MHC class II molecules for recognition by CD4+ T cells. Intracellular proteins can access the vacuolar compartment by sequestration in double-membraned organelles named autophagosomes, which then fuse with vesicles of the endosomal/lysosomal compartment where antigen processing and peptide loading onto MHC class II molecules take place (5, 6). Processing of endogenous proteins can also be initiated by cytosolic proteases, and the resulting peptide fragments are then transferred from the cytoplasm to the endosomal/lysosomal compartment by transport mechanisms that require the function of proteins as LAMP-2 and HSC-70 (3).

It has been shown that loss of some APM components, such as TAP1, causes marked MHC class I antigen down-regulation in human cells as well as defective antigen presentation to CTLs (7). Furthermore, qualitative analysis of surgically removed tumors, with immunohistochemical techniques, has shown frequent defects in HLA class I and II and APM components expression in neoplastic cells (8–17), but the evaluation of the potential effect of these defects on clinical outcome has not provided concordant result in all tumors investigated. In fact, in melanoma and head and neck carcinomas, down-regulation or loss of expression of HLA class I or of some APM components is significantly associated with disease progression or with shorter patients’ survival (8, 10–13). In contrast, down-regulation or loss of HLA class I antigens has been shown to correlate with better prognosis in colorectal and breast cancers (14, 15). Discordant results have been obtained even by looking at the effect on clinical outcome of HLA class II antigen expression of tumor cells. Thus, in metastatic melanoma, based on a specific pattern of expression of HLA class I and II on neoplastic cells, early results suggested that a high expression of HLA class II antigens may be associated with shorter patients’ survival (8). On the other hand, in large B-cell lymphoma and colorectal carcinomas, HLA-DR expression on neoplastic cells is associated with improved survival (16, 17).
In addition, the relationship between quantitative variations in the overall APM component, HLA class I and II expressions, and clinical outcome has not been investigated previously in any human tumor. Here, by taking advantage of a recently developed panel of APM component-specific monoclonal antibodies (mAbs; refs. 18, 19) and of appropriate intracellular staining technique (20, 21), we have evaluated APM component and HLA class I and II expressions in a large panel of human melanomas by flow cytometry. Melanoma was selected for this study based on the evidence that immunologic events are involved in the clinical course of this disease (22, 23) and that HLA and APM defects are frequently found in this tumor (8, 10, 13). The results of this quantitative analysis were evaluated for the potential association of the APM and HLA class I and II profiles with relevant clinical variables.

Materials and Methods

Patients and tumors. Melanoma cells were isolated from primary vertical growth phase (VGP) tumors and from lymph node metastases surgically removed from 11 and 74 patients, respectively, at Istituto Nazionale Tumori (Milan, Italy) between 1985 and 1999. Sixty-six patients were in American Joint Committee on Cancer (AJCC) stage III and 19 in AJCC stage IV when tumors were removed. The melanoma cell lines used in this study were established from surgical specimens and maintained as described previously (23). All melanoma cell lines were evaluated before the tenth in vitro passage. Tissue sections from metastatic lesions of an unrelated group of 52 melanoma patients in AJCC stage III and IV were also used for immunohistochemical analysis.

Monoclonal antibodies. The Delta(Y)-specific mAb SY-4 and SY-5, the MB-1(X)-specific mAb SJ-3, the LMP2-p2-specific mAb SY-1, the LMP7-specific mAb HB-2, the LMP10-specific mAbs TO-6 and TO-7, the calnexin-specific mAb TO-5, the tapasin-specific mAb TO-3, the mAb HC-10 that recognizes a determinant expressed on all classical HLA-I and C heavy chains and on nonclassic HLA-A10, HLA-A28, HLA-A29, HLA-A30, HLA-A31, HLA-A32, and HLA-A33 heavy chains, the β2-M-free mAb L360, the HLA-DR, HLA-DQ, and HLA- DP-specific mAb LGF612-14, and the MHC class I-related molecule B (MHCBI)-specific mAb SJ-5 were developed and characterized as described (18, 19, 24–26). The TAP1-specific mAb NOB-1 and the TAP2-specific mAb NOB-2 are secreted by hybridomas derived from the corresponding metastatic lesions. The results of this analysis were evaluated for the potential association of the APM and HLA class I and II profiles with relevant clinical variables.

