A R T I C L E S

Association of HLA Class I and Class II Antigen Expression and Mortality in Uveal Melanoma

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PURPOSE. Malignant transformation of cells is frequently associated with abnormalities in human leukocyte antigen (HLA) expression. These abnormalities may play a role in the clinical course of the disease, because HLA antigens mediate interactions of tumor cells with T cells and NK cells. Uveal melanoma is a highly malignant tumor of the eye and is characterized by a hematogenic spread to the liver. Little is known about the role of HLA expression in progression of this malignant disease.

METHODS. In the present study HLA class I antigen, β_2-microglobulin (β_2-m), and HLA class II antigen expression was analyzed in primary uveal melanoma lesions by immunoperoxidase staining with monoclonal antibodies of 65 archival clinical samples. The results were correlated with the clinical course of the disease.

RESULTS. HLA class I antigen expression and β_2-m expression were downregulated in 40 and 35 lesions, respectively. HLA class II antigens were expressed in 30 lesions. Patients with high HLA class I, including β_2-m, and HLA class II antigen expression in their primary melanoma lesions had a significantly decreased survival ($P = 0.009$, $P < 0.001$, and $P = 0.006$, respectively).

CONCLUSIONS. The findings argue against a major role of cytotoxic T-lymphocyte (CTL)–mediated control of tumor growth in the clinical course of uveal melanoma and are compatible with a potential role of NK-cell–mediated control of hematogenic metastatic spread. (*Invest Ophthalmol Vis Sci.* 2001;42: 2153–2156)

Uveal melanoma is the most common primary intraocular malignant tumor in adults, with an annual incidence of six cases per million in the white population.[1] Metastases from uveal melanoma have a preference for the liver and cause a high mortality rate.[2] No effective treatment of metastatic disease is yet available.

As in other types of malignant melanoma, immunologic events are believed to play an important role in the clinical course of uveal melanoma. This possibility has stimulated interest in the analysis of the expression of HLA antigens in uveal melanoma lesions, because these molecules are thought to play a major role in the interactions of melanoma cells with the host's immune system. HLA class I antigens, which consist of a polymorphic heavy chain noncovalently associated with β_2-microglobulin (β_2-m), restrict the interaction of target cells with cytotoxic T lymphocytes (CTLs).[3] HLA class II molecules, predominantly expressed on immunologically active cells, are required for antigen presentation of peptides to helper T cells.[3] This process is a key event in the activation of immune responses.

Major histocompatibility complex (MHC) class I antigen downregulation is common among human tumors and is believed to be an important factor in their escape from recognition and destruction by HLA class I antigen–restricted, tumor-associated, antigen-specific CTLs.[4] In contrast, MHC class I antigen loss may result in increased sensitivity of malignant cells to NK cells.[5] Ma and Niederkorn[6] have reported that TGF-β can alter MHC class I antigen expression and the susceptibility of ocular melanoma cells to NK-cell–mediated cytolysis. The association of HLA class I antigen downregulation in malignant lesions with poor prognosis in various types of malignancies suggests that CTLs play a major role in tumor growth control. HLA class II antigens have been found in a number of malignancies, although with variable frequency.[7] The role of HLA class II antigens in the interaction of malignant cells with immune cells remains to be determined, because the evidence of the association of HLA class II antigen expression in malignant lesions with the clinical course of the disease is conflicting.[8]

Analysis of a limited number of frozen primary uveal melanoma lesions has shown an association between HLA class I antigen downregulation and favorable prognosis,[9] and Crowder et al.[10] have demonstrated that immunoreactivity to HLA class I and II was greater in epithelioid than spindle cell uveal melanoma. Because these findings are unexpected, in the present study we retrospectively determined HLA class I and HLA class II antigen expression in a large number of primary uveal melanoma lesions by immunoperoxidase staining of formalin-fixed, paraffin-embedded tissue sections with monoclonal antibodies (mAbs). Furthermore, we correlated the immunostaining results with the clinical course of the disease to assess their clinical significance.

