Ki-67 Immunostaining in Uveal Melanoma

The Effect of Pre-enucleation Radiotherapy

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Abstract: The reactivity of 33 choroidal and ciliary body melanomas with monoclonal antibody Ki-67, which recognizes a proliferation associated nuclear antigen, has been assessed and compared with clinicopathologic parameters. In 23 cases, 8 Gy irradiation was given 2 days before enucleation. Nonirradiated melanomas had a significantly higher proliferation rate as defined by staining with monoclonal antibody Ki-67 as compared with irradiated tumors (P = 0.007). Similarly, a strong relationship was found between pre-enucleation irradiation and low mitotic activity (P = 0.001). There was no significant correlation between the presence of Ki-67-positive nuclei and histologic classification, largest tumor diameter, localization of the tumor, age, sex, scleral invasion, pigmentation, and lymphocytic infiltration. The relevance of Ki-67 immunohistochemistry for the assessment of the life prognosis of patients with uveal melanoma has to be studied prospectively. Ophthalmology 1990; 97:1275–1280

The monoclonal antibody Ki-67 reacts with a DNA-associated antigen in the nuclei at all phases of the cell cycle except the resting phase.1–3 Immunocytochemical labeling with this antibody has been shown to correlate with generally accepted indices of cell proliferation such as autoradiography,4 flow cytometry,5 bromodeoxyuridine labeling index,6 and thymidine labeling index7 and therefore provides a simple and reliable means of rapidly evaluating the growth fraction of normal and neoplastic human cell populations. In some malignancies,8–11 it has been shown that Ki-67 labeling can serve as a prognostic parameter and recently its potential use as monitor for therapy in hormone-dependent human prostatic cancer has been described.12 We have used immunostaining with monoclonal antibody Ki-67 in a series of ciliary body and choroidal melanomas to assess the proliferative index and to investigate the effect of pre-enucleation irradiation. This was done by comparison of eyes that had been irradiated with 2 × 4-Gy electron beam irradiation on the last 2 days before enucleation and nonirradiated eyes with ciliary body and choroidal melanoma. We also studied the possible relationship between Ki-67 immunostaining and various conventional clinical and pathological prognostic parameters.

MATERIALS AND METHODS

Specimens were obtained from 24 patients treated in Rotterdam and from 9 patients treated in Amsterdam between January 1987 and October 1988. As part of another study, melanoma patients in Rotterdam receive, since 1978, local radiotherapy in two fractions of 4 Gy on the 2 days before enucleation. Thus, all eyes from Rotterdam, except one, were irradiated. The nine patients treated in Amsterdam were not irradiated. The enucleated eyes were transported immediately to the pathology de-
HISTOPATHOLOGIC EXAMINATION

Conventional histologic sections stained with hematoxylin-eosin were prepared from the paraffin-embedded tissue. We determined in these the following parameters: cell type (spindle, mixed, or epithelioid type), mitotic rate, largest tumor diameter, scleral invasion (absent; slight, <25% of the scleral thickness; moderate, approximately 50%; deep, >75%; episcleral growth), pigmentation (absent; slight, 25%; moderate, approximately 50%; heavy, >75%), and lymphocytic infiltration (absent, moderate, or marked). Mitoses were counted in 15 consecutive high-power fields with a total magnification of ×400.

IMMUNOHISTOCHEMISTRY

Frozen sections of 6-μm thickness were air dried and fixed for 10 minutes in acetone. Thereafter, slides were rinsed in phosphate-buffered saline (PBS, pH 7.4) and incubated with the monoclonal antibody Ki-67 (Dako Immunoglobulins Ltd, Copenhagen, Denmark). Slides were incubated with Ki-67 for 60 minutes at room temperature in PBS containing 0.01% gelatin and 0.1% sodium azide. As second-step reagent, a peroxidase-conjugated polyclonal rabbit anti-mouse immunoglobulin serum was applied (Dakopatts, Denmark). Subsequently, slides were rinsed in PBS to remove the unbound portion of the second reagent. After a final thorough washing in PBS, antigen–antibody binding was visualized by incubation in an acetate buffer solution (pH 4.6) that contained 3-aminio-9-ethylcarbazole, dimethylformamide, and hydrogen peroxide. Sections were counterstained with Mayer's hematoxylin for exactly 15 seconds to obtain a discrete nuclear staining pattern without obscuring the Ki-67 reactivity. Positive reactions produced a red color, making differentiation with brown/black endogenous melanin pigment possible. Of all samples analyzed immunohistochemically with the Ki-67 antibody, consecutive frozen sections were stained routinely with hematoxylin-eosin for microscopic examination to evaluate the pathologic parameters.

