Human Leukocyte Antigen Class I Expression
Marker of Poor Prognosis in Uveal Melanoma

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Purpose. Because the expression of human leukocyte antigen (HLA) antigens is important for immunologic recognition of tumor cells, we determined expression of locus-specific HLA class I antigens in uveal melanoma and tested whether the level of HLA expression was related to prognosis or associated with known prognostic parameters.

Methods. Expression of HLA-A and -B antigens was determined on 30 formalin-fixed and paraffin-embedded sections of uveal melanoma by immunohistochemistry with locus-specific monoclonal antibodies and scored semiquantitatively.

Results. The level of expression of HLA-A and -B varied between uveal melanomas. Expression levels of HLA-A and -B were significantly correlated \( (P = 0.02) \). High HLA-B expression was significantly correlated with the presence of epithelioid cells \( (P = 0.04) \) in the tumor. Expression levels of HLA-A as well as of HLA-B, cell type, mitotic rate, Mib-1 score, and largest tumor diameter were significant predictive factors for survival. High expression of HLA-A and -B was associated with a decreased survival. Multiple Cox regression analysis with stepwise selection of covariates showed that the contribution of HLA-A expression to survival \( (P = 0.0003) \) exceeded that of tumor diameter \( (P = 0.02) \) and Mib-1 score \( (P = 0.04) \).

Conclusions. Lack of expression of HLA-A as well as of HLA-B antigens on uveal melanoma is correlated with a better patient survival. Our data suggest that shedding of uveal melanoma micrometastases with a low expression of HLA class I into the systemic circulation may facilitate their removal and prevent the development of metastases. These findings support a protective role for natural killer cells in the development of metastatic disease in uveal melanoma. Invest Ophthalmol Vis Sci. 1997;38:1865–1872.

Uveal melanoma is the most common primary malignant intraocular tumor in adults, with an annual incidence of six cases per million in white populations. Uveal melanoma has a high mortality rate due to a high incidence of metastases, which have a preference for the liver. No effective treatment of metastases is available as yet, and the role of the immune system in the development of metastases is unclear. Several tumor-specific antigens that were previously identified on skin melanomas were recently also detected on uveal melanoma and may be a target for cytotoxic T-lymphocytes (CTLs). Expression of human leukocyte antigen (HLA) class I antigens is essential for presentation of such tumor-specific antigens and an effective antitumor T-cell response. Lack of expression of the specific HLA molecules might lead to “tumor escape.” However, expression of the HLA class I antigens may play a dual role with regard to recognition of tumor cells by the immune system: whereas tumor-cell recognition by CTLs needs expression of HLA class I molecules, the presence of these molecules turns off natural killer (NK) cells. Therefore, changes in expression of the class I molecules are likely to have a direct effect on the susceptibility of the tumor cells to lysis by either CTLs or NK cells and hence can influence the development of metastases.
Studies have been performed to investigate a possible relation between HLA expression and patient survival in a wide range of tumors. For instance, an analysis of cutaneous melanomas showed that patients with HLA-A-, HLA-B-, and HLA-C-negative tumors had shorter survival times than patients with lesions in which more than 50% of tumor cells were stained by anti-HLA class I antibodies. Similarly, in a study of primary breast carcinoma, Concha et al reported worse survival times in patients with HLA-A-, HLA-B-, and HLA-C-negative tumors than in those with positive tumors. However, reports about the influence of HLA expression on patient survival vary: studies on colorectal cancer, non-small cell lung carcinoma, and prostate cancer failed to demonstrate a significant prognostic influence of HLA-A, -B, and -C expression.

Previous studies in uveal melanoma showed variable levels of expression of monomorphic HLA molecules, as well as of polymorphic HLA antigens. In a recent study on uveal melanoma, De Waard-Siebinga showed that expression of HLA class I antigens, as determined by immunohistology using the antibody W6/32, did not correlate with survival. However, that study involved only monomorphic HLA class I expression. Because presentation of tumor-specific peptides occurs only in association with specific HLA alleles, it may be that quantitative differences in expression of the different polymorphic HLA alleles is of greater importance to the effective presentation of tumor-specific peptides than expression of backbone molecules, and therefore has a greater impact on survival.