MATERIALS AND METHODS

Patients and Tumors

Formalin-fixed, paraffin-embedded lesions from 70 patients with uveal melanoma were available for this study. Five lesions were deemed necrotic and thus disregarded from further evaluation. The remaining 65 lesions were from 23 female and 42 male patients with an average age of 61 years (range, 23–87). All patients had been treated solely by enucleation of the eye harboring the tumor. Thirty patients died of uveal melanoma (metastatic disease), 14 patients died of causes unre-

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lated to uveal melanoma, and 22 were still alive at the end of follow-up. The mean follow-up time was 17.5 years (range, 10.3–21.7) for the surviving group, 9.7 years (range, 2.3–17.8) for the death-of-other-causes group, and 4.1 years (range, 0.5–17.6) for the death-of-uveal-melanoma group.

The study was reviewed and approved by the local ethics committee at the Karolinska Institute, and the committee deemed that it conformed to the generally accepted principles of ethics in research, in accordance with the Helsinki Declaration.

Monoclonal and Polyclonal Antibodies

The mAb HC-10, which recognizes a determinant expressed on HLA-A10, -A28, -A29, -A30, -A31, -A32, and -A33 heavy chains and on virtually all HLA-B heavy chains; the anti-β2-m mAb L368; and the anti-HLA class II mAb LGII-612.14 were developed and characterized as described elsewhere.[11–13] The hybridoma secreting the anti-CD44 mAb IM-7 was purchased from the American Type Culture Collection (Rockville, MD). The biotinylated anti-mouse IgG antibodies were purchased from Vector Laboratories (Burlingame, CA).

Immunostaining Protocol

Immunostaining of tissue sections was performed using the standard avidin-biotin complex technique. Briefly, 4-μm formalin-fixed, paraffin-embedded tissue sections were cut from each of the selected 70 tumor specimens. Tissue sections were then deparaffinized and rehydrated, and endogenous peroxidase was blocked with H_2O_2 for 30 minutes at room temperature. No antigen retrieval was performed before the antibody incubation. Tissue sections were then rinsed in Tris and phosphate-buffered saline (Tris-PBS, pH 7.6) and incubated with blocking serum (1% bovine serum albumin) for 20 minutes at room temperature followed by an overnight incubation at 8°C with an excess of anti-HLA mAb. Biotinylated anti-mouse IgG antibodies were then added, and incubation was continued for an additional 30 minutes at room temperature. The avidin-biotin complex (Vector Laboratories) was then added. The peroxidase reaction was developed for 6 minutes at room temperature using 0.6 mg/ml 3′3-diaminobenzidine tetrahydrochloride (DAB) with 0.03% hydrogen peroxide. Counterstaining was performed with Mayer's hematoxylin. Tris-PBS was used for rinsings between the different steps.

Staining Assessment

Tissue sections were read independently by two investigators (SS, CE) without knowledge of the results obtained by the other investigator or of the survival data. Furthermore, each investigator read all the slides twice without knowledge of the results obtained in the previous reading. All stained cells were considered positive, irrespective of staining intensity. The immunoreactivity was differentiated from melanin pigment, as reported previously.[14] Specifically, the dark brown, finely granular appearance of the immune-reaction product could be separated from the coarse granular appearance of the melanin pigment. Results were scored as low when less than 30% of melanoma cells were stained and high when more than 30% of melanoma cells were stained. Concordance between observers was 93% for HLA class 1 antigens, 87% for β2-m, and 93% for HLA class II antigens. The interobserver reproducibility using the κ test was 0.90 for HLA class I antigens, 0.78 for β2-m, and 0.88 for HLA class II antigens. When the results obtained by the investigators were not concordant, those obtained by the first investigator (SS, who is a senior pathologist) were used for the analysis.