As a negative control, specimens were stained with the second-step reagent only. Frozen sections of two primary and one metastatic cutaneous melanomas served as a positive control.

RESULTS

Immunostaining with Ki-67 was observed in all tumors tested, but the percentage of positively staining cells varied from case to case. Staining with monoclonal antibody usually showed a speckled pattern in the nuclei (Fig 1), but in some cases staining of the nuclear matrix was prominent. Only rarely, a weak cytoplasmic staining was seen. The percentage of Ki-67-positive cells in all melanomas ranged from 0.16 to 3.80%, and one with a high score of 18.30% (Table 1).

The Ki-67 score of irradiated and nonirradiated eyes differed significantly: a reduction in the Ki-67 score was observed after pre-enucleation irradiation (P = 0.007) (Fig 2). To quantitate this reduction, the natural logarithm of Ki-67 was taken because of its positive skewness and the unequal variance in the irradiated and the nonirradiated group. By comparing the mean logarithm of the Ki-67 score in both groups, it can be estimated that irradiation resulted into approximately a threefold reduction of the Ki-67-defined proliferative fraction. The 95% confidence limits of this reduction are 1.3 and 9.8. Similarly, decreased mitotic rate was found after pre-enucleation irradiation (P = 0.001). A significant correlation was found between Ki-67 score and mitotic rate in the irradiated group, using the Spearman rank correlation test (S = 0.58; P = 0.002) (Fig 3), but not in the nonirradiated group (S = 0.12; P = 0.37) (Fig 3).

No significant correlation could be demonstrated between the Ki-67 score and largest tumor diameter (Fig 4), tumor localization or pigmentation, scleral invasion, and age and sex of the patient. Histologic classification ob-
Fig 1. Frozen section of an epithelioid cell-type melanoma incubated with the monoclonal antibody Ki-67 (original magnification, ×150). Notice the speckled pattern of Ki-67 immunostaining.

Stained on frozen sections as well as on paraffin-embedded tissue (Fig 5) also did not have a significant correlation with the Ki-67 score.

In 12 cases, a discrepancy existed in histologic typing of the tumor between frozen sections and the paraffin-embedded tissue, which can be explained partly by sampling and partly by the unreliability of tumor typing on frozen sections.

To compare our results with bromodeoxyuridine uptake in uveal melanomas, we calculated the mean Ki-67 count in 32 high-power fields in nonirradiated melanomas. The mean count was 128.

The percentage of Ki-67-positive nuclei of the control cutaneous melanomas varied from 1.4 (metastatic cutaneous melanoma) to 4.2%.

**COMMENT**

Proliferation rates in uveal melanomas have been retrospectively studied by DNA flow cytometry and by incorporation of bromodeoxyuridine. Both methods have disadvantages: when flow cytometry is used, the proliferative index only can be assessed reliably in diploid tumors. The reported varying incidence of aneuploidy in uveal melanomas (between 4 and 77%)

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LTD = largest tumor diameter; P = posterior; C = ciliary body; E = equator; A = anterior.
* + = 8 Gy pre-eucleation irradiation; = no irradiation.
† 1 = absent; 2 = <25%; 3 = 25-75%; 4 = >75%; 5 = episcopal growth.
‡ 1 = absent; 2 = <25%; 3 = 25-75%; 4 = >75%.
§ 1 = absent; 2 = moderate; 3 = marked.
Cell type scored on paraffin-embedded tissue.
influence the proliferative index. In addition, flow cytometry cannot distinguish between tumor cells alone and tumor cells clumped with nontumor cells. In vivo or in vitro incorporation of the potentially mutagenic DNA bromodeoxyuridine is another frequently used technique to detect cells in the S-phase. A disadvantage of this technique may be that sufficient bromodeoxyuridine incorporation may not occur as a result of poor vascular supply to the tumor or due to limited tissue diffusion. Therefore, we have assessed the growth fraction of ocular melanomas using the monoclonal antibody Ki-67 on frozen sections, which is a simple and reliable method of rapidly evaluating the growth fraction of normal and neoplastic human cell populations.

Under in vitro conditions, some authors have observed discrepancies between Ki-67 labeling and bromodeoxyuridine incorporation. Nevertheless, in a series of 20 human solid tumors a consistent ratio was found between Ki-67 labeling index (cycling cells/total tumor cells) and bromodeoxyuridine labeling index (S-phase cells/total tumor cells). Comparing the mean Ki-67 count in nonirradiated uveal melanomas in 32 high-power fields with the results of a previous study using bromodeoxyuridine uptake in nonirradiated uveal melanomas, the findings are in keeping with this ratio.

