Cell proliferation activity in posterior uveal melanoma after Ru-106 brachytherapy: an EORTC ocular oncology group study

Jacob Pe’er, Fritz H Stefani, Stefan Seregard, Tero Kivela, Peter Lommatzsch, Jan U Prause, Beate Sobottka, Bertil Damato, Itay Chowers

Abstract

Aim—To evaluate the cell proliferation activity in posterior uveal melanomas after Ru-106 brachytherapy and which had enough melanoma tissue to enable histological assessment, were included. The 57 eligible specimens were divided into a group of 44 eyes that were enucleated because of tumour regrowth, and a non-recurrent group of 13 eyes that were enucleated because of complications such as neovascular glaucoma. 46 non-irradiated eyes harboured uveal melanoma served as a control group. All specimens underwent routine processing. They were cut into 5 µm sections, and were stained with two main cell proliferation markers: PC-10 for PCNA and MIB-1 for Ki-67. The stained sections were assessed, and the cells that were positive in the immunostaining were counted in each section. The results were evaluated by various statistical methods.

Results—The PC-10 score showed a statistically significant difference across the three groups (p = 0.002). The control group showed the highest PC-10 score (median 31.0 PCC/HPF) followed by the tumour regrowth group (median 4.9 PCC/HPF). The lowest PC-10 scores were found in the non-recurrent tumours (median 0.05 PCC/HPF). The MIB-1 score in the control group (median 5.77 PCC/HPF) was similar to the regrowth group (median 5.4 PCC/HPF). In contrast, the MIB-1 score in the non-recurrent tumours was statistically significantly lower (median 0.42 PCC/HPF). The PC-10 and MIB-1 scores were similar in tumours composed of either spindle cells or epithelioid cells in all groups.

Conclusions—The non-recurrent melanomas demonstrate significantly lower cellular proliferation activity than melanomas that showed regrowth or that were not irradiated at all. In our hands, PCNA gave more meaningful information than Ki-67. Our findings strongly support the need for treating regrowing posterior uveal melanoma either by enucleation or re-treatment by brachytherapy. On the other hand, also in the non-recurrent uveal melanomas there are viable cells with potential for proliferation, although fewer in number, with unknown capacity for metastatic spread. Therefore, the irradiated tumours should be followed for many years, probably for life.

The most common eye conserving way of treating posterior uveal melanoma is by radiotherapy. This is usually delivered by means of episcleral radioactive plaques. Cobalt-60,1 ruthenium-106,2 and iodine-1253 are the radioactive materials most commonly used. Other methods of radiotherapy of posterior uveal melanomas include teletherapy using charged particles of proton4 and helium ion beams5; recently, stereotactic radiosurgery using the Leksell gamma knife has been described as being efficient.6–7

After radiotherapy, most uveal melanomas show significant reduction in size, although complete disappearance is not common. Histological evaluation of residual tumours have demonstrated that most of the tumours harbour viable melanoma cells8–16 and some of these cells show proliferating activity using immunohistochemical markers.17–20

Previous studies have reported that between 6% to 34% of eyes treated for posterior uveal melanoma by radiotherapy are eventually enucleated, either because of regrowth of the tumour or because of complications caused by the radiotherapy, mainly secondary neovascular glaucoma.21

The purpose of the present multicentre, multinational study was to evaluate the cell proliferation activity in posterior uveal melanoma after Ru-106 brachytherapy in eyes that were enucleated for various reasons. We have used two common cell proliferation markers, PC-10 for PCNA and MIB-1 for Ki-67. We also compared the findings in irradiated posterior uveal melanomas with those in tumours that were enucleated without being previously irradiated.

Methods

Eyes enucleated because of choroidal or ciliary body melanoma first treated with Ru-106 brachytherapy, and which had enough melanoma tissue to enable histological assessment, were included. Eyes that were enucleated after brachytherapy, and which had enough melanoma tissue to enable histological assessment, were included. The 57 eligible specimens were divided into a group of 44 eyes that were enucleated because of tumour regrowth, and a non-recurrent group of 13 eyes that were enucleated because of complications such as neovascular glaucoma. 46 non-irradiated eyes harboured uveal melanoma served as a control group. All specimens underwent routine processing. They were cut into 5 µm sections, and were stained with two main cell proliferation markers: PC-10 for PCNA and MIB-1 for Ki-67. The stained sections were assessed, and the cells that were positive in the immunostaining were counted in each section. The results were evaluated by various statistical methods.