Statistical Analysis

Survival data without loss to follow-up were obtained, according to the tenets of the Declaration of Helsinki, for all patients with uveal melanoma from the Swedish National Causes of Death Registry. Time from the date of surgery to death or the end of 1996 was considered censored if the patient was alive at the end of 1996 or had died of any other than a melanoma-related cause. The log-rank test was used to assess survival differences. The χ^2 test was used to measure association of HLA antigen expression with metastasis development and cell type.
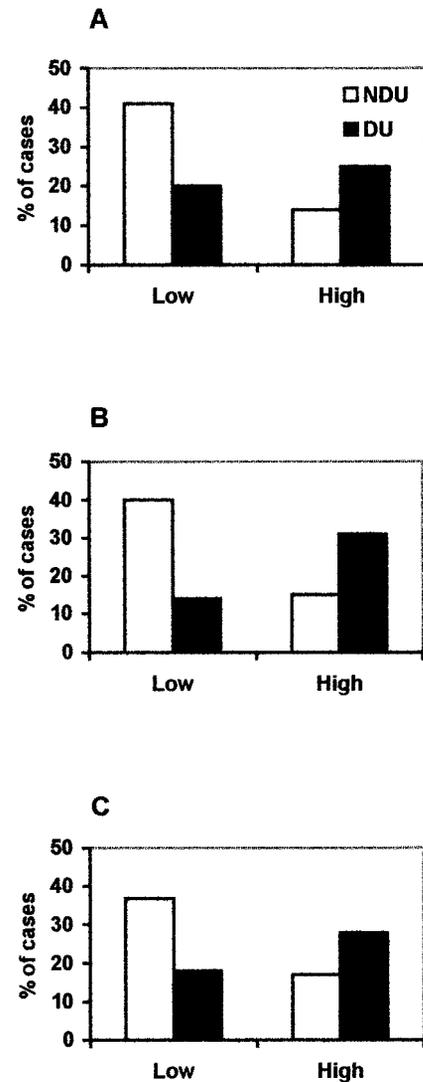

FIGURE 1. Expression of HLA antigens in uveal melanomas is correlated with clinical outcome. Patients were grouped according to staining of their lesions as high (>30% positive cells) and low (<30% positive cells) as analyzed with anti-HLA class I (**A**), -β_2-m (**B**), and -HLA class II (**C**) mAbs. The groups of those who had not died of uveal melanoma (NDU) and those who had died (DU) comprised 36 and 29 patients, respectively. There was a significant correlation between the expression of HLA class I antigens, β_2-m, and HLA class II antigens and the development of metastases ($P = 0.013$, $P < 0.001$, and $P = 0.021$, respectively).

The κ test was used for assessing interobserver reproducibility. The significance level was set at 0.05. Calculations were performed by computer (Statistica, ver. 5.5; StatSoft, Tulsa, OK; and MedCalc; MedCalc Software, Mariakerke, Belgium).

Results

Immunoperoxidase staining of 65 formalin-fixed, paraffin-embedded primary uveal melanoma lesions with anti-HLA class I heavy chain mAb HC-10 and with anti-β_2-m mAb L368 detected HLA class I antigen downregulation in 62% (40/65) and 54% (35/65) of the lesions, respectively (Figs. 1A, 1B). Immunoperoxidase staining with mAb LGII-612.14 detected high levels of HLA class II antigens in 46% (30/65) of the lesions (Fig. 1C). The low-expression groups (Figs. 1A–C) also involved cases that were negative for the antigens analyzed. For each antigen, 14 cases were negative (data not shown). The expres-

sion of the three molecules analyzed is significantly correlated. The probabilities for HLA class I antigen and β_2-m association, HLA class I and HLA class II antigen association, and β_2-m and HLA class II antigen association were $P = 0.014$, $P < 0.001$, and $P < 0.001$, respectively. These findings are in agreement with other observations that suggest that HLA class I and II antigens share a common regulatory pathway.[15]