In this study, we retrospectively determined the expression of the HLA-A and -B locus-specific products on 30 uveal melanomas and investigated whether expression of these alleles was of prognostic significance.

MATERIALS AND METHODS

Patients and Tumors

A retrospective analysis of 30 formalin-fixed, paraffin-embedded uveal melanomas was undertaken to determine expression of HLA-A and -B. These 30 uveal melanomas were part of a previously studied group of 51 uveal melanomas. From 1973 to 1987, consecutive cases were entered in the study on the basis of availability of adequate histologic material. This research protocol followed the tenets of the Declaration of Helsinki. Enough material was available in the paraffin blocks to obtain material for the current study in 30 of the 51 cases. Follow-up data were obtained by contacting the general practitioner or the local ophthalmologist, and these data were reviewed to define tumor-related death or death as a result of other causes.

Of the 30 patients studied, 9 were female and 21 were male. Age varied between 17 and 78 years, with a mean age at diagnosis of 57 years. Sixteen patients died of a tumor-related cause, 5 died of other causes, 6 were still alive, and 3 were lost to follow-up at the time of our study. The mean follow-up time was 84 months.

Histologic Specimens

The tumors were histologically classified according to the presence or absence of epithelioid cells (spindle cell melanoma versus nonspindle—that is, a combination of mixed and epithelioid cell types in one tumor). The cell type of the tumors was classified as spindle in 11 cases and as nonspindle in 19 cases. Two tumors were small (largest tumor diameter [LTD] <10 mm), 12 were medium (LTD 10 to 15 mm), and 16 were large (LTD >15 mm) (Table 1). Mitoses were counted in 15 high-power fields with a total magnification ×400, using an eyepiece grid. This procedure was repeated four times, and the number of mitoses was averaged.

Immunohistochemistry

Formalin-fixed and paraffin-embedded 5-μm sections were mounted on glass slides coated with aminopropyltriethoxysilane (APES, Sigma, St. Louis, MO) and dried overnight at 37°C. After deparaffinization and rehydration, the slides were immersed in 3% H2O2 in methanol for 20 minutes. After washing in distilled water and phosphate-buffered saline (PBS), the slides were placed in a sequencer. The sections were washed in PBS, which was also used for all subsequent washes, and preincubated with normal goat serum in a dilution of 1:10 PBS with 5% BSA. After a wash in PBS–0.05% Tween 20 (PBS-T), the slides were incubated for 30 minutes with biotinylated goat anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark) in a dilution of 1:50. The slides were incubated for 30 minutes with the monoclonal antibody (mAb) HCA2 with a specificity for HLA-A at a dilution of 1:1,600, with mAb HC10 with a specificity for HLA-B in a dilution of 1:12,800, or with PBS as negative control. After washing in PBS–0.05% Tween 20 (PBS-T), the slides were incubated for 30 minutes with biotinylated goat anti-mouse immunoglobulin (Dakopatts, Glostrup, Japan) in a dilution of 1:100, 2% normal human serum, and 2% normal goat serum in PBS with 5% BSA. After a wash in PBS-T, the slides were incubated for 30 minutes with peroxidase-conjugated avidin–biotin complex (Biogenex, San Ramon, CA) in a dilution of 1:50. The slides were removed from the sequencer and after a wash with PBS for 5 minutes were placed in acetate buffer (0.1 M, pH = 4.0) for 30 minutes. Finally, the slides were placed in 10% AEC (3-amino-9-ethyl-carbazole) in acetate buffer with 200 μl H2O2 for 30 minutes in the dark. Hereafter, the sections were washed in...
TABLE 1. Clinicopathologic Parameters

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<th>Patient's Age (years)</th>
<th>Patient's Sex</th>
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<th>Follow-up (months)</th>
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LTD = largest tumor diameter; Mib-1 = percentage of cells reacting with the proliferation-associated antigen Ki-67; — = no follow-up after enucleation.

* Patient survival: 0 = still alive; 1 = death due to tumor; 2 = other cause of death; 9 = lost to follow-up.

acacetate buffer for 30 minutes and rinsed in running water, counterstained with Mayer’s hematoxylin, and mounted in Kaiser’s glycerin-gelatin. All incubations and staining were performed at room temperature. As positive control for HLA expression, sections of spleen and lymphoid tissue were used. As a negative control, specimens were stained using the same incubation protocol without use of the primary mAbs.