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Table 1 Clinical and histological features of the uveal melanomas in the study

<table>
<thead>
<tr>
<th>Brachytherapy</th>
<th>Non-recurrent</th>
<th>Regrowth</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location (choroid/ciliary body)</td>
<td>11/2</td>
<td>41/3</td>
<td>42/5</td>
</tr>
<tr>
<td>Tumour height (mm)(SD)</td>
<td>5.2 (3)</td>
<td>6 (3)</td>
<td>6.7 (3.4)</td>
</tr>
<tr>
<td>Largest tumour diameter (mm)(SD)</td>
<td>10 (2.3)</td>
<td>11.4 (4.5)</td>
<td>12.5 (3.9)</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>5/8</td>
<td>22/22</td>
<td>19/28</td>
</tr>
<tr>
<td>Age (years)(SD)</td>
<td>56 (12)</td>
<td>56 (14)</td>
<td>57 (12)</td>
</tr>
<tr>
<td>Cell type (number of tumours):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spindle</td>
<td>8</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>Epithelioid</td>
<td>3</td>
<td>20</td>
<td>26</td>
</tr>
<tr>
<td>Necrotic</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

Tumour location, height and largest tumour diameter were determined according to the histological sections after enucleation.

enough tissue to enable reliable immunostaining, were excluded.

Tissue blocks from 143 cases of uveal melanoma were collected from the ophthalmologic pathology laboratories of six European departments of ophthalmology. All eyes enucleated in these departments during the years 1980–97, after being previously treated with Ru-106 plaque brachytherapy for uveal melanoma, were evaluated for the study. Of the 143 specimens collected, 57 were included in the study.

The other 86 tumours were excluded because the paraffin block did not contain sufficient residual tumour. Two groups were formed according to the indication for enucleation: the first included 44 eyes that were enucleated because of tumour regrowth after the brachytherapy; the second group included the remaining 13 eyes that were enucleated following brachytherapy due to complications such as glaucoma or a blind and painful eye without evidence of tumour regrowth—the non-recurrent group. Forty six non-irradiated consecutive uveal melanomas were obtained from the ophthalmic pathology laboratory of the Hadassah University Hospital for use as the control group.

Tumours from all laboratories had been fixed for at least 24–48 hours in 10% neutral buffered formalin before original tissue processing. Formalin fixed, paraffin embedded sections were stained with haematoxylin and eosin. Tumour location was categorised as follows: ciliary body melanoma was defined as a uveal melanoma of which more than two thirds of the tumour mass was located in the ciliary body; other tumours were defined as choroidal melanoma. Tumour cell typing was defined as epithelioid (containing epithelioid cells), or spindle (tumour without epithelioid cells). In order to evaluate similar areas of the tumour, serial sections of paraffin tissue blocks were cut at 5 µm thickness, and immunostainings were performed on sections adjacent to that of the haematoxylin and eosin stain. Bleaching was used in sections from heavily pigmented tumours to enable immunostaining assessment.

Sections were deparaffinised in xylene and alcohols and placed for 15 minutes in 3% alcoholic H₂O₂ to block endogenous peroxidase. In order to reveal masked antigen, slides stained with MIB-1 were placed in 10 mM citrate buffer (pH 6.0), treated in the microwave for 15 minutes, and then the container was removed from the microwave for cooling for 15 minutes. Slides stained with PC-10 were not treated in a microwave. Both MIB-1 and PC-10 stained slides were placed in phosphate buffered saline (PBS, pH 7.6). Sections were then treated with bovine serum albumin (BSA) to prevent background staining, and incubated for 1 hour with the primary antibody, either MIB-1 or PC-10 (Zymed Laboratories, Inc, San Francisco, CA, USA), at room temperature in a humidified chamber. Slides were rinsed in PBS for 3–4 minutes and incubated with biotin linked secondary antibody for 30 minutes and with the labelling reagent peroxidase conjugated streptavidin for 30 minutes (Bio Genex Laboratories, San Ramon, CA, USA). After rinsing, the peroxidase label was demonstrated using 3-amino-9-ethylcarbazole (AEC) for 15 minutes, and counterstained with Mayer’s haematoxylin. A negative control was run using the same technique but omitting the primary antibody and adding the streptavidin-biotin complex.

The MIB-1 immunostaining was assessed by two observers who were masked to the light microscopic classification of the tumour as described by Seregard and his colleagues. In each section, 10 high power fields (>40) in areas of maximal immunoreactivity were assessed; all melanoma cells that contained a distinct positive nuclear stain were regarded as positive. The mean MIB-1 and PC-10 positive cell count (PCC) for high power field (HPF) were calculated for each observer in each section.