Expression of HLA antigens in primary uveal melanoma lesions is correlated with their histopathologic characteristics. Low expression of HLA class I antigens and HLA class II antigens is significantly ($P = 0.006$ and $P = 0.010$, respectively) more frequent in spindle cell melanoma lesions than in other types of uveal melanomas (data not shown). This finding is in agreement with results previously obtained by one of us,[16] by testing frozen uveal melanoma lesions with the anti-HLA class I mAb W6/32 and with the anti-β_2-m mAb NAMB-1. In that study the latter two mAbs were found to stain only uveal melanomas of the mixed and prognostically unfavorable epithelioid type.

HLA class I antigen, β_2-m, and HLA class II antigen expression in the 65 primary uveal melanoma lesions was associated with poor clinical outcome (Figs. 1A–C). Thus, high expression of these markers in the lesions correlated significantly with development of metastases ($P = 0.013$, $P < 0.001$, and $P = 0.021$, respectively). These associations appear to be specific, because expression in primary uveal melanoma lesions of CD44, which is also a cell surface protein, was not correlated with clinical outcome ($P = 0.334$; data not shown). None of the patients ($n = 14$) who were negative for HLA class II died of uveal melanoma (data not shown).

DISCUSSION

Expression of HLA class I antigens, β_2-m, and HLA class II antigens in primary uveal melanoma lesions has prognostic significance. Analyses with a log-rank test and Kaplan-Meier graphs have shown a significant correlation between high expression of any one of the three HLA molecules and uveal melanoma–related mortality (Fig. 2). This correlation was highest in tumors with high β_2-m expression ($P < 0.001$; for HLA class I and II, $P = 0.009$ and $P = 0.006$, respectively). Blom et al.[17] found high HLA class I antigen expression in primary uveal melanoma lesions, detected by staining frozen tissue sections with HLA locus-specific mAbs, to be correlated with death from metastases. In our studies a similar correlation was found by staining formalin-fixed, paraffin-embedded tissue sections, which represent a useful substrate to monitor HLA class I antigen expression in tissue and to assess the clinical significance of abnormalities in HLA class I antigen expression in malignant lesions. In contrast to our findings, De Waard-Siebinga et al.[9] reported in an earlier study the expression of HLA class I antigens in all 23 investigated ocular melanoma lesions, as measured by staining frozen tissue sections with the mAb W6/32, which recognizes a monomorphic determinant expressed on β_2-m–associated HLA-A, -B, and -C heavy chains.