Data analysis and statistics. Spearman correlation analysis was used to correlate immunohistochemical data with flow cytometry analysis of melanoma cells and to correlate expression of APM components and HLA class I and II antigens in short-term melanoma cell lines. Expression of markers, in terms of percentage positive cells and of MFI, was compared in primary tumors versus metastases by Mann-Whitney test. Correspondence analysis (28) and hierarchical clustering of APM and HLA phenotype were carried out as described previously (27, 29) using the J-Express Pro 2.6 software (http://www.Molmine.com). MFI values were used for hierarchical clustering of APM and HLA profiles. The flow cytometry data were subjected to normalization before carrying out hierarchical clustering because each marker had a distinct range of fluorescence intensity values in the panel of cell lines. To this end, the ratio of the MFI of each marker to the median MFI of the same marker in the panel of 85 cell lines was evaluated. Normalized expression values were then clustered by both tumor and antigen. Survival analysis was done by the Kaplan-Meier method, and survival curves were compared by the log-rank test. The Cox proportional hazards model and the Wald statistic, by Biometric Research Branch Array Tools version 3.3.0, developed by Dr. Richard Simon and Dr. Amy Peng Lam (Biometric Research Branch, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MD) were used to test for association of single antigens with patients’ survival. Permutation P values for significant antigens were computed based on 10,000 random permutations.

Results and Discussion

Close correlation between melanoma lesions and corresponding short-term cell lines in the expression of APM components and HLA class I and II antigens. Preliminary experiments compared the results of the analysis of APM component, HLA class I antigen, β2-M, and HLA class II antigen expression in surgically removed metastatic lesions, assessed by immunohistochemical staining with mAbs and, in the corresponding short-term (<10 in vitro passage) cell lines, assessed by flow cytometry analysis following intracellular staining with mAbs. A positive and significant correlation was found between the results, expressed as percentage stained melanoma cells, obtained by flow cytometry and immunohistochemistry (see Fig. 1 for representative results from one patient; see Supplementary Fig. S1 for results from two additional patients). These results indicate that the phenotype of the short-term melanoma cell lines is representative of that of the corresponding metastatic lesions.

Heterogeneity and defects in the expression of APM components and HLA class I and II antigens in a panel of short-term melanoma cell lines. Eighty-five short-term cell lines from 11 primary VGP melanoma lesions and 74 lymph node metastases were permeabilized and then stained with APM component-specific, HLA class I-specific, HLA class II-specific, and MHCIBI-specific mAbs. To provide an initial classification of the phenotypic profiles of all tumors, the results were analyzed by correspondence analysis as described (27). Correspondence analysis is an exploratory technique for multivariate data analysis that reduces the complexity of multidimensional data sets to a low-dimensional projection of the data called “biplot” (28). Correspondence analysis provided a two-dimensional projection of the expression patterns of each of the markers (Fig. 2A) and
identified six phenotypic subsets of tumors (subsets 1-6 in Fig. 2A). Each subset contained tumors with similar overall APM and HLA profiles. The APM and HLA profiles of the 85 tumors were then visualized (Fig. 2B) according to the six subsets identified by correspondence analysis. Expression of all markers, although with quantitative variations in the level of most of them, characterized subset 1 (6 lesions). A progressive reduction in LMP2, LMP7, LMP10, ERp57, and MICB expression characterized subsets 2 and 3 (seven and eight lesions, respectively). Heterogeneity for MB1, calreticulin, and tapasin expression, frequent LMP2, LMP7, LMP10, ERp57, or MICB loss, and high HLA class II antigen expression characterized subset 4 (23 lesions). Similarity to the phenotype of subset 4, in terms of several APM components, but progressive reduction in ERp57 and HLA class II antigen expression, and increasing heterogeneity in HLA class I expression characterized subsets 5 and 6 (24 and 17 lesions, respectively). Furthermore, comparison of the phenotype of the cell lines originated from primary lesions and metastases showed a significantly higher Delta, calreticulin, and \( \beta_2 \)M expression, in terms of both percentage stained cells and MFI, in the latter cell lines than in the former ones (Supplementary Table S1).