The mAb Mib-1, reacting with the proliferation-associated antigen Ki-67 (Dianova-Immunotech, Hamburg, Germany), which is expressed on proliferating cells, was used in a dilution of 1:200 in an overnight incubation protocol at 4°C. The Mib-1 score was determined as the percentage of Mib-1-positive cells relative to the total number of cells per high-power field.

Assessment of Results

Immunohistochemical results were evaluated without access to the follow-up data. Two independent investigators (D-JRB, CMM) determined the HLA-A and -B scores semiquantitatively as a percentage of positive cells (0, 1% to 25%, 26% to 50%, 51% to 75%, 76% to 100%). Interobserver disagreements did not exceed one class. Differences were reevaluated until consensus was reached.

Statistical Analysis

Spearman’s correlation coefficient was used to quantify and to test the association between the different variables. A two-sided P < 0.05 was considered significant. The association between death secondary to uveal melanoma (with death from other causes censored) and expression of HLA-A and -B, cell type, LTD, Mib-1 score, and mitotic rate were assessed with Kaplan–Meier survival analysis and with the log rank test and Cox proportional hazards regression model. The group of 30 patients was compared to the group of 21 patients from the previous study (who were excluded because of lack of material) with Student's t-test, the chi-square test, the Mann-Whitney test, or the log rank test, where appropriate. Comparison between the group of 30 included patients and the group of
21 excluded patients revealed that these groups did not differ with regard to cell type, LTD, age, sex, mitotic rate, Mib-1 score, median follow-up, or patient survival.

RESULTS
Immunohistochemistry
Expression of HLA-A and -B was not observed on the tumor cells of 10 and 12 tumors, respectively (Fig. 1). On other tumors, variable levels of expression were noted: five tumors had HLA-A on at least 75% of the cells and two tumors had HLA-B on at least 75% of the cells. Expression levels of HLA-A and -B were significantly correlated (p = 0.42, P = 0.02). A significant relation existed between expression of HLA-B and cell type: a high expression of HLA-B was related to the presence of nonspindle cells (Table 2). No significant correlations were observed among HLA-A or -B expression and LTD, Mib-1 score, and mitotic rate.

Survival
In the 30 patients studied, 5- and 10-year tumor-related survival rates were 62% (standard error = 10%) and 46% (standard error = 11%), respectively. Of the 20 patients with tumors expressing HLA-A, 15 died of uveal melanoma metastases, 2 died of another cause, 1 was still alive, and 2 were lost to follow-up. Of the 10 patients with tumors not staining for HLA-A, 2 died of uveal melanoma metastases, 2 died of another cause, 5 were still alive, and 1 was lost to follow-up. Of the 18 patients with tumors expressing HLA-B, 11 died of uveal melanoma metastases, 2 died of another cause, 3 were still alive, and 2 were lost to follow-up. Of the 12 patients with tumors not staining for HLA-B, 5 died of uveal melanoma metastases, 3 died of another cause, 3 were still alive, and 1 was lost to follow-up. A clear difference in the 5- and 10-year tumor-related survival rates was observed between patients with tumors with and without HLA-A and -B expression (Figs. 2A, 2B). The hazard rate of the extent of HLA-A expression for tumor-related survival was 2.21 (95% confidence interval [ci], 1.34 to 3.63; P = 0.004). An effect on tumor-related survival was also found for HLA-B expression (hazard rate = 1.76; 95% ci, 0.98 to 3.15; P = 0.04). In addition, a significant association was observed between survival and the parameters LTD and cell type (Table 3, Figs. 2C, 2D). In stepwise analysis according to the Cox model, HLA-A expression was found to be the strongest independent predictor of tumor-related survival (Table 4): the adjusted hazard rate of HLA-A expression was 2.10 (95% ci, 1.38 to 3.19; P = 0.0003), followed by LTD and Mib-1 score. HLA-B expression was not found to be an independent predictor of survival.