The association between high HLA class I antigen expression in primary lesions and poor prognosis we, as well as Blom et al.,[17] found in patients with uveal melanoma is contrary to that found in cutaneous melanoma and in other types of malignancies. In the latter diseases high HLA class I antigen expression in primary lesions has been shown to be associated with a favorable clinical course of the disease.[18] This has been suggested to reflect the major role played by HLA class I antigen–restricted, tumor-antigen–specific CTLs in the control of tumor growth. From this, it follows that HLA class I antigen downregulation may provide tumor cells with an escape from CTL recognition and destruction. In uveal melanoma, the association of low HLA class I antigen expression in primary lesions with a favorable clinical course may reflect the suscep-
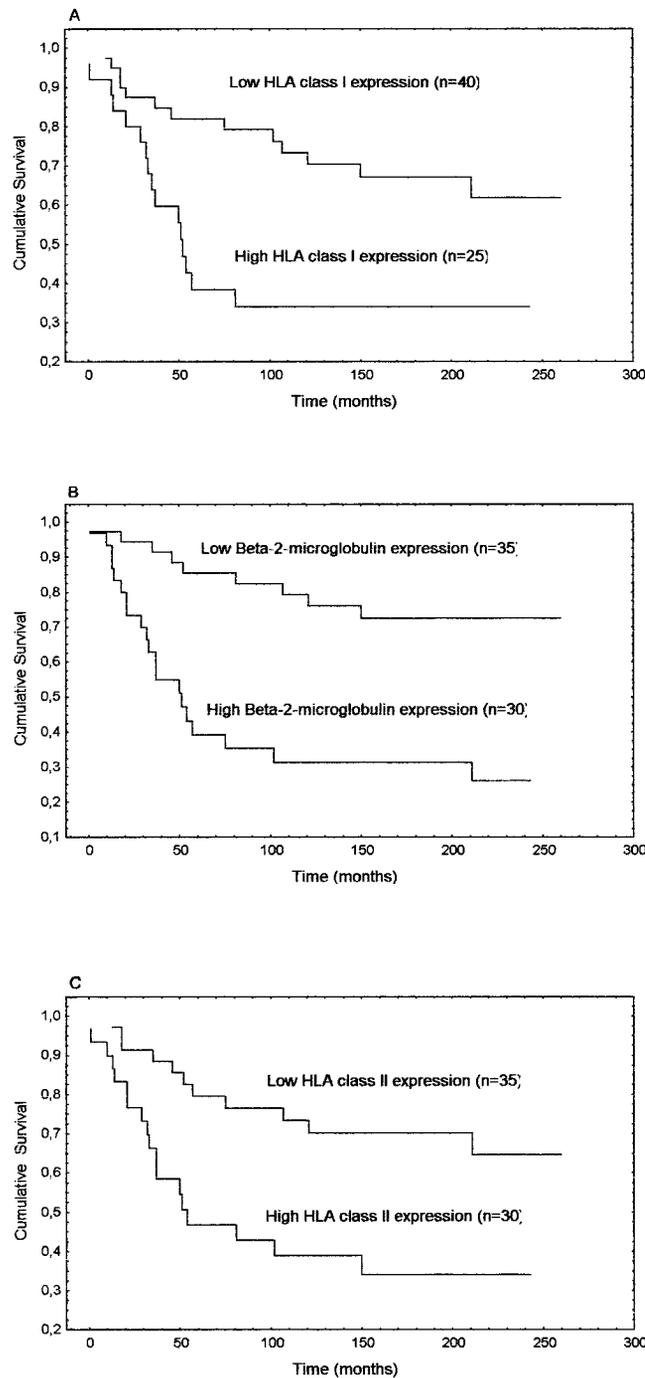

FIGURE 2. Kaplan-Meier graphs of the cumulative survival proportion after enucleation for uveal melanoma. High expression (>30% immunopositive tumor cells) of HLA class I antigens (**A**), β_2-m (**B**), and HLA class II antigens (**C**) were all associated with tumor-related death ($P = 0.009$, $P = 0.001$, and $P = 0.006$, respectively).

tibility to NK-cell–mediated lysis of low HLA class I– expressing melanoma cells invading blood vessels, as proposed by Blom et al.[17] If this interpretation is correct, NK cells may be particularly important in tumors that spread hematogenously, but may play less of a role in tumors that spread through the lymphatic system.

Similar to our findings, experimental melanoma cells that express high MHC class I antigen levels are more resistant to intravenous lysis by NK cells than those with low MHC class I antigen levels.[19] Also, intravenous injection of MHC class I-positive clones from a murine fibrosarcoma was reported to

be oncogenic, whereas subcutaneous injection of MHC class I–negative clones from the same tumor was more oncogenic than that of their MHC class I–positive counterparts.[20] The critical role of NK-cell–mediated cytotoxicity in uveal melanoma is also supported experimentally by the observation that NK-cell depletion results in increased number and growth of hepatic micrometastases.[21] Furthermore, Ma et al.,[22] using a nude mice model, showed that disruption of NK-cell activity increased the metastatic spread of uveal melanoma cells. Uveal melanoma cells appear, however, to have evolved other means to escape NK-mediated surveillance, including production of a macrophage-inhibitory factor that prevents lysis by NK cells.[23]