In order to estimate the correlation between two quantitative parameters (such as tumour height and proliferative activity), the Spearman correlation coefficient was calculated, and its significance was assessed. When the mean levels of a quantitative parameter were compared in different categories of a qualitative parameter (such as different groups) with more than two categories, the ANOVA classic test was
Table 2 The positive MIB-1 and PC-10 cell count per high power field according to the cell type

<table>
<thead>
<tr>
<th>Group/cell type</th>
<th>Spindle</th>
<th>Epithelioid</th>
<th>p Value (Mann-Whitney)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regrowth</td>
<td>MIB-1</td>
<td>1.8 (0–48.8)</td>
<td>8.8 (0–48.15)</td>
</tr>
<tr>
<td></td>
<td>PC-10</td>
<td>5.7 (0–88.4)</td>
<td>4.92 (0–157.6)</td>
</tr>
<tr>
<td>Non-recurrent</td>
<td>MIB-1</td>
<td>0.7 (0–2.6)</td>
<td>0.15 (0–119.8)</td>
</tr>
<tr>
<td></td>
<td>PC-10</td>
<td>0.32 (0–49.15)</td>
<td>0 (0–46.1)</td>
</tr>
<tr>
<td>Control</td>
<td>MIB-1</td>
<td>5.82 (0–35.65)</td>
<td>6.05 (0–35.35)</td>
</tr>
<tr>
<td></td>
<td>PC-10</td>
<td>30 (0–202.05)</td>
<td>27.95 (0–145.75)</td>
</tr>
<tr>
<td>Total (all groups)</td>
<td>MIB-1</td>
<td>1.12 (0–48.8)</td>
<td>6.07 (0–119.8)</td>
</tr>
<tr>
<td></td>
<td>PC-10</td>
<td>4.35 (0–88.4)</td>
<td>1.97 (0–157.6)</td>
</tr>
</tbody>
</table>

Discussion

The diagnosis of uveal melanoma as well as the follow up of these tumours after conservative, usually irradiation, treatment, is done by non-invasive methods, mainly funduscopy and B-scan and A-scan ultrasonography. As opposed to most malignancies, in uveal melanoma we often do not have tissue diagnosis, and we do not have histological follow up of the residue of the tumour. The reason for this is the fear of spreading tumour cells beyond the
Eye, through the site of a biopsy entrance or through tumour vessels damaged by the needle. By fundus examination we can see the reaction of the posterior uveal and MIB-1 to the treatment. On the other hand, we cannot ignore the apparent proliferating activity, although low, of part of the non-recurrent tumours that were enucleated because of ocular complications, the group that comprises the tumours that reacted well to irradiation. Apparently, these regrowing uveal melanomas. Our findings strongly support this attitude, since regrowing tumours may have a compartment of cycling cells as large as non-irradiated tumours, and since the presence of such proliferating cell population was previously shown to be associated with death from metastatic disease.

It is obvious that clinically detectable regrowth of tumours indicate activity of the tumour, and our results are not surprising. Without having histological markers, ocular oncologists tend to enucleate or to re-treat regrowing uveal melanomas. Our findings strongly support this attitude, since regrowing tumours may have a compartment of cycling cells as large as non-irradiated tumours, and since the presence of such proliferating cell population was previously shown to be associated with death from metastatic disease.

Both immunostainings demonstrated that the non-recurrent tumours had significantly lower cellular proliferation activity than the control group and the regrowth group. The regrowth group showed significantly less proliferative activity than the control group by PC-10 staining for PCNA. However, we could not show a difference between the control group and the regrowth group when we used the Ki-67 immunostaining. The reason for this may be manifold. Firstly, the Ki-67 and PCNA proteins are distinctly different cell cycle associated antigens. Secondly, the long half life of PCNA will in most cases generate a larger proportion of immunopositive cells than Ki-67. Compared with flow cytometry, PC-10 tends to overestimate cell proliferation, whereas MIB-1 underestimates cell proliferation. PC-10 will stain cells even after they have left the cell cycle, because of long half life. In this sense it is more “sensitive” to cells that are cycling or recently left the cell cycle. This also explains why more cells stain with PC-10 than with MIB-1.

This study is the first to compare the usefulness of these markers in uveal melanoma after failed brachytherapy. PC-10 was the only immunostaining to detect a difference between regrowing tumours and controls, owing to its higher sensitivity, and therefore in our hands it provides more meaningful information. Our results are comparable with previous studies that examined cell proliferation activities by one of the markers we used in our study. The measurements by both markers supplement each other and give a fuller picture than does either alone.

Counting the number of tumours in which proliferating tumour cells were not detected, we found significantly more tumours with zero proliferating cells in the non-recurrent group. Also in this counting, the results with PCNA marker proved to be more meaningful. These results are also comparable with previous studies.

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On the other hand, we cannot ignore the apparent proliferating activity, although low, of part of the non-recurrent tumours that were enucleated because of ocular complications, the group that comprises the tumours that reacted well to irradiation. Apparently, these viable tumours remain a potential for later regrowth, and should be followed carefully for many years and probably for life. It remains unclear if these tumours, successfully treated by clinical standards, maintain a capacity for metastatic spread.


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