DISCUSSION
Because CTLs are considered of great importance in the immune response against malignant cells, we expected to find that a low level of HLA class I expression on uveal melanoma cells would be associated with “tumor escape” and therefore with death resulting from metastases.2223 However, in this study, we observed the opposite: High expression of HLA-A and -B molecules in uveal melanoma was significantly correlated with

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<th>1-25%</th>
<th>26-50%</th>
<th>51-75%</th>
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<tr>
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<td>1-25%</td>
<td>26-50%</td>
<td>51-75%</td>
<td>76-100%</td>
</tr>
</tbody>
</table>

**TABLE 2.** Association Among Immunohistochemical Parameters, Spearman Correlation Coefficients (r), and Two-Sided P Values

- **Tumor Diameter**
  - Cell type: r = 0.08, P = 0.67
  - Tumor diameter: r = 0.36, P = 0.02
- **Mitotic Rate**
  - Cell type: r = 0.23, P = 0.22
  - Tumor diameter: r = 0.32, P = 0.08
  - Mitotic rate: r = 0.53, P = 0.003
- **HLA-A**
  - Cell type: r = 0.26, P = 0.17
  - Tumor diameter: r = 0.09, P = 0.09
  - Mitotic rate: r = 0.23, P = 0.23
- **HLA-B**
  - Cell type: r = 0.38, P = 0.04
  - Tumor diameter: r = 0.63
  - Mitotic rate: r = 0.30
  - HLA-A: r = 0.42, P = 0.02

Mib-1 = percentage of cells reacting with the proliferation-associated antigen Ki-67; HLA = human leukocyte antigen.

* Significant value.
death from metastases. We also found a significant relation between expression of HLA-B and cell type: a high expression of HLA-B was related to the presence of the prognostically unfavorable nonspindle cells (Fig. 3). This finding is in line with the observation of Natali et al., who noticed that in 12 surgically removed uveal melanomas, the anti-HLA class I mAb W6/32 and the anti-β2-microglobulin mAb BBM.1 did not stain the spindle A and B lesions (n = 3) but stained all the mixed (n = 4) and prognostically unfavorable epithelioid type (n = 5) lesions.

Our data show an association between expression of HLA antigens on a primary uveal melanoma and the presence of metastases. It may be that not CTLs but NK cells play an essential role in immune responses directed against uveal melanoma metastases. It is generally accepted that the presence of class I molecules protects cells from NK cell-mediated lysis by generating a negative signal to the NK cell receptor.24 NK cells recognize HLA class I molecules and can discriminate between groups of HLA loci and alleles.25 As a consequence, expression of HLA class I on uveal melanoma cells may block NK cell-mediated lysis, and therefore it may be that shedding of uveal melanoma cells with a high expression of HLA class I into the systemic circulation especially facilitates the development of tumor metastases. Although it is possible that the expression of HLA class I on tumor cells at the time of enucleation (when we looked) is not representative of tumor cells at the time they migrate from the eye, our theory is supported by the recent finding that an excellent correlation exists between the presence of HLA class I antigen expression and poor NK cell lysis of uveal melanoma cell lines in vitro.26 In addition, a good correlation was observed between lysability and the absence of hepatic metastases when uveal melanoma cell lines were injected into nude mice.27

With regard to other tumors, evidence has accumulated that in humans, NK activity might be important in the control of metastases and that patients with advanced metastatic disease often have abnormalities in NK cell function or numbers.27 Animal models have shown that NK cells are especially suited for eliminating tumor cells in the circulation and thus serve as the earliest cellular effector mechanism against dis-

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**FIGURE 2.** Kaplan–Meier survival curves for 30 patients, depicting the probability of death due to uveal melanoma (with death from other causes censored) versus expression of HLA-A (A), HLA-B (B), cell type (C), and largest tumor diameter (D). The level of expression was scored semiquantitatively as a percentage of positive cells (0, 1% to 25%, 26% to 50%, 51% to 100%).
semination of blood-borne metastases. Because uveal melanoma metastases specifically spread hematogenously, NK cell lysis may play an essential role; however, in other types of cancer, spreading may occur lymphatically and NK cells may not be relevant. This hypothesis is experimentally supported by data from Algarra et al, who found that major histocompatibility complex class I-positive clones from a heterogenous fibrosarcoma were oncogenic after intravenous injection in mice, but after subcutaneous inoculation, MHC class I-negative tumor cells were more oncogenic than their class I-positive counterparts.