In the present study, HLA class II antigens were detected in a significantly higher percentage of primary uveal melanoma lesions than that reported by Jager et al.[24] Although it cannot be excluded that this reflects differences in the sensitivity of the immunohistochemical assays used in the two studies, we favor the possibility that the low HLA class II antigen expression described by Jager et al.[24] is caused by the exposure to x-ray irradiation before enucleation of the uveal melanoma analyzed in the study. Our interpretation is supported by the correlation found in another study between HLA-DQ expression in a uveal melanoma and a ciliary body localization of the tumor and between a low HLA-DQ and -DP expression and an intact Bruch's membrane.[9]

HLA class II antigen expression in primary uveal melanoma lesions has clinical significance, because none of the patients who did not express HLA class II antigens in their primary lesions (14 cases) died of uveal melanoma. In contrast, 18 of the 30 patients with high HLA class II antigens died of uveal melanoma. To the best of our knowledge, this is a novel finding. Its mechanism is not readily apparent. Conflicting information is available about the clinical significance of HLA class II antigen expression in cutaneous melanoma.[25]

It has been demonstrated that HLA class II antigen–bearing melanoma cells induce the secretion of immunosuppressive cytokine IL-10 by T cells, resulting in T-cell anergy.[26] This may explain the association between high HLA class II antigen expression in primary uveal melanoma lesions and poor prognosis. Alternatively, the observed correlation may reflect the resistance of hematogenously spreading melanoma cells with high HLA class I as well as HLA class II antigen expression to NK-cell lysis, because HLA class I and class II antigens may share a common regulatory pathway.[15] Regardless of the underlying mechanisms, assessment of HLA antigen expression in uveal melanoma lesions may be of value for determining whether immunotherapeutic strategies in individual patients should be directed toward T cells or NK cells.

Acknowledgments

References

1. Österlind A. Trends in incidence of ocular malignant melanoma in Denmark 1943-1982; *Int J Cancer.* 1987;40:161-164.

2. Rajpal S, Moore R, Karakousis CP. Survival in metastatic ocular melanoma. *Cancer.* 1983;52:334–336.

3. Boon T, Coulie PG, Van den Eynde B. Tumor antigens recognized by T cells. *Immunol Today.* 1997;18:267–268.

4. Garrido F, Cabrera T, Concha A, Glew S, Ruiz-Cabell F, Stern PL. Natural history of HLA expression during tumour development. *Immunol Today.* 1993;14:491–499.

5. Kärre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2 deficient lymphoma variants suggests alternative immune defense strategy. *Nature.* 1986;319:675–678.

6. Ma D, Niederkorn JY. Transforming growth factor-β down-regulates major histocompatibility complex class I antigen expression and increases the susceptibility of uveal melanoma cells to natural killer cell-mediated cytolysis. *Immunology.* 1995;86:263–269.

7. Pellegrino MA, Natali PG, Ng AK, et al. Unorthodox expression of Ia-like antigens on human tumor cells of nonlymphoid origin: immunological properties, structural profile, and clinical relevance. In: Nakamura RM, Dito WR, Tucker ES, eds. *Immunologic Analysis. Recent Progress in Diagnostic Laboratory Immunology.* New York: Mason; 1982:159–173.

8. Seliger B, Maeurer MJ, Ferrone S. Antigen-processing machinery breakdown and tumor growth. *Immunol Today.* 2000;21:455–464.

9. De Waard-Siebinga I, Houbiers JGA, Hilders CGJ, de Wolff-Rouendal D, Jager MD. Differential expression of HLA-A and B-alleles on uveal melanoma as determined by immuno-histology. *Ocular Immunol Inflamm.* 1996;4:1–14.

10. Crowder J, Dithmar S, Jager MJ, Hurks HMH, Vigneswaran N, Grossniklaus HE. HLA class I antigen expression correlates with epithelioid cell type in uveal melanoma [ARVO Abstract]. *Invest Ophthalmol Vis Sci.* 2000;41(4):**S**384. Abstract nr 2028.