The phenotypic heterogeneity in the panel of melanoma cell lines analyzed resulted from variations not only in percentage stained cells but also in MFI as documented by the wide range of MFI values observed for markers, such as Delta, TAP calnexin, calreticulin, tapasin, HLA class I and II antigens, and \( \beta_2 \)M (Supplementary Table S1). For some of these markers, MFI values were even more heterogeneous (therefore, more discriminative of the different phenotypes) than percentage stained tumor cells. For example, when the phenotype of tumor cells was expressed as percentage stained melanoma cells, markers, such as Delta and calnexin, showed a narrow range of variation in their expression, being expressed on >90% of malignant cells in most tumors (Fig. 2C). In contrast, MFI values for the same markers showed a wide range of values (26-201 for Delta and 29-777 for calnexin).
The results of the correspondence analysis showed similar expression patterns of distinct markers as indicated by clustering of Delta, calnexin, TAP subunits, HLA class I antigens, and β2M in the biplot (Fig. 2A). To evaluate the statistical significance of the similarities in the patterns of expression of several markers, we then carried out a Spearman correlation analysis of all 136 possible correlations generated by evaluating all combinations of the 17 markers analyzed, two at a time. MFI values were used for this purpose, as in several instances this variable was more discriminative of the phenotypes of the tumors than percentage stained cells. Sixty-seven of the 136 possible correlations were significant (Supplementary Fig. S2). Interestingly, 61 of the significant correlations had a positive coefficient (r) and involved proteasome subunits, TAP subunits, peptide-loading complex chaperones, HLA class I antigens, and β2M. This finding suggests that several components of the HLA class I APM are coordinately expressed in melanoma cells. This conclusion is corroborated by the lack of significant correlation between level of HLA class I APM components and that of HLA class II antigens, with the exception of a positive and significant correlation between level of HLA class I and II (Supplementary Fig. S2).

Taken together, these results indicate that the APM and HLA class I and II phenotype of a large panel of short-term melanoma cell lines, as evaluated by quantitative assays, is characterized by marked heterogeneous expression of most of the markers analyzed and by coordinated expression of several of the HLA class I APM components.

Hierarchical clustering of the HLA and APM profiles of 85 tumors identifies two clusters of patients characterized by significantly different overall survival. As a first approach to evaluate the potential association of APM component and HLA phenotype of the tumors analyzed with clinical variables, we carried out a hierarchical clustering as described (27, 29). In this analysis, MFI values were used after normalization to the median value of each marker in the whole panel of tumors (see Materials and Methods). The resulting clusters were then compared for association with relevant clinical variables, as clinical data and long-term survival information were available for 84 of the 85 patients investigated. This analysis (Fig. 3A) identified two main clusters of tumors, (cluster 1, 37 lesions; cluster 2, 48 lesions) and two main sets of antigens (antigen set 1 and antigen set 2; Supplementary Fig. S2).

**Figure 2.** Phenotypic heterogeneity and defective expression of HLA and APM components in 85 short-term melanoma cell lines. A, correspondence analysis of the phenotypic profiles of 85 tumors characterized by flow cytometry in permeabilized cells with a panel of 17 mAbs to HLA and APM components. Correspondence analysis results were plotted in a two-dimensional (Dim.1 versus Dim.2) scatter plot. ○, tumors; ■, antigens. Density plot, dotted lines, based on their HLA and APM profiles, the 85 tumors defined six subsets (1-6). Two different anti-LMP10 and two different anti-Delta mAbs (1 and 2) were used. B, HLA and APM profiles of the 85 tumors for each of the markers recognized by the panel of 17 mAbs. P, primary VGP tumor; M, metastasis. Tumors were grouped according to the six subsets identified by correspondence analysis in (A). Gray, results expressed as % positive cells for each marker. ●, tumors belonging to cluster 1 of Fig. 3A. C, Delta and calnexin expression in the panel of 85 tumors. Results expressed as % positive cells or as MFI. Horizontal lines, median value in each plot.
Fig. 3. Two clusters of tumors, identified by hierarchical clustering of the HLA and APM profiles, are associated with distinct patients’ survival. A, hierarchical cluster analysis of APM and HLA class I and II antigen profiles of 85 tumors. Normalized antigen expression values (based on MFI values) were clustered by both tumor and antigen. Two different anti-LMP10 and two different anti-Delta mAbs (1 and 2) were used. *, VGP primary tumor (light gray) and metastasis (dark gray); stage IIIb (light gray); stage IIc (dark gray); stage IV (black). +, IFN-α (green); chemotherapy (orange); IFN-α followed by chemotherapy (brown); TP5 (red); surgery only (white). B, Kaplan-Meier survival analysis of patients according to the two main clusters identified in (A). Survival was evaluated as time from surgical removal of the neoplastic lesions used for generating the short-term cell lines characterized in (A).