The significant relation between HLA expression and death that we observed may also be due to the fact that hardly any response is possible against the primary tumor. It seems probable that the immunologic response against the primary intraocular tumor is inhibited because of local circumstances. Although T lymphocytes are often present in uveal melanomas in small numbers, they are probably not intraocularly active and do not contribute to tumor cell elimination. Ksander et al demonstrated that T cells freshly isolated from uveal melanomas are not directly cytotoxic; this may be due to the characteristics of the intraocular fluids. Kaiser et al showed that the aqueous humor from mice and rabbits inhibits T-lymphocyte proliferation, and Apte and Niederkorn and Ma and Niederkorn showed that aqueous humor also blocks NK cell function. Both findings may be related to the high concentration of transforming growth factor-β found in the aqueous humor (e.g., 2.0 ng/ml). Taken together, these findings indicate that CTL and NK immune responses may not play an important role in the defense against tumors inside the eye, allowing growth to occur, regardless of the level of HLA expression on the tumor.

Our observation that significance of HLA-B for patient survival did not appear in multivariate analysis suggests that HLA-B is not an independent factor for survival and that its significance for prognosis as determined in univariate analysis might be attributed to its correlation to HLA-A. The observation in a previous study on uveal melanoma that no correlation between expression of HLA antigens and patient survival was discernible probably can be attributed to the use of the antibody W6/32, with a specificity for monomorphic HLA-A, -B, and -C. Our data show that with regard to survival, expression levels of the different polymorphic HLA antigens are more important than expression of backbone molecules as determined with mAb W6/32.

In summary, in this study we demonstrate that the absence of HLA antigens on uveal melanoma is correlated with better patient survival. This may be cause by the hematogenous spreading of micrometastases, which may increase the role of NK cell-mediated lysis in removing HLA class I-negative cells from the peripheral blood. In the search for the most effective clinical treatment of uveal melanoma, attention is focused on two goals with regard to survival, expression levels of the different polymorphic HLA antigens are more important than expression of backbone molecules as determined with mAb W6/32.

TABLE 3. Univariate Hazard Rates of Prognostic Parameters of Tumor-Related Survival

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<td>1.18</td>
<td>1.00-1.39</td>
<td>0.045</td>
</tr>
<tr>
<td>Cell type</td>
<td>4.05</td>
<td>1.14-14.39</td>
<td>0.05</td>
</tr>
<tr>
<td>Mitotic rate</td>
<td>1.13</td>
<td>0.99-1.30</td>
<td>0.07</td>
</tr>
<tr>
<td>Mib-1 score</td>
<td>1.29</td>
<td>0.88-1.89</td>
<td>0.19</td>
</tr>
</tbody>
</table>

HR = hazard rate; CI = confidence interval; HLA = human leukocyte antigen; Mib-1 = percentage of cells reacting with the proliferation-associated antigen Ki-67.

* Log rank test.

TABLE 4. Multivariate Cox Regression Analysis of Prognostic Parameters of Uveal Melanoma in 30 Patients, With Death From Causes Other Than Uveal Melanoma Censored

<table>
<thead>
<tr>
<th>Multiple Cox Regression Analysis</th>
<th>HR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-A</td>
<td>2.10</td>
<td>1.38-3.19</td>
<td>0.0003*</td>
</tr>
<tr>
<td>Largest tumor diameter</td>
<td>1.23</td>
<td>1.03-1.47</td>
<td>0.02</td>
</tr>
<tr>
<td>Mib-1 score</td>
<td>1.58</td>
<td>1.03-2.43</td>
<td>0.04</td>
</tr>
</tbody>
</table>

HR = hazard rate; CI = confidence interval; HLA = human leukocyte antigen; Mib-1 = percentage of cells reacting with the proliferative-associated antigen Ki-67.

* When death from other causes was also included, no significant relation was found between death from all causes and expression of HLA-A (P = 0.18).
and second, specific CTLs might be induced to remove tumor cells expressing HLA class I once they have spread to peripheral sites.

**Key Words**

human leukocyte antigen expression, patient survival, uveal melanoma

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