11. Stam NJ, Spits H, Ploegh HL. Monoclonal antibodies raised against denatured HLA-B locus heavy chains permit biochemical characterization of certain HLA-C locus products. *J Immunol.* 1986;137:2299–2306.

12. Lampson LA, Fisher CA, Whelan JP. Striking paucity of HLA-A, B, C and β_2-microglobulin on human neuroblastoma cell lines. *J Immunol.* 1983;130:2471–2478.

13. Temponi M, Kekish U, Hamby CV, Nielsen H, Marboe CC, Ferrone S. Characterization of anti-HLA class II monoclonal antibody LGII-612.14 reacting with formalin fixed tissues. *J Immunol Methods.* 1993;161:239–256.

14. Kivelä T. Immunohistochemical staining followed by bleaching of melanin: a practical method for ophthalmic pathology (Letter). *Br J Biomed Sci.* 1995;52:325.

15. Van den Elsen PJ, Gobin SJP, van Eggermond MCAJ, Peijneburg A. Regulation of MHC class I and II gene transcription: differences and similarities. *Immunogenetics.* 1998;48:208–221.

16. Natali PG, Bigotti A, Nicotra MR, et al. Analysis of the antigenic profile of uveal melanoma lesions with anti-cutaneous melanoma-associated antigen and anti-HLA monoclonal antibodies. *Cancer Res.* 1989;49:1269–1274.

17. Blom DJR, Luyten GPM, Mooy C, Kerkvliet S, Zwinderman AH, Jager MJ. Human leukocyte antigen class I expression, marker of poor prognosis in uveal melanoma. *Invest Ophthalmol Vis Sci.* 1997;38:1865–1872.

18. Hicklin DJ, Marincola FM, Ferrone S. HLA class I antigen down-regulation in human cancers: T-cell immunotherapy revives an old story. *Mol Med Today.* 1999;5:178–186.

19. Taniguchi K, Petersson M, Kiessling R, Klein G, Kärre K. Gamma-interferon induced lung colonization and metastasis by mouse B16 melanoma associated with enhanced expression of class i MHC antigens and decreased NK sensitivity. *Proc Natl Acad Sci USA.* 1987;84:3405–3409.

20. Algarra I, Ohlen C, Perez M. NK sensitivity and lung clearance of MHC-class-I-deficient cells within a heterogenous fibrosarcoma. *Int J Cancer.* 1989;44:675–680.

21. Dithmar SA, Rusciano DA, Armstrong CA, Lynn MJ, Grossniklaus HE. Depletion of NK cell activity results in growth of hepatic micrometastases in a murine ocular melanoma model. *Curr Eye Res.* 1999;19:426–431.

22. Ma D, Luyten GP, Luider TM, Niederkorn JY. Relationship between natural killer cell susceptibility and metastasis of human uveal melanoma cells in a murine model. *Invest Ophthalmol Vis Sci.* 1995;36:435–441.

23. Repp CA, Mayhew ES, Apte S, Niederkorn JY. Human uveal melanoma cells produce macrophage migration-inhibitory factor to prevent lysis by NK cells. *J Immunol.* 2000;165:710–715.

24. Jager MJ, Van der Pol JP, De Wolff-Rouendaal D, De Jong PTVM, Ruiter DJ. Decreased expression of HLA class II antigens on uveal melanoma cells after in vivo X-ray irradiation. *Am J Ophthalmol.* 1988;105:78–86.

25. Ruiter DJ, Mattijssen V, Broecker EB, Ferrone S. MHC antigens in human melanomas. *Semin Cancer Biol.* 1991;2:35–45.

26. Brady MS, Lee F, Petrie H, Eckels DD, Lee JS. CD4$^+$ T cells kill HLA-class- II-antigen positive melanoma cells presenting peptide in vivo. *Cancer Immunol Immunother.* 2000;48:621–628.