No significant differences were found between the two clusters of tumors for all the relevant clinical variables, including (a) stage of progression (primary lesion versus metastatic; $P = 0.335$), (b) clinical stage of the disease at time tumors were surgically removed (AJCC stage IIIb versus stage IIc versus stage IV; $P = 0.192$), and (c) type of therapy administered after surgery ($P = 0.364$). In contrast, Kaplan-Meier survival analysis and log-rank test showed that patients, whose tumors fell in cluster 1, had a significantly better overall survival, measured from the time of surgical removal of the tumor to death, than patients in cluster 2 (Fig. 3B; $P = 0.0063$). This significant difference persisted even when survival was measured as time from the initial diagnosis of primary melanoma to death ($P = 0.0147$; data not shown). The potential association with patients’ survival of any of the APM components and HLA antigens was then evaluated by the Cox proportional hazards model. β2M (permutation $P = 0.0175$; hazard ratio = 0.467) and HLA class II antigens (permutation $P = 0.0164$; hazard ratio = 0.699) resulted to be significantly associated with patients’ survival. To corroborate the validity of these results, the association of HLA class II molecule expression with patients’ survival was further investigated in two sets of experiments. First, HLA class II antigen expression was evaluated by immunostaining with mAb LGII-612.14 in the tumors used to isolate the short-term melanoma cell lines. The 24 lesions available for this purpose were from 12 patients in cluster 1 and from 12 in cluster 2. Most of the lesions from the latter group of patients were either not stained or weakly stained with mAb LGII-612.14 (Supplementary Fig. S3). In contrast, all the tumors from patients in cluster 1 were stained with the mAb LGII-612.14 with a homogeneous or a heterogeneous pattern in agreement with HLA class II expression in the corresponding cell lines. The only exception was the tumor from patient 66 (Supplementary Fig. S3). The short-term cell lines corresponding to these two subsets of lesions differed significantly in terms of both percentage stained cells (mean 76.8% for the 12 cell lines from cluster 1 versus 15.5% for the 12 cell lines from cluster 2; $P < 0.0001$) and MFI (mean 146.1 for the 12 cell lines from cluster 1 versus 7.3 for the 12 cell lines from cluster 2; $P < 0.0001$).

Furthermore, the association of HLA class II antigen expression in melanoma cells with patients’ survival was evaluated using metastatic lesions from an unrelated group of 52 AJCC stage III or IV melanoma patients (Supplementary Table S2). To this end, tissue sections were stained by immunohistochemistry with mAb
Based on HLA class II antigen expression, lesions where classified into four subsets (see legend to Supplementary Table S2). Kaplan-Meier survival analysis and log-rank test showed that patients, whose tumors where classified as “positive” or “heterogeneous,” had a significantly longer overall survival, measured as time from surgical removal of the metastatic lesion to death, than patients whose tumors were classified as either “weak” or “negative” (Supplementary Fig. S4; \( P = 0.0115 \)). No significant differences were found between these two groups of patients in terms of clinical stage (i.e., AJCC stage IIIb, IIIc, or IV) at time when tumors were removed \((P = 0.471)\) and type of therapy administered after surgery \((P = 0.376)\).

These results suggest that quantitative variations in the expression level of APM components and HLA class I and II antigens in melanoma cells from AJCC stage III and IV patients may have an effect on the clinical course of the disease. The mechanism underlying this association is likely to be immunologic because patients’ survival is associated with the expression in neoplastic lesions of HLA class II antigens and \(\beta_2\)M, which play a key role in the generation of HLA class II antigen-peptide and HLA class I antigen-peptide complexes recognized by CD4+ and CD8+ T cells, respectively. One possibility is that the T-cell–mediated antitumor response may be more effective (at either the priming and/or effector phases) in metastatic lesions with retained or higher levels of expression of HLA class II antigens and \(\beta_2\)M. In fact, defective generation of HLA class II-peptide and HLA class I-peptide complexes might impair the initial activation of antitumor immunity if such phase relies on direct T-cell priming by tumor cells instead of being mediated by dendritic cell–dependent cross-priming (30). Similarly, expansion at tumor site of previously primed T cells and effective recognition of neoplastic cells by effector T cells may be affected by the extent of expression of HLA-peptide complexes by neoplastic cells. Interestingly, by looking at frequency and differentiation phenotype of melanoma-specific T cells from tumor-invaded lymph nodes, we found the highest frequency of tumor antigen-specific T cells (and with a differentiated phenotype) in the lesions that retained expression of the tumor antigens and of the appropriate HLA allele acting as restricting element for such T cells (23). Moreover, recent results have indicated that presence of melanoma-specific T cells at tumor site is a significant prognostic factor predicting improved survival in advanced-stage melanoma (31).