In our study, the Ki-67 score of nonirradiated uveal melanomas was low (i.e., <1 to 3.08%) as compared with the Ki-67 score in primary malignant tumors elsewhere in the body, which varies from <5 to 65%. Furthermore, our results indicate that the Ki-67 score of the nonirradiated choroidal melanomas is generally lower than the Ki-67 score of nonirradiated primary cutaneous melanomas. Recently, a higher Ki-67 score was reported on five choroidal melanomas. These findings are not in keeping with the results using bromodeoxyuridine uptake in choroidal melanomas. Their discrepant findings can be attributed to differences in techniques and selection of patients. Unfortunately, no additional data were provided in their heterogeneous series allowing their results for comparison.

The question still remains if Ki-67 immunostaining is relevant for the assessment of prognosis of patients with ocular melanomas. In our study, there was no correlation between Ki-67 score and conventional prognostic parameters such as histologic classification and largest tumor diameter. These results are similar to the findings for bromodeoxyuridine uptake in uveal melanomas. In this preliminary study, we did not attempt to assess clinical outcome because the follow-up time for the patients from whom we obtained frozen melanoma material was too

Fig 2. Percentage of Ki-67 positive nuclei in a group of patients who received 8-Gy pre-enucleation irradiation (closed circle) as compared with a group of patients who did not receive pre-enucleation irradiation (open triangle).

Fig 3. A statistically significant correlation \((P < 0.001)\) was found between Ki-67 positivity and mitotic rate. Closed circle indicates pre-enucleation irradiation (8 Gy). Open triangle indicates no pre-enucleation irradiation. HPF = high-power field.
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Fig 4. Lack of correlation between largest tumor diameter (LTD) and the percent of Ki-67 positive nuclei. Closed circle indicates pre-enucleation irradiation (8 Gy). Open triangle indicates no pre-enucleation irradiation.

short. The antigen recognized by Ki-67 does not survive conventional fixation and thus is not suitable for retrospective studies on stored paraffin-embedded tissue specimens. It should be noted, however, that 1 of the 33 examined patients died only 6 months after enucleation of disseminated choroidal melanoma (epithelioid cell type).

Before enucleation, there was no clinical evidence of metastasis. The irradiated choroidal melanoma of this patient had the highest proliferative index (18.3%) in this study, which is comparable with the proliferative index reported for malignant tumors elsewhere in the body. It might be that selection of patients who are at high risk for tumor metastasis could be achieved by looking for a high proliferative index in addition to conventional prognostic parameters as histologic type, tumor size and mitotic rate.

The prognostic significance of the Ki-67 staining remains to be proven. However, conventional counting of mitotic figures yields a poor reflection of the proliferative activity of a given tumor, because only a minor fraction of proliferating cells is in actual mitosis. The mitotic score may be unreliable, due to problems with identification of mitotic figures, variation in cell cycling time or to different handling of the tissue. Nevertheless, in our study a good rank correlation ($S = 0.71; P < 0.001$) was noted between the Ki-67 score and the mitotic rate in the irradiated group, similar to findings in mammary carcinoma, but in contrast to findings in cervical carcinoma.

In patients treated with 20 Gy in 5 fractions over 5 to 7 days before enucleation, pre-enucleation radiotherapy has been shown to decrease the mitotic rate of choroidal melanomas to zero in 15 of 21 patients. In our study, the mitotic rate was reduced to zero in only 3 of 23 patients.

After more than 60 GyE of helium ion charged-particle therapy, the proliferation rate measured by uptake of bromodeoxyuridine was zero in six of eight patients. A dose of 20 Gy pre-enucleation irradiation gave a 100-fold reduction of bromodeoxyuridine incorporation. The reduction of Ki-67 score in this study was approximately threefold after 8 Gy pre-enucleation dose, which is a small difference with respect to the variability of the Ki-67 score in the irradiated group.

The lesser reduction of proliferative activity in our study can be explained by the much smaller radiation dose and the short interval between irradiation and enucleation.

The aim of the low-dose irradiation of 8 Gy was to reduce the risk of hematogenous metastases during the enucleation procedure and was not meant to be curative. Whether radiation will only be effective when the number
of cells synthesizing DNA is reduced to near zero is a point of discussion.\textsuperscript{15}

In conclusion, we demonstrated with Ki-67 immunostaining a reduction of the proliferative activity of uveal melanomas after pre-enucleation irradiation. The proliferative activity of uveal melanomas was low in comparison with cutaneous melanomas\textsuperscript{34} and primary malignant tumors elsewhere in the body. The relevance of Ki-67 immunostaining for the life prognosis of patients with uveal melanomas remains to be established.

REFERENCES