The significant association of HLA class II antigen expression with longer survival, found in this study, agrees with the results obtained in large B-cell lymphomas and colorectal carcinomas (16, 17) but not with previous results obtained by immunohistochemistry in metastatic melanoma patients, where a low expression of HLA class II on neoplastic cells was associated with longer survival (8). However, in the latter study, survival was evaluated by comparing groups of patients defined by differential expression in neoplastic cells of both HLA class I and HLA class II antigens and not only of HLA class II antigens. This may contribute to explain the discrepancy with the current study.

A few of the HLA and APM markers that we evaluated in the present study are progressively down-regulated during tumor progression in melanoma and other tumors (9, 10). In addition, mutations of \(\beta_2\)M gene, resulting in loss of functional protein, have been described in human metastatic melanomas (32). Moreover, expression of HLA class I and TAP proteins has been shown previously to correlate with survival in carcinomas of the head and neck and in primary melanomas (11, 13). Taken together, these results suggest that the HLA and APM component profiles may be relevant to the clinical outcome in different human tumors. In addition, we found that LMP2 and LMP7 immunoproteasome subunits and Erp57 were the markers most frequently not detectable or expressed at very low levels among 85 tumors. In agreement with these findings, reduced expression of LMP proteasomal subunits has been reported in melanoma and other tumors (10, 12, 33). In contrast, most tumors investigated in this study showed frequent, although heterogeneous expression of constitutive proteasomal component Delta, of TAP subunits, and of different components of the peptide-loading complex, such as the chaperones, calnexin, calreticulin, and tapasin. Interestingly, several of these molecules involved in the HLA class I antigen-processing and presentation pathway showed significant correlations in the extent of their expression in melanoma cells. This suggests that HLA class I and APM component expression is, to some extent, coordinated in melanoma cells as proposed also by Giorda et al. (34). The retention of several APM components even in late-stage metastases may be explained even on the basis of the function that these molecules exert, in addition to antigen-processing and MHC peptide-loading complex. In fact, these molecules do also several relevant functions needed for cell survival, cell cycle progression, and inhibition of apoptosis, such as the quality control of newly synthesized proteins in the ER (35) and the degradation by the proteasome of proteins tagged by the ubiquitin system (36). Thus, two opposing selection forces may contribute to shape the HLA and APM phenotype of neoplastic cells in advanced tumors. On one hand, neoplastic cells may attempt to maintain protein degradation and ER function pathways relevant for cell survival. On the other hand, the function of these pathways will favor the production of MHC-peptide complexes recognized by T cells, thus exposing the tumors to negative selection by the immune system. Interestingly, among the 85 cell lines used in this study, 39 were HLA-A2+, allowing to evaluate these tumors for recognition by HLA-A2-restricted CTLs directed to two different melanoma antigens (Melan-A/Mart-1 and gp100) and to correlate the results with antigen expression and with the HLA and APM phenotype of the neoplastic cells. In this subset of tumors, we found reduced or absent melanoma lysis by Melan-A/Mart-1-specific and gp100-specific CTLs in 13 of 39 and in 17 of 39 melanomas, respectively. Such impaired CTL-mediated recognition was explained by loss/defective expression of the melanoma antigens Melan-A/Mart-1 and gp100 in the neoplastic cells, although some significant correlations with defective APM component expression were also found (data not shown). These results support a model in which “immune editing” of the tumor phenotype (37) during tumor progression may promote selection of those neoplastic cells, whose genes coding for TAAs (not essential for cell survival) were silenced in preference to tumor cells with widespread defects in most of their APM components